Targeting SNHG3/miR-186-5p reverses the increased m6A level caused by platinum treatment through regulating METTL3 in esophageal cancer

Mingxin Zhang1†, Minghua Bai2†, Li Wang3†, Ning Lu1, Jia Wang1, Rong Yan1, Manli Cui1*, Honglin Yan1* and Lingmin Zhang4*

Abstract

Background: Platinum-based chemotherapy is a mainstay for treating esophageal cancer patients. In this manuscript, we have provided clues for influence of platinum on overall m6A level and further investigated the potential regulatory mechanism.

Methods: qRT-PCR was used to measure SNHG3 and miR-186-5p expression; ELISA and western blot were used to measure the expression of METTL3. CCK8 was used to measure the cell proliferation rate. Caspase 3/7 activity was used to measure the apoptosis rate. Dual luciferase reporter gene assay and RNA pull down assay were used to investigate the potential crosstalk between miR-186-5p and SNHG3; and miR-186-5p and METTL3.

Results: m6A level was increased when treated with platinum (CDDP, CPB and L-OHP). Besides, SNHG3 expression was induced and miR-186-5p expression was suppressed by platinum. Furthermore, SNHG3 could promote the m6A level, however miR-186-5p inhibited the m6A level through targeting METTL3. SNHG3 interacts with miR-186-5p to negatively regulate the expression of miR-186-5p; and miR-186-5p might bind to the 3′UTR of METTL3 to regulate its expression.

Conclusion: Platinum can increase the overall m6A level of esophageal cancer. SNHG3/miR-186-5p, induced by platinum, was involved in regulating m6A level by targeting METTL3. Our manuscript has provided clues that regulating m6A level might be a novel way to enhance the platinum efficacy.

Keywords: m6A level, METTL3, SNHG3, miR-186-5p, Platinum

Introduction

Esophageal carcinoma is one of the most common malignant tumors. It ranks the seventh in incidence and sixth in mortality all over the world [1]. In China, esophageal carcinoma is the leading cause of cancer death [2]. Esophageal cancer is mainly composed of two pathological types (adenocarcinoma and squamous cell carcinoma), which have significant different etiologies and treatment strategies.

Platinum-based drug plus 5-fluorouracil (FP) is the first line regimen for esophageal squamous cell carcinoma,
especially for patients at advanced stages. It was reported that paclitaxel plus lobaplatin showed satisfying therapeutic efficiency and less toxicity in ESCC patients [3]. Platinum-based drugs include carboplatin, cisplatin and oxaliplatin [4], which target the DNA to interfere the cell cycle progression and replication, by forming adducts to DNA strand crosslinks [4]. Although platinum-based agents are widely applied in the clinical treatment in ESCC, platinum resistance frequently leads to local recurrence and poor prognoses of patients. Therefore, it is urgent to explore the specific mechanisms of platinum resistance in ESCC.

N6-methyladenosine (m6A) is one of the most abundant RNA modification types in mammals [5]. m6A is induced by the methyltransferase complex, including METTL3–METTL14 and erased by demethylases, including ALKBH5 and FTO [6]. Accumulating evidence has shown that LncRNAs plays important roles in regulating various biological functions of cancers, including chemotherapy resistance [11]. SNHG3 was previously found to exert oncogenic roles in a plethora of cancers: Based on current studies, SNHG3 was involved in TGF-β, NOTCH, JAK2/STAT3 and HGF pathway to promote cancer cell proliferation, invasion and inhibit apoptosis rate [12–14]. Besides, Xuan et al. has presented evidences that SNHG3 interacts with EZH2 to regulate the methylation status of MED18 and finally suppress MED18 expression, leading to gastric cancer progression [15]. However, the specific role of SNHG3 in esophageal cancer was not reported yet. In this manuscript, we hypothesized that SNHG3 down regulated in esophageal cancer tissues and cells, and the m6A level in ESCC cells. And we hypothesized that enhanced m6A level might result in platinum resistance. SNHG3 was previously found to exert oncogenic roles in a plethora of cancers: Based on current studies, SNHG3 was involved in TGF-β, NOTCH, JAK2/STAT3 and HGF pathway to promote cancer cell proliferation, invasion and inhibit apoptosis rate [12–14]. Besides, Xuan et al. has presented evidences that SNHG3 interacts with EZH2 to regulate the methylation status of MED18 and finally suppress MED18 expression, leading to gastric cancer progression [15]. However, the specific role of SNHG3 in esophageal cancer was not reported yet. In this manuscript, we hypothesized that SNHG3 down regulated in esophageal cancer tissues and cells, and the m6A level in ESCC cells. And we hypothesized that enhanced m6A level might result in platinum resistance.

**Method**

**Patients**

348 esophageal cancer patients were recruited from 2013 to 2015 in First affiliated hospital of Xi’an medical University. All patients were pathologically diagnosed after esophagectomy. All patients were informed of cancer and normal esophageal tissues and signed the consent. This study meets the standard of Helsinki declaration and is approved by First affiliated hospital of Xi’an medical University.

**Cell culture and transfection**

KYSE-150 and Eca-9706 were purchased from Cell Bank of Chinese Academy of Sciences (Shanghai, China). RPMI 1640 supplemented with 10% fetal bovine serum (Gibco, USA) were used for cell culture. The culture condition is 5% CO2 and 37 °C. Cells were transfected with SNHG3 down regulated lentivirus, METTL3 and miR-186-5p up and down regulated lentivirus, which were purchased from Genechem (Shanghai, China) according to the manufacturer’s instructions.

**ELISA**

SimpleStep ELISA® (ab270552, Abcam, Shanghai, China) was purchased to perform ELISA tests. Around 1 × 10⁶ cells were used to resuspend at 3 mL medium; 50 μL cell lysates were added at each well for evaluation. 50μL METTL3 antibody (1:1000, Invitrogen, Shanghai, China) Then 100 μL detection reagent was added and incubated for 15 min at room temperature. The absorbance results were read at 450 nm.

**qRT-PCR**

Total RNA form esophageal cancer patients and cells were extracted with TRIzol reagent (Invitrogen, Shanghai, China). We used ReverTra Ace (Toyobo Co., Ltd., Osaka, Japan) and LightCycler 2.0 (Roche Molecular Systems, Inc., Pleasanton, CA, USA) were used to perform the qPCR process. 2−ΔΔCt method was used to calculate the relative expression. GADPH and U6 were used as the control.
Western blot
Protein samples were obtained from KY-SE150 and Eca-9706 cell lines. The extracted protein samples were then suspended with loading buffers and deployed on the SDS-PAGE. After that, they were transferred onto PVDF membranes. In the end the blotting was visualized by ECL. The primary antibody of METTL3 was purchased from Abcam.

Cell proliferation ability
Cell viability was evaluated using a CCK-8 assay. Cells were resuspended and plated in a 96-well plate at the concentration of $1 \times 10^5$. As for treatment group, platinum (IC50) was added to treatment group and incubated for 24 h. After cultured for 24, 48, 72, 96 and 120 h respectively, 10 µl CCK-8 was added to each well and incubated for 2 h; PBS was used as the blank control. The absorbance was detected at 450 nm.

Cell apoptosis
FAM-FLICA® Caspase-3/7 Assay Kit was used to detect the apoptosis rate. 1:35 FLICA was added and incubated for 1 h according to the manufacturer's guidelines. The caspase3/7 activity was evaluated by the fluorescence microscope (FAM-FLICA excites at 492 nm and emits at 520 nm).

Dual luciferase reporter gene assay
The pGL3-SHG3 and pGL3-METTL3 promoter (Promega, MA, USA) was transfected with pcDNA3.1/MIR-186-5p and pcDNA3.1 vector or sh-MIR-186-5p and sh-NC into KY-SE150 cell lines. The wild-type (WT) and mutant (Mut) binding sites of SNHG3 or METTL3 sequence was cloned into pmirGLO luciferase vector (Promega) to construct SNHG3 or METTL3-Wt and SNHG3 or METTL3-Mut, which were then co-transfected respectively with miR-186-5p mimics or NC mimics into KY-SE150 cell line. The luciferase activity was detected using Dual-Luciferase Reporter Assay System (Promega).

RNA pull-down assay
The miR-186-5p-WT, miR-186-5p-Mut and NC were synthesized and biotin labeled by Genecreate (Wuhan, China). Then target cells were incubated with streptavidin-coated magnetic beads with RNase free bovine serum albumin for 48 h. Pull-down assay was carried out in biotin-coupled RNA complex. qRT-PCR was used to detect the expression of SNHG3.

Xenografts
We have recruited 10 nude mice who were supplied by Animal center of Xi’an Jiao Tong University. We used $1 \times 10^6$ SNHG3 knocked down KY-SE150 cells and NC lentivirus to inject into the right flank of mice to generate xenografts. Four weeks later, xenograft tumors were harvested for further analysis.

Statistics
GraphPad Prism 8.2.1 was used for statistically analysis and data visualization (La Jolla, CA, USA). Student’s t test and one-way ANOVA were used to test the difference among different groups. P < 0.05 was considered as statistically significance. All experiments were repeated six times.

Result
SNHG3, miR-186-5p and m6A associated enzymes expression esophageal cancer
qRT-PCR showed that SNHG3 was highly expressed and miR-186-5p was lowly expressed in 348 esophageal cancer patients compared with their normal esophageal tissues; moreover, we noticed that SNHG3 expressed highly in recurrent esophageal cancer patients, however, miR-186-5p expressed lowly in recurrent esophageal cancer patients (Fig. 1a–d). Then, ELISA result showed that METTL3 and METTL14 were highly expressed in esophageal cancer, whereas FTO and ALKBH5 expressed lowly in esophageal cancer (Fig. 1e). According to Fig. 1f, we found that METTL3 expressed highly in recurrent esophageal cancer patients, however, METTL14, FTO and ALKBH5 were not differentially expressed between recurrent and non-recurrent esophageal cancer patients. After that, we have found that SNHG3 was highly expressed and miR-186-5p was lowly expressed in esophageal cancer in vitro (Fig. 1g, h).
Cisplatin (CDDP), carboplatin (CPB) and oxaliplatin (L-OHP) could enhance the m6A level through up regulating SNHG3, miR-186-5p and METTL3

The IC50 for CDDP is 0.76 μg/mL in KY-SE150 and 0.72 μg/mL in Eca-9706 (Fig. 2a). The IC50 for CPB is 29.53 μg/mL (KY-SE150) and 23.17 μg/mL (Eca-9706) (Fig. 2b). Besides, the IC50 for oxaliplatin is 3.19 μg/mL (KY-SE150) and 3.03 μg/mL (Eca-9706) (Fig. 2c). Then we treated KY-SE150 with IC50 for CDDP, CPB and L-OHP respectively for 24 h. After that, we found that CDDP, CPB and L-OHP could enhance the m6A level in KYSE-150 and Eca-9706 (Fig. 2d–f).

In KY-SE150 and Eca-9706, after treated with CDDP, we have found increased expression of SNHG3 and METTL3, but miR-186-5p expression was inhibited (Fig. 2g–i). Besides, CPB and L-OHP induced the expression of SNHG3 and METTL3, and suppressed the expression of miR-186-5p as well (Fig. 2j–o).

SNHG3 and miR-186-5p regulates the m6A level of esophageal cancer by targeting METTL3

We have successfully knocked down SNHG3 in both KY-SE150 and Eca-9706 (Fig. 3a).

After down-regulating SNHG3, we have found suppressed m6A level, down-regulated METTL3 and up-regulated miR-186-5p (Fig. 3b–d). Moreover, miR-186-5p overexpression resulted in suppressed m6A level and down-regulated METTL3 as well (Fig. 3e–g), however, the expression of SNHG3 was not significantly influenced (Fig. 3h). Then we treated SNHG3 down regulated KYSE-150 cell with CDDP, CPB and L-OHP, m6A level could be rescued by SNHG3 down regulated (Fig. 3i). Besides, increase in m6A level caused by platinum could be rescued by miR-186-5p overexpression and METTL3 knock down. (Fig. 3j, k). Therefore, we hypothesized that SNHG3 and miR-186-5p could regulate m6A level by targeting METTL3.

SNHG3 promotes esophageal cancer proliferation and inhibits apoptosis by targeting miR-186-5p

In KYSE-150 and Eca-9706, we knocked down SNHG3 and found decreased proliferation rate and increased apoptosis rate (Fig. 4a–d). Besides, miR-186-5p overexpression led to decreased proliferation rate and increased apoptosis rate as well (Fig. 4e–g). Then, rescue experiments showed that the proliferation and apoptosis rate is not significantly different between miR-186-5p knock down plus SNHG3 knock down and control group (Fig. 4h, i).

SNHG3 directly interacts with miR-186-5p

Previously, we have shown that SNHG3 knock down could lead to overexpression of miR-186-5p (Fig. 3c). Then Dual luciferase Reporter gene assay showed that relative luciferase activity was significantly lower in SNHG3-WT group than that in SNHG3-Mut group (Fig. 5a; Supplementary Figure1). We then transfected SNHG3-Mut and SNHG3-Mut in KYSE-150 respectively and found that miR-186-5p was significantly suppressed in SNHG3-Mut group but not significantly influenced in SNHG3-Mut group (Fig. 5b, c). Furthermore, we have performed RNA-pull down assay and found that SNHG3 was significantly enriched in miR-186-5p-WT group (Fig. 5d). Therefore, we assumed that SNHG3 directly interacts with miR-186-5p.

miR-186-5p binds to the 3’UTR of METTL3 to inhibit its expression

We found that miR-186-5p overexpression resulted in inhibited expression of METTL3, however, miR-186-5p knock down led to overexpression of METTL3 (Fig. 6a, b). Further dual luciferase reporter gene assay showed that relative luciferase activity was significantly suppressed in METTL3-WT group than that in METTL3-Mut group (Fig. 6c; Supplementary Figure 2). Then we have knocked miR-186-5p and METTL3 in the meantime, and found that METTL3 expression was not significantly different from that in control group (Fig. 6d). These results indicated that SNHG3 could interact with miR-186-5p to regulate the expression of METTL3.

After that, we noticed that SNHG3 knock down could decrease the xenografts growth in vivo (Fig. 6e, f). Then we harvested the xenografts and found aberrant high expression of miR-186-5p and low expression of METTL3 in SNHG3 knocked down xenografts (Fig. 6g, h).
Discussion

It was reported that m6A methylation took part in various biological functions, including chemotherapy resistance, by modifying target RNAs [7]. It is found 0.1%-0.4% of adenosines from isolated RNA are modified by m6A, which accounts for 50% of total methylated ribonucleotides [16]. Previous studies have demonstrated that m6A methylation affects the physiological processes, including DNA damage repair, embryogenesis, heat shock responses, metastasis and proliferation [17–20]. In this manuscript, we found that platinum (CDDP, CPB and L-OHP) significantly induced the m6A level in KY-SE150 and Eca-9706 cell lines. Therefore, we assume that m6A regulation might be a novel way to control platinum resistance.

Furthermore, our study indicated that although METTL3, METTL14, FTO and ALKBH5 differentially expressed in esophageal cancer patients’ tissues and normal esophageal tissues, only METTL3 expression was related to esophageal cancer recurrence. It is reasonable to hypothesize that METTL3 might be key platinum resistance gene. In previous study, Taketo et.al
showed that METTL3 knockdown sensitized pancreatic cancer to multiple anti-cancer reagents, including gemcitabine, 5-fluorouracil, cisplatin and irradiation as well [21]. In esophageal carcinoma, FTO [a demethylates N(6)-methyladenosine (m6A) RNA] and ALKBH5 were risk factors for poor prognoses as well [21]. It was shown that ALKBH5 knockdown could suppress proliferation and migration of ESCC cells [22]. Yang et al. found that knockdown of FTO could sensitize tumors to anti-PD-1 treatment [23]. Yan et al. found that overexpression of FTO could lead to tyrosine kinase inhibitor resistance in leukemia cells [8].

Moreover, we have found aberrant high expression of SNHG3 and low expression of miR-186-5p in esophageal cancer both in vitro and in vivo. We assumed that SNHG3/miR-186-5p played important role in regulating esophageal cancer progression. More importantly, we found that SNHG3/miR-186-5p expression was associated with m6A level in esophageal cancer. Further mechanism study showed that SNHG3 directly interacted with miR-186-5p to regulate the expression of METTL3. SNHG3 has been found to be necessary for cell growth: SNHG3 is vital for keeping embryonic stem cells’ self-renewal and pluripotency in embryonic stem cells [24]. Lu et al. stated that SNHG3 expression was higher in highly metastatic HCC cells (HCCLM3) than lowly metastatic HCC cells, such as Hep3B and PLC/PRF/5. Besides, SNHG3 also plays important roles in regulating drug resistance: high SNHG3 expression leads to poor survival and sorafenib resistance in hepatocellular carcinoma [25]. Fei et al. reported that in glioma SNHG3 overexpression could promote the cells proliferation, inhibit apoptosis rates, and accelerate the cell cycle progress by recruiting enhancer of zeste homolog 2 to the promoter of KLF2 and p21 [26]. Previous studies have indicated that SNHG3 could acted as ceRNA to regulate cancer biological process through binding microRNA [27]. For example, SNHG3 could promote laryngeal carcinoma proliferation and migration by binding miR-384 [27]. In osteosarcoma, SNHG3 could promote the progression of SNHG3 by absorbing miRNA-151a-3p and then upregulating RAB22A expression [28]. In this study, we assumed that SNHG3 could regulate the expression of METTL3 by sponging miR-186-5p. With the help of Dual Luciferase Reporter gene assay and RNA pull down assay, we found that SNHG3 could directly interact with miR-186-5p, which could bind to METTL3 to suppress its expression.
Conclusion
We found that overall m6A level of esophageal cancer can be increased by platinum treatment, which would be an essential and important aspect for clarifying the molecular mechanism for platinum resistance. Furthermore, SNHG3/miR-186-5p, which can be induced by platinum, played an important part in involved in regulating overall m6A level by targeting METTL3 (Additional files 1, 2).

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s12935-021-01747-9.

Additional file 1. Figure S1: Luciferase activity for SNHG3 and miR-196-3p
Additional file 2. Figure S2: Luciferase activity for METTL3 and miR-196-3p

Abbreviations
CDDP: Cisplatin; CPB: Carboplatin; L-OHP: Oxaliplatin; m6A: N6-methyladenosine; ESCC: Esophagal squamous cell carcinoma.
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Authors’ contributions
MZ and YX wrote the manuscript and performed the most experiments. NL and JW assisted in performing the experiments. Ry, MC and Hy were in charge of clinical parts. Lz sponsored and designed the study. All authors read and approved the final manuscript.

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Availability of data and materials
Data and materials would be made available on request.

Ethics approval and consent to participate
Patients recruited have signed the informed consent. This study met the requirement of the Declaration of Helsinki and was approved by the Ethics Committee of the First Affiliated Hospital of Xi’an Medical University.

Consent for publication
All the authors listed have approved the manuscript that is enclosed.

Competing interests
All the authors declared no conflicts of interest.

Author details
1 Department of Gastroenterology, The First Affiliated Hospital of Xi’an Medical University, No. 48 Feng Hào West Road, Xi’an 710077, Shaanxi, China. 2 Department of Health, Liaocheng People’s Hospital, Liaocheng 252000, Shandong, China. 3 Department of Scientific Research, The First Affiliated Hospital of Xi’an Medical University, Xi’an, Shaanxi, China. 4 Department of Anesthesiology, The First Affiliated Hospital of Xi’an Jiaotong University, No. 277 Yanta West Road, Xi’an 710061, Shaanxi, China.

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