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N^6-methyladenosine mediates the cellular proliferation and apoptosis via microRNAs in arsenite-transformed cells

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ABSTRACT

N^6-methyladenosine (m^6A) modification is implicated to play an important role in cellular biological processes, but its regulatory mechanisms in arsenite-induced carcinogenesis are largely unknown. Here, human bronchial epithelial (HBE) cells were chronically treated with 2.5 μM arsenite sodium ($NaAsO_2$) for about 13 weeks and these cells were identified with malignant phenotype which was demonstrated by increased levels of cellular proliferation, percentages of plate colony formation and soft agar clone formation, and high potential of resistance to apoptotic induction. Our results firstly demonstrated that m^6A modification on RNA was significantly increased in arsenite-transformed cells and this modification may be synergistically regulated by methyltransferase-like 3 (METTL3), methyltransferase-like 14 (METTL14), Wilms tumor 1-associated protein (WTAP) and Fat mass and obesity-associated protein (FTO). In addition, knocking down of METTL3 in arsenite-transformed cells can dramatically reverse the malignant phenotype, which was manifested by lower percentages of clone and colony formation as well as higher rates of apoptotic induction. Given the critical roles of miRNAs in cellular proliferation and apoptosis, miRNAs regulated by m^6A in arsenite-transformed cells were analyzed by Venn diagram and KEGG pathway in this study. The results showed that these m^6A-mediated miRNAs can regulate pathways which are closely associated with cellular proliferation and apoptosis, implicating that these miRNAs may be the critical bridge by which m^6A mediates dysregulation of cell survival and apoptosis in arsenite-transformed cells. Taken together, our results firstly demonstrated the significant role of m^6A in the prevention of tumor occurrence and progression induced by arsenite.

1. Introduction

Arsenite distributes ubiquitously in drinking water, food and air, and has a serious threat to about 19.6 million people in China (Rodríguez-Lado et al., 2013). Eepidemiological studies have shown that arsenite is carcinogenic and associated with skin, liver and lung cancers (Oberoi et al., 2014). The carcinogenic mechanism of arsenite has not yet been fully elucidated although plenty of studies have been done on DNA and protein aspects (Reichard and Puga, 2010; Bach et al., 2016; Kim et al., 2015). The strategies that target specific DNA and/or protein were also not fully successful to reverse the tumor occurrence and progression induced by arsenite. This suggested that there are other biological molecules involved in the arsenite carcinogenesis in addition to DNA and protein. RNA, an essential bridge between DNA and protein, has attracted a lot of interest in the process of tumor occurrence and progression in recent years (Luo et al., 2017; Xue et al., 2017). At present, RNA epigenetic has become a fast moving research field in biomedical science and shown a great promise in the preventive and therapeutic strategy for human diseases (Klungland and Dahl, 2014; Wang et al., 2017; Jaffrey and Kharas, 2017; Wei et al., 2017). Among RNA epigenetic, the various RNA modifications, particularly the N^6-methyladenosine (m^6A) is the most attractive and has become a hot spot.

m^6A has been identified as a most abundant and conservative reversible post-transcriptional modification existing in various eukaryotic RNA including mRNA, tRNA, rRNA, pri-microRNA and long non-coding RNA (Yue et al., 2015; Roignant and Soller, 2017). Although discovered in 1974, the physiological significance of m^6A modification on RNA has only been realized in recent years because of the breakthrough findings in m^6A methyltransferases and demethylases (Liu et al., 2014; Jia et al., 2011; Ping et al., 2014; Zheng et al., 2013; Meyer and Jaffrey, 2017). Methyltransferase-like 3 (METTL3), methyltransferase-like 14 (METTL14) and Wilms tumor 1-associated protein (WTAP) form the core methyltransferase complex which catalyzes the formation of m^6A modification in mammalian cells (Liu et al., 2014; Ping et al., 2014; Meyer and Jaffrey, 2017). The m^6A modification can be also removed

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by RNA demethylases, of which the two known ones are Fat mass and obesity-associated protein (FTO) and alkylated DNA repair protein alkB homolog 5 (ALKBH5) (Jia et al., 2011; Zheng et al., 2013; Meyer and Jaffrey, 2017). Currently, accumulating studies have demonstrated that m^6A can regulate a variety of biological events, including tissue development, cell proliferation and apoptosis, stem cell self renewal and differentiation, control of heat shock response, circadian clock, microRNA biogenesis and so forth (Wang et al., 2017; Jaffrey and Kharas, 2017; Wei et al., 2017; Yue et al., 2015; Roignant and Soller, 2017; Alarcón et al., 2015a; Alarcón et al., 2015b). These findings have implicated that m^6A modification may be involved in the disease initiation and progression. Supporting for this speculation, the latest researches show that perturbation of m^6A modification have been involved in type 2 diabetes mellitus, obesity, cancer, infertility, developmental arrest (Jaffrey and Kharas, 2017; Shen et al., 2015; Wei et al., 2017; Wang et al., 2017). Specifically, the role of m^6A in tumor has attracted the most attention and emerging evidence has demonstrated a causative link between m^6A modification and cancer progression (Jaffrey and Kharas, 2017; Wang et al., 2017). For instance, hypoxia-induced the activation of ALKBH5 was required for acquisition of the breast cancer stem cell phenotype (Zhang et al., 2016). Likewise, several components of the m^6A methyltransferase complex, including methyltransferase-like 3 and 14 (METTL3 and METTL14) were all highly expressed in myeloid leukemia and their alterations were linked with poor prognosis (Jaffrey and Kharas, 2017). In addition, METTL14 down-regulation was an adverse prognosis factor for recurrence free survival of hepatocellular carcinoma and notably associated with tumor metastasis in vitro and in vivo (Ma et al., 2017). Also, METTL3 was reported to improve the expression of several vital proteins which can regulate the proliferation, survival, apoptosis and invasion of lung cancer cells (Lin et al., 2016). These studies have suggested that m^6A modification appears to be actively involved in cancer initiation and progression. In view of the fact that a typical phenotype of transformed cells induced by carcinogen is uncontrolled cell proliferation and apoptosis (Li et al., 2012) and that m^6A modification can regulate the survival and apoptosis of cancer cells (Jaffrey and Kharas, 2017; Lin et al., 2016; Ma et al., 2017; Wang et al., 2017; Zhang et al., 2016;), m^6A may also play a regulatory role in the process of cell malignant transformation. However, whether m^6A and its relevant enzymes involve in the process of malignant transformation induced by carcinogen? How does m^6A regulate cellular proliferation and apoptosis during this process? Many unanswered questions remain and are worth to study.

miRNAs are a class of small non-coding RNAs that regulate expression of genes at the post-transcriptional level and thereby influence fundamental biological process including cellular differentiation, proliferation and apoptosis (Paul et al., 2018). So far, about 3000 miRNAs have been identified and these miRNAs have similar biogenesis process (MacDonagh et al., 2015). Latest studies have shown that METTL3, a major enzyme which catalyzes m^6A formation, is sufficient to enhance miRNA maturation in a global and non-cell-type-specific manner, indicating that m^6A may act as a key post-transcriptional mark to promote the initiation of miRNA biogenesis (Alarcón et al., 2015a; Alarcón et al., 2015b). The another key component of methyltransferase complex METTL14 is able to interact with the microprocessor protein and positively modulates miRNA-126 level in an m^6A-dependent manner (Ma et al., 2017). These studies have implicated that m^6A modification is an essential step for determining the biogenesis of different miRNAs. Since studies have demonstrated that miRNAs play an important role in the regulation of cell proliferation and apoptosis (Paul et al., 2018; MacDonagh et al., 2015), m^6A may mediate cellular survival and apoptosis through affecting the miRNA species and levels. However, which miRNAs are regulated by m^6A in arsenite-induced carcinogenesis? And what mechanisms underlying these miRNAs are involved in regulating the arsenite-induced dysregulation of cellular proliferation and apoptosis? All these questions are still largely unknown.

In this study, m^6A modification and its methyltransferases (METTL3, METTL14 and WTAP) were all up-regulated and demethylase (FTO) was down-regulated in arsenite-transformed cells. Upon decreasing the m^6A level by knocking down METTL3 protein, the malignant phenotype induced by arsenite was reversed. Subsequently, nine miRNAs mediated by m^6A in arsenite-transformed cells were identified using Venn diagram and these m^6A-mediated miRNAs may regulate the pathways which were closely related to cellular proliferation and apoptosis, such as p53 signaling pathway, mTOR signaling pathway and so forth. These results together demonstrated that miRNAs may be the important bridge by which m^6A mediates abnormalities of cell survival and apoptosis in arsenite-transformed cells. Collectively, our results demonstrated for the first time that m^6A modification plays a vital role in the malignant transformation induced by arsenite, and this is also the first time to explore the carcinogenic mechanism of arsenite in the area of RNA epigenetic.

2. Materials and methods

2.1. Cell culture

Human bronchial epithelial (HBE) cell line is generously provided by Stem Cells and Tissue Engineering Laboratory, State Key Laboratory of Biotherapy, Sichuan University, China. The cells were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM, Life Technologies/Gibco, Grand Island, NY, USA) supplemented with 10% (v/v) fetal bovine serum, 100 units/mL penicillin and 100 μg/mL streptomycin. Cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 and 95% air.

2.2. Cell treatment and transfection

HBE cells were continuously maintained in the medium containing 0 or 2.5 μM sodium arsenite ($NaAsO_2$, Sigma, St. Louis, MO, purity: 99.0%) for 24 h per passage. This process was continued for 40 passages (about 13 weeks) and these $NaAsO_2$ treatment cells were termed as HBE-T cells. The cell transfection asasy was carried out according to manufacturer's instructions of the reagents of METTL3 siRNA (h) and siRNA negative control (Santa Cruz biotechnology, inc. USA). Briefly, HBE-T cells were seeded in 6-well plates at a density of 2×10^5 per well in antibiotic-free medium. Following overnight incubation, cells were respectively transfected with METTL3-siRNA and its negative control with siRNA transfection reagents (Santa Cruz biotechnology, inc. USA) in fresh medium without serum and antibiotics for 5 h. Transfection medium was then replaced by fresh medium with serum and cells were incubated for additional 72 h and harvested for experiments.

2.3. MTT assay

The cellular proliferation and viability were detected by MTT assay. For the determination of cell proliferation rate, HBE and HBE-T cells were seeded in 96-well plates at 2×10^3 cells/well and incubated overnight, and then cultured for additional 0 h, 24 h, 48 h, 72 h and 96 h. For the detection of cell viability, 1×10^4 cells were seeded in 96-well plates. After attachment, cells were exposed to different concentrations of arsenite sodium (0 μM, 5 μM, 10 μM, 15 μM, 20 μM, 40 μM, 60 μM, 80 μM, 100 μM) for 24 h. At the end of each designed time, cells were incubated with 100 μl of 0.5 mg/mL MTT at 37 °C for additional 4 h in the dark. Subsequently, 100 μl of dimethylsulfoxide was added to dissolve the formazan crystals. Absorbance at 570 nm (A_{570}) was measured with a micro-plate reader (Multiskan™GO, Thermo Fisher Scientific, Inc., Waltham, MA, USA). The cell proliferation rate was calculated using the formula: cell proliferation rate (%) = $T_nA_{570}/T_0A_{570} \times 100\%$. In this formula, T_0A_{570} was the absorbance at 570 nm of cells cultured overnight and T_nA_{570} was the absorbance at 570 nm of cells incubation for 24 h, 48 h, 72 h and 96 h after overnight incubation. The cell viability was calculated using the