Toxicology and Applied Pharmacology 304 (2016) 30–41

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

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

Epigenetic silencing of miR-218 by the lncRNA CCAT1, acting via BMI1, promotes an altered cell cycle transition in the malignant transformation of HBE cells induced by cigarette smoke extract

Lu Lu [1], Hui Xu [1], Fei Luo, Xinlu Liu, Xiaolin Lu, Qianlei Yang, Junchao Xue, Chao Chen, Le Shi, Qizhan Liu *

Institute of Toxicology, School of Public Health, Nanjing Medical University, Nanjing 211166, Jiangsu, People's Republic of China
The Key Laboratory of Modern Toxicology, Ministry of Education, School of Public Health, Nanjing Medical University, Nanjing 211166, Jiangsu, People's Republic of China

ARTICLE INFO

Article history:
Received 11 February 2016
Revised 2 May 2016
Accepted 18 May 2016
Available online 19 May 2016

Keywords:
Cigarette smoke extract (CSE)
lncRNAs
miRNAs
Cell cycle
Carcinogenesis

ABSTRACT

Cigarette smoking is the strongest risk factor for the development of lung cancer, the leading cause of cancer-related deaths. However, the molecular mechanisms leading to lung cancer are largely unknown. A long-noncoding RNA (lncRNA), CCAT1, regarded as cancer-associated, has been investigated extensively. Moreover, the molecular mechanisms of lncRNAs in regulation of microRNAs (miRNAs) induced by cigarette smoke remain unclear. In the present investigation, cigarette smoke extract (CSE) caused an altered cell cycle and increased CCAT1 levels and decreased miR-218 levels in human bronchial epithelial (HBE) cells. Depletion of CCAT1 attenuated the CSE-induced decreases of miR-218 levels, suggesting that miR-218 is negatively regulated by CCAT1 in HBE cells exposed to CSE. The CSE-induced increases of BMI1 levels and blocked by CCAT1 siRNA were attenuated by an miR-218 inhibitor. Moreover, in CSE-transformed HBE cells, the CSE-induced cell cycle changes and elevated neoplastic capacity were reversed by CCAT1 siRNA or BMI1 siRNA. This epigenetic silencing of miR-218 by CCAT1 induces an altered cell cycle transition through BMI1 and provides a new mechanism for CSE-induced lung carcinogenesis.

1. Introduction

In mammalian cells, most transcripts are noncoding RNAs, ranging from short RNAs (including microRNAs, miRNAs) to long RNAs spanning up to hundreds of kb (Carninci et al., 2005; Cheng et al., 2005; Birney et al., 2007; Washietl et al., 2007). Long noncoding RNAs (lncRNAs), which are >200 nt in length and are not translated into proteins, have a functional role in a variety of diseases (Muers, 2011). The lncRNA, CCAT1 (colon cancer-associated transcript-1), also known as CARlo-5 (cancer-associated region long noncoding RNAs), located in the vicinity of the transcription factor, *C*-Myc (Kim et al., 2014), is up regulated in colon cancer (Nissan et al., 2012), hepatocellular carcinoma (Zhu et al., 2015), gallbladder cancer (Ma et al., 2015), gastric carcinoma (Yang et al., 2013), and lung cancer (Luo et al., 2014).

While the actions of miRNAs as destabilizers and repressors of the translation of protein-coding transcripts (mRNAs) have been studied in detail, the influence of lncRNAs upon miRNA function is being elucidated. These lncRNA-miRNA interactions modulate gene expression patterns that drive cellular processes such as cell differentiation, proliferation, and death (Yoon et al., 2014). Little is known about the mechanism through which CCAT1 exerts its oncogenic activity, but the interaction between CCAT1 and miRNAs provides a new insight into cancer biology.

Tobacco use, primarily associated with cigarette smoking, remains the leading cause of preventable deaths (Mader et al., 2015). Apart from environmental and genetic factors, cigarette smoking is the strongest risk factor for development of lung cancer, an aggressive and heterogeneous disease and the leading cause of cancer-related deaths worldwide (Balogh et al., 2014). Although evidence for the carcinogenicity of cigarette smoke in humans is strong, the process of malignant transformation of cells remains largely unclear. In particular, the molecular mechanisms by which lncRNA-miRNA interactions participate in cigarette smoke-induced carcinogenesis remain to be established.

Uncontrolled cell proliferation resulting from deregulated cell cycle progression, and enhanced invasion and migration, are main events in neoplastic transformation (Fernandez et al., 2002). Although our previous studies showed that exposure of human bronchial epithelial (HBE) cells to cigarette smoke extract (CSE) induces an altered cell cycle transition and metastasis during cell transformation (Liu et al., 2016; Wang et al., 2016), the mode of lncRNA-miRNA regulation and how these regulatory mechanisms are involved in cigarette smoke-induced cell cycle changes and metastasis are still unknown.

* Corresponding author at: Institute of Toxicology, School of Public Health, Nanjing Medical University, Nanjing 211166, Jiangsu, People's Republic of China.
E-mail address: drqzliu@hotmail.com (Q. Liu).
[1] Authors contributed equally.

http://dx.doi.org/10.1016/j.taap.2016.05.012
0041-008X/

In the present study, we investigated the roles of CCAT1 and the relationship between CCAT1 and miR-218 in the malignant transformation induced by CSE. The results establish a link, through BMI1, between the lncRNA CCAT1 and malignant transformation, and present a mechanism for CSE-induced carcinogenesis.

2. Materials and methods

2.1. Subjects and epidemiological data

This study was approved by the Institutional Review Board of Nanjing Medical University. Written informed consent was obtained from each participant. On the basis of China Non-communicable Disease Surveillance in 2010, the study population in this cross-sectional study was a subset population recruited in Jiangsu Province, using a complex, multistage, probability sampling design (Wang et al., 2015). First, 6 counties were first selected from 106 counties using stratified random sampling according to the population, gross domestic product, degree of urbanization, and geographic setting. Second, 4 towns from each county and 3 villages from each town were selected with the probability proportional to the population size, using cluster random sampling. Third, 1 residential group including at least 50 households was selected from each village using simple random cluster sampling. The random digit function in Excel was applied in the selection of these households from each residential group. Finally, one family member aged 18 years or over was randomly selected from each household using the KISH Grid method (Smith et al., 1995). A total of 1568 men were included in the cohort.

The smoking history included age at smoking initiation, years of smoking, number of cigarettes smoked per day, and smoking cessation. An individual who never smoked or smoked fewer than 100 cigarettes in his lifetime was defined as a never smoker. Current smokers included those presently smoking and those who quit smoking <12 months before the interview. The pack·years of smoking were calculated according to the number of packs of cigarettes smoked per day and smoking duration (years). According to baseline data and previous studies (Ezzati and Lopez, 2003), current smokers were separated into three groups including mild smokers (<15 pack·year), medium smokers (15–30 pack·year), and heavy smokers (>30 pack·year). From each group, 60 men were randomly selected, and their sera were used for our study.

2.2. Isolation of miRNA from sera

From each participant, a venous blood sample (4–5 mL) was collected in tubes without any additives and, after at least 20 min of storage at room temperature, was centrifuged at 4000 rpm and 20 °C for 5 min. The serum samples were subsequently stored at −80 °C until further processing. For RNA isolation, 100 μL of serum was processed with *mir*Vana™ PARIS™ Kits (Life Technologies). In brief, cel-miR-39 was added as a control, then 100 μL of serum was mixed with 200 μL of acid phenol, 200 μL of chloroform, and 300 μL of RNase-free water. The mixture was vortex-mixed at room temperature for 1 min and centrifuged for 5 min at 12,000 *g*. After phase separation, the aqueous layer was removed and mixed with 1.5 volumes of isopropyl alcohol and 0.1 volume of 3 μmol/L sodium acetate (pH 5.3), and the solution was stored at −20 °C for 1 h. The RNA pellet was collected by centrifugation at 16,000 *g* for 20 min at 4 °C and then washed once with ethanol and dried for 10 min at room temperature. Finally, the pellet was dissolved in 20 μL of RNase-free water and stored at −80 °C until further analysis.

2.3. Extraction of total RNA from sera

Blood samples were centrifuged at 15,000 *g* to separate the sera. The samples were collected, stored at −70 °C, and thawed immediately before assay. Total RNA was isolated from serum using TRIzol LS reagent (Invitrogen, Life Technologies, Paisley, UK) according to manufacturer's instructions with the following modifications: In brief, each 250 μL serum sample was mixed with 750 μL TRIzol LS Reagent. After 5 min incubation at room temperature, 200 μL of chloroform was added, followed by 15 s of shaking and 10 min of incubation at room temperature. The mixture was centrifuged at 12,000 *g* for 15 min at 4 °C in a concentrator (Eppendorf-Netheler-Hinz, Hamburg, Germany). The aqueous layer containing RNA was transferred into a new tube, then RNA was precipitated for 16 h at −20 °C with 0.5 mL of isopropyl alcohol and washed with 1 mL of 75% ethanol. Finally, the RNA pellet was dried for 5–10 min at room temperature, dissolved at 60 °C in 15 μL of RNase-free water. The RNA concentration was measured with a NanoDrop spectrophotometer (Thermo Fisher Scientific). The final concentrations of RNA ranged from 319–782 ng/μL. The samples were stored at −80 °C until analysis. The TRIzol method has been previously described (Luo et al., 2016).

2.4. Cell culture and reagents

Simian virus 40 (SV40)-transformed HBE cells are nontumorigenic and retain features of parent HBE cells. They are useful for studies of multistage bronchial epithelial carcinogenesis (Reddel et al., 1988). HBE cells and BEAS-2B cells were obtained from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China) and were maintained under 5% CO_2 at 37 °C in Eagle's minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS, Life Technologies/Gibco, Grand Island, NY), 100 U/mL penicillin, and 100 μg/mL streptomycin (Life Technologies/Gibco, Gaithersburg, MD). HBE cells were exposed to CSE at a concentration of 20 μg/mL, the maximum concentration causing no changes in cell viability (Zhao et al., 2013). For chronic exposure, 1×10^6 cells were seeded into 10-cm (diameter) dishes for 24 h and exposed to 0 or 20 μg/mL of CSE for 24–48 h per passage. This process was continued for 40 passages (about 20 weeks). The cells in passage 0 (normal HBE cells) were not exposed to CSE. CSE-transformed HBE (T-HBE) cells were those exposed to 20 μg/mL of CSE for 40 passages, during which they undergo malignant transformation (Zhao et al., 2013; Liu et al., 2015). This process was performed as described previously (Zhao et al., 2013).

2.5. Preparation of CSE

Aqueous CSE was used to mimic the effects of cigarette smoke. CSE was generated from the University of Kentucky Reference Cigarette 1R4F (9 mg tar and 0.8 mg nicotine/cigarette), adapting the previously described procedure (Hsu et al., 1991). CSE was prepared as previously reported (Zhao et al., 2013). Briefly, the 'tar' or particulate phase of smoke was collected under standard Federal Trade Commission conditions (once each minute by a 2-s 35-mL puff) (Pillsbury and Bright, 1972; Narayan et al., 2004). The whole smoke was bubbled through 10 mL of serum-free MEM. The resulting suspension was adjusted to pH 7.4 and then filtered through a 0.22-μm pore filter (Schleicher & Schuell GmbH, Dassel, Germany) to remove bacteria and large particles. The CSE was standardized by monitoring the absorbance at 320 nm. This solution was defined as the original CSE at the concentration of 1 mg/mL. To prevent possible inactivation of compounds, the CSE was aliquoted into small vials and stored in the dark at −80 °C. Before each experiment, the frozen CSE stock solution was defrosted and diluted to the desired concentrations with cell medium.

2.6. Reverse-transcriptase polymerase chain reaction (RT-PCR)

Total RNA (2 μg) was transcribed into cDNA by the use of AMV reverse transcriptase (Promega, Madison, Wisconsin, USA). Forward (F) and reverse (R) primers were as follows: *CCAT1-F*, *5′-TTTATGCTTGAGCCTTGA-3′*; *CCAT1-R*, *5′-GAAGCCAGACCCAGTAAG-3′*; *18S-F*, *5′-GTAACCCGTTGAACCCCATT-3′*; and *18S-R*, *5′-*