Toxicology Letters 240 (2016) 60–67

Contents lists available at ScienceDirect

Toxicology Letters

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

Epigenetic silencing of p21 by long non-coding RNA HOTAIR is involved in the cell cycle disorder induced by cigarette smoke extract

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HIGHLIGHTS

- CSE enhances transfer of H3K27me3 to the p21 promoter via HOTAIR in HBE cells.
- CSE accelerates the G1/S transition by inhibiting p21 expression in HBE cells.
- Epigenetic silencing of p21 by HOTAIR is involved in CSE-induced cell cycle disorder.

ARTICLE INFO

Article history:
Received 4 August 2015
Received in revised form 13 October 2015
Accepted 17 October 2015
Available online 23 October 2015

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

ABSTRACT

Long noncoding RNAs (lncRNAs), which are epigenetic regulators, are involved in human malignancies. Little is known, however, about the molecular mechanisms for lncRNA regulation of genes induced by cigarette smoke. We recently found that, in human bronchial epithelial (HBE) cells, the lncRNA, Hox transcript antisense intergenic RNA (HOTAIR), is associated with changes in the cell cycle caused by cigarette smoke extract (CSE). In the present study, we report that increased expression of HOTAIR and enhancer of zeste homolog 2 (EZH2), and tri-methylation of Lys 27 of histone H3 (H3K27me3), affect cell cycle progression during CSE-induced transformation of HBE cells. Inhibition of HOTAIR and EZH2 by siRNAs attenuated CSE-induced decreases of p21 levels. Further, ChIP assays verified that HOTAIR and EZH2 were needed to maintain the interaction of H3K27me3 with the promoter regions of p21; combined use of a HOTAIR plasmid and EZH2 siRNA supported this observation. Thus, HOTAIR epigenetic silencing of p21 via EZH2-mediated H3K27 trimethylation contributes to changes in the cell cycle induced by CSE. These observations provide further understanding of the regulation of CSE-induced lung carcinogenesis and identify new therapeutic targets.

1. Introduction

Long RNA transcripts that have no protein-coding capacity are referred to as long (or large) non-coding RNAs (lncRNAs) (Moran et al., 2012). As a class, lncRNAs are involved in various biologic processes, including regulation of transcription, regulation of epigenetic genes, and diseases (Ponting et al., 2009). Furthermore, lncRNAs affect cellular processes, such as the cell cycle, survival, migration, and metabolism (Zhang et al., 2013). Dysregulated lncRNAs are involved in various diseases, including the development and progression of cancer. Thus, they provide opportunities to elucidate the molecular mechanisms associated with carcinogenesis (Gibb et al., 2011; Zhang et al., 2013). Dysregulation of miRNAs is now recognized as a feature of many types of cancer, and it is likely that lncRNAs affecting cancer initiation, progression, and treatment remain to be discovered (Tsai et al., 2011). In particular, the molecular mechanisms by which lncRNAs participate in cigarette smoke-induced carcinogenesis remain to be established.

Among the cancer-related lncRNAs, HOX transcript antisense intergenic RNA (HOTAIR) functions in cancer progression. HOTAIR, discovered in 2007, recruits polycomb repressive complex 2 (PRC2), a transcriptional co-repressor, to repress expression of the homeobox gene D cluster (HOXD) (Rinn et al., 2007). The

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http://dx.doi.org/10.1016/j.toxlet.2015.10.016
0378-4274/

human HOTAIR gene is located in the intergenic region between HOXC11 and HOXC12 in the HOXC cluster on chromosome 12 (Loewen et al., 2014). PRC2 binds to the 5' end of HOTAIR, and its 3' end binds to the LSD1 complex (Tsai et al., 2010; Wu et al., 2013). The PRC2 complex, which is conserved from *Drosophila* to mammals, comprises four components: EZH1/2, SUZ12, EED, and RbAp46/48 (Margueron and Reinberg, 2011). Enhancer of zeste homolog 2 (EZH2) is a histone methyltransferase, which, as a component of PRC2, epigenetically controls gene transcription (Cao et al., 2002). The PRC2 complex is involved in tri-methylation of Lys 27 of histone H3 (H3K27me3), and PRC2–EZH2 establishes cellular H3K27me3 levels through its EZH2-mediated methyltransferase activity (Simon and Kingston, 2009). Although the relationship between HOTAIR/PRC2 and cancer has been investigated (Liu et al., 2013; Ma et al., 2014; Rinn et al., 2007), it remains to be determined if HOTAIR and the composition of PRC2 are modified as a consequence of, or during the processes of, tumorigenesis.

Tobacco use, primarily associated with cigarette smoking, is the largest preventable cause of cancer mortality, being responsible for about one-third of all cancer deaths (Balogh et al., 2014). Approximately 85% of lung cancers result from smoking, with an additional fraction caused in nonsmokers by exposure to second-hand smoke (Warren and Cummings, 2013). In the 1950s, a causal relationship of cigarette smoking with lung cancer was established (Gandini et al., 2008). Although the evidence that cigarette smoke induces lung cancer is strong, the process of malignant transformation of cells remains unclear. Recently, lncRNAs have emerged as regulators of lung cancer and environmental carcinogens (Gao et al., 2013; Sun et al., 2014), however, the molecular mechanisms by which lncRNAs participate in cigarette smoke-induced carcinogenesis remain to be established.

Deregulated cell cycle progression can result in uncontrolled cell proliferation, a main event in neoplastic transformation (Fernandez et al., 2002). Effects on genes related to the cell cycle can result in proliferative advantages and increased susceptibility to the accumulation of additional genetic alterations that contribute to tumor progression and acquisition of more aggressive phenotypes (Malumbres and Barbacid, 2009). The cyclin-dependent kinase inhibitor p21 is a p53-dependent protein implicated in cell cycle checkpoints at the G1 and S phases. By inhibiting activities of cyclin E-CDK2 and cyclin A-CDK2 complexes (el-Deiry et al., 1993), p21 reduces cell cycle progression primarily through inhibition of CDK2 activity (Zhu et al., 2005). The disordered cell cycle mediated by aberrant p21 is involved in cancer progression (Sikdar et al., 2015). The function of p21 in tumor suppression was established in a study demonstrating such its role in a genomically unstable background (Shen et al., 2005), in which cells become more susceptible to malignant transformation. Although our previous studies showed that transformation of human bronchial epithelial (HBE) cells by CSE was mediated by HOTAIR (Liu et al., 2015), the mechanisms involved in the process have not been established.

In the present study, the roles of lncRNAs in regulating the cell cycle and in cigarette smoke extract (CSE)-treated HBE cells were investigated. The results provide a link, through EZH2, between lncRNAs and cell cycle aberrations and present a mechanism for CSE-induced carcinogenesis. They also contribute to an understanding of lung oncogenesis caused by smoking.

2. Materials and methods

2.1. Cell culture and reagents

Simian virus 40 (SV40)-transformed HBE cells are nontumorigenic and retain features of HBE cells. They are useful for studies of multistage bronchial epithelial carcinogenesis (Reddel et al., 1988). These 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). All other reagents used were of analytical grade or the highest grade available.

2.2. Western blots

Total cell lysates were prepared with a detergent buffer, as described previously (Zhao et al., 2013). Protein concentrations were measured with the BCA Protein Assay according to the manufacturer's manual (Beyotime Institute of Biotechnology, Shanghai, China). Equal amounts (80 μg) of protein were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and were transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA). Membranes were incubated overnight at 4 °C with a 1:1000 dilution of anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Sigma), histone3 (H3), and antibodies (Abs) for EZH2 (BD Biosciences, San Diego, USA), H3K27me3 (Millipore), p21, CyclinD1, Cyclin B1, CDK4 (Cell Signaling Technology, Beverly, MA). After additional incubation with a 1:1000 dilution of an anti-immunoglobulin horseradish peroxidase-linked Ab for 1 h, the immune complexes were detected by enhanced chemiluminescence (Cell Signaling Technology). Blots were quantified by densitometry and normalized by use of GAPDH or H3 to correct for differences in loading of proteins. For densitometric analyses, the bands on the blots were measured by Eagle Eye II.

2.3. 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). Primers used: 18s forward, 5′-*GTAACCCGTTGAACCCCATT*-3′, and reverse, 5′-*CCATCCAATCGGTAGTAGCG*-3′; HOTAIR forward, 5′-*CAGTGGGGAACTCTGACTCG*-3′, and reverse, 5′-*GTGCCTGGTGCTCTCTTACC*-3′. PCR was evaluated by checking the products on 2% w/v agarose gels. Bands were quantified by densitometry and normalized by the use of 18S ribosomal RNA to correct for differences in loading. For densitometric analyses, the mRNA bands on the gels were measured by Eagle Eye II.

2.4. Cell Transfection

Transfections of HBE cells were performed with the N-TER™ and AccuTarget TM Nanoparticle siRNA Transfection System (Sigma, BIONEER) following the manufacturer's protocol. Briefly, 5×10^5 cells were seeded into each well of 6-well plates, 18–24 h prior to transfection. The siRNA nanoparticle preparations were made by adding target gene siRNA dilutions to N-TER or AccuTarget peptide dilutions. The preparations were incubated at room temperature for 30 min. NFS transfection medium (2 mL) containing target gene siRNA was transferred to each well of the culture plates, and, after 24 h, cells were treated and harvested for analysis. For cotransfection of EZH2 siRNA and the lncRNA HOTAIR plasmid, cells were seeded into 6-well plates at 5×10^5 cells per well. At 24 h later, cells were cotransfected with the HOTAIR plasmid and EZH2 siRNA as indicated. Control siRNA and EZH2 siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Control siRNA and HOTAIR siRNA were purchased from BIONEER. Control and