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Let-7a inhibits proliferation and induces apoptosis by targeting EZH2 in nasopharyngeal carcinoma cells

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Abstract. Let-7a is frequently downregulated in various types of human cancer including nasopharyngeal carcinoma. However, the underlying mechanism of let-7a action in nasopharyngeal carcinoma remains elusive. In this study, we show that the enhancer of zeste homolog 2 (EZH2) is a direct target of let-7a in human nasopharyngeal carcinoma cells. The inhibition of EZH2 *in vitro* by let-7a, EZH2 siRNA, attenuated nasopharyngeal carcinoma cell growth, inhibited cell proliferation and induced cell apoptosis. In addition, for each biological process we identified ontology-associated transcripts that significantly correlate with EZH2 expression. Finally, the expression of EZH2 significantly abrogated let-7a-mediated cell proliferation and apoptosis in the nasopharyngeal carcinoma cells. Taken together, our results suggest that let-7a and EZH2 may be potential therapeutic targets for nasopharyngeal carcinoma.

Introduction

Nasopharyngeal carcinoma is a common head and neck malignancy. Despite recent advances in cancer treatment, the prognosis for patients with nasopharyngeal carcinoma, particularly for those at the advanced stages of the disease, remains poor. Therefore, it is essential to investigate the mechanisms involved in the development and progression of nasopharyngeal carcinoma.

microRNAs (miRNAs) comprise a large group of endogenous non-coding RNAs that can block mRNA translation onegatively regulate mRNA stability and thereby play a central role in the regulation of gene expression. miRNAs can function as oncogenic miRNAs or tumor suppressor miRNAs, playing crucial roles in the development and progression of cancer (1,2). Recent studies have indicated that deregulated miRNA expression is a common feature of human nasopharyngeal carcinoma (3-5). Let-7a has been shown to be significantly downregulated in human nasopharyngeal squamous cancer tissues, and functions as a potential tumor suppressor in human nasopharyngeal cancer (6). However, the involvement of deregulated miRNAs in nasopharyngeal carcinoma remains unknown and warrants further investigation.

In the present study, we determined the function of let-7a in nasopharyngeal carcinoma and investigated the mechanims involved. Our results demonstrate that the enhancer of zeste homolog 2 (EZH2) expression in nasopharyngeal carcinoma is directly and negatively regulated by let-7a. Let-7a is downregulated in nasopharyngeal carcinoma cells, resulting in increased EZH2 expression, thus leading to enhanced proliferation and the inhibition of apoptosis in nasopharyngeal carcinoma cells.

Materials and methods

Clinical sample collection. The 10 nasopharyngeal carcinoma tissues used in this study were obtained from the Taizhou People's Hospital in China. Specimens were snap-frozen in liquid nitrogen. Fresh normal nasopharyngeal mucosal tissues were obtained from another 5 patients undergoing biopsies for other non-neoplastic diseases. The collection and use of the patient samples were reviewed and approved by the Institutional Ethics Committees and written informed consent from all patients was appropriately obtained.

In silico analysis. mRNA expression profiling containing 31 nasopharyngeal carcinoma and 10 normal tissues samples (GSE12452) was carried out (http://www.ncbi.nlm.nih.gov/

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geo/query/acc.cgi?acc=GSE12452). Using the AmiGO tool (7) of the Gene Ontology project (8), lists of transcripts associated with the biological processes, proliferation (GO:0008283) and apoptosis (GO:0006915), were obtained. Lists of unique transcripts were prepared by removing duplicate entries. Subsequently, the microarray dataset was queried for the genes in each of these ontologies. Samples were sorted according to the EZH2 expression level for the nasopharyngeal carcinoma and 10 normal tissues samples separately and the average expression levels scaled on a gene by gene basis for genes significantly correlating with EZH2 expression (absolute value Pearson's correlation >0.6) were plotted as a heatmap.

Cell culture and transfection. CNE-2 cells maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum. Hsa-let-7a, siEZH2 and negative control oligonucleotides were purchased from GenePharma Co., Ltd. (Shanghai, China). For the expression plasmid construct, wild-type EZH2 cDNA sequence without 3'UTR was selected and cloned into the pGenesil-1 vector. Cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) at 50-60% confluency. Forty-eight hours after transfection, the cells were harvested for further experiments.

Quantitive RT-PCR. Real-time quantification of hsa-let-7a was performed by stem-loop RT-PCR. All the primers of miRNAs for TaqMan miRNA assays were purchased from GenePharma Co., Ltd. and were as follows: human EZH2 forward, 5'-CCTG AAGATGTCGGCATCGAAAGAG-3' and reverse, 5'-TGCAA AAATTCACTGGTACAAAACACT-3'; and human GAPDH forward, 5'-GTCGGAGTCAACGGATT-3' and reverse, 5'-AAG CTTCCCGTTCTCAG-3'. Real-time PCR was performed according to the manufacturer's instructions. All experiments were performed using biological triplicates and experimental duplicates. The relative expression was calculated via the 2-ΔΔCt method.

MTT assay. Cells were plated at 10^4 cells/well in 96-well plates with 6 replicate wells. After transfection as previously described, 20 μl of MTT (5 g/l, Sigma, St. Louis, MO, USA) were added into each well on 4 consecutive days after treatment and the cells were incubated for an additional 4 h. The supernatant was subsequently discarded. DMSO (200 μl) was added to each well to dissolve the precipitate. Optical density (OD) was measured at a wavelength of 550 nm. The data are presented as the means ± SD, which are derived from triplicate samples of at least 3 independent experiments.

Cell cycle analysis. Cells were washed with PBS and fixed with 70% ethanol for at least 1 h. After extensive washing, the cells were suspended in Hank's balanced salt solution (HBSS) containing 50 μg/ml PI and 50 μg/ml RNase A and incubated for 1 h at room temperature and analyzed by FACScan (Becton-Dickinson, Franklin Lakes, NJ, USA). Cell cycle analysis was analyzed by ModFit software. Experiments were performed in triplicate. The results were presented as a percentage of cells in a particular phase.

Cell apoptosis assay. Cells were plated into 6-well plates at $1x10^5$ cells/well. Forty-eight hours after transfection, the cells were harvested by trypsinization and washed with PBS. Annexin V and PI double-staining (BD Biosciences, San Jose, CA, USA) were used to detect and quantify cellular apoptosis by flow cytometry. Annexin V^- and PI^- cells were used as the controls. Annexin V^+ and PI^- cells were designated as apoptotic; Annexin V^+ and PI^+ cells were considered necrotic. All the tests were performed in triplicate.

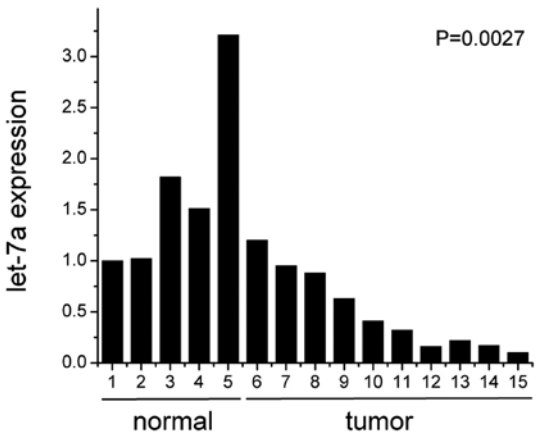

Figure 1. Let-7a expression in nasopharyngeal carcinoma. Real-time PCR was employed to determine the expression of let-7a in human nasopharyngeal carcinoma specimens and normal nasopharyngeal mucosal tissues.

Western blot analysis. Equal amounts of protein per lane were separated by 8% SDS-polyacrylamide gel and transferred onto a PVDF membrane. The membrane was blocked in 5% skim milk for 1 h and then incubated with a specific antibody for 2 h. The antibodies used in this study were as follows: antibodies to EZH2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The antibody against GAPDH (Santa Cruz Biotechnology) was used as the control. The specific protein was detected by using a SuperSignal protein detection kit (Pierce, Rockford, IL, USA). The band density of specific proteins was quantified after normalization with the density of GAPDH.

Luciferase reporter assay. The human EZH2 3'UTR were amplified and cloned into the *Xba*I site of the pGL3-control vector (Promega, Madison, WI, USA), downstream of the luciferase gene, to generate the plasmids pGL3-WT-EZH2-3'UTR. pGL3-MUT-EZH2-3'UTR plasmids were generated from pGL3-WT-EZH2-3'UTR by deleting the binding site for let-7a 'CUACCUC'. For the luciferase reporter assay, cells were cultured in 96-well plates, transfected with the plasmids and let-7a using Lipofectamine 2000. Forty-eight hours after transfection, luciferase activity was measured using the Luciferase assay system (Promega).

Statistical analysis. Statistics was determined by ANOVA, or the t-test using SPSS11.0. A value of $P<0.05$ was considered to indicate a statistically significant difference.

Results

Let-7a expression in nasopharyngeal carcinoma. To explore let-7a expression in nasopharyngeal carcinoma, we examined

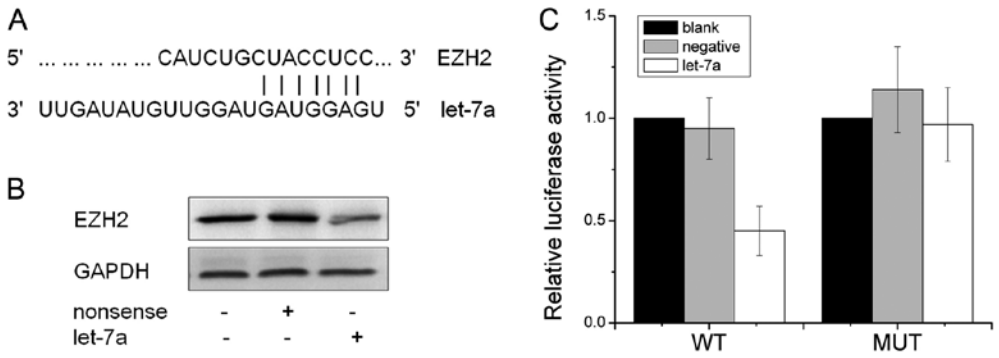

Figure 2. EZH2 was identified as a target gene of let-7a. (A) A schematic representation showing the putative target site for let-7a in the 3'UTR of EZH2 mRNA. (B) Cells were transfected with let-7a and the expression of EZH2 was analyzed by western blot analysis. The expression of GAPDH was used as the loading control. (C) Luciferase constructs were transfected into cells transduced with let-7a. Luciferase activity was determined 48 h after transfection. The ratio of normalized sensor to control luciferase activity is shown. Data are expressed as the means ± SD of 3 independent experiments.

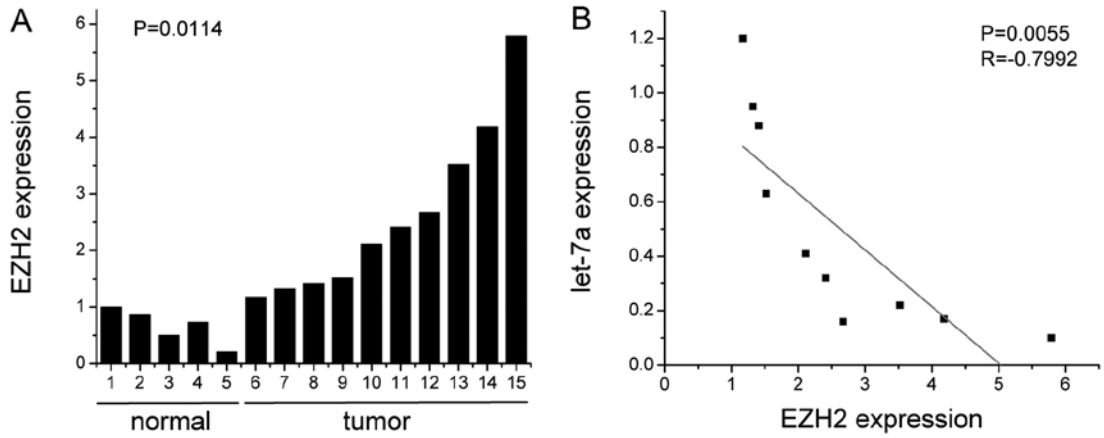

Figure 3. Let-7a inversely correlates with EZH2 expression in nasopharyngeal carcinoma tissues. (A) Expression levels of EZH2 in nasopharyngeal carcinoma tissues were measured by real-time PCR and quantified as described in Materials and methods. (B) Inverse correlation of let-7a expression with EZH2 expression in nasopharyngeal carcinoma tissues by Pearson's correlation analysis.

10 human nasopharyngeal carcinoma specimens and 5 normal nasopharyngeal mucosal tissues using real-time PCR. As shown in Fig. 1, the levels of let-7a decreased markedly in the nasopharyngeal carcinoma in comparison to the normal tissue samples (P<0.01).

EZH2 is a direct target of let-7a. By performing bioinformatics analysis of let-7a potential target genes, we found that the tumor suppressor EZH2 contained the highly conserved putative let-7a binding sites. To determine whether EZH2 is directly regulated by let-7a (Fig. 2A), western blot analysis and luciferase reporter assay were employed. Western blot analysis demonstrated a significant reduction in EZH2 expression following the overexpression of let-7a in the CNE-2 cells (Fig. 2B). Furthermore, we created the pGL3-WT-EZH2-3'UTR and pGL3-MUT-EZH2-3'UTR plasmids. Reporter assay revealed that the overexpression of let-7a triggered a marked decrease in the luciferase activity of the pGL3-EZH2-EZH2-3'UTR plasmid in the CNE-2 cells; however, no change in the luciferase activity of the pGL3-MUT-EZH2-3'UTR plasmid was observed (Fig. 2C).

Inverse correlation of expression of let-7a and EZH2 in nasopharyngeal carcinoma tissues. We further explored the correlation between let-7a and EZH2 expression in nasopharyngeal carcinoma. We examined 10 human nasopharyngeal carcinoma specimens using real-time PCR. The levels of EZH2 increased markedly in the nasopharyngeal carcinoma in comparison to the normal tissues (P<0.02) (Fig. 3A). Additionally, Pearson's correlation showed that a significant negative correlation existed between let-7a and EZH2 expression in the nasopharyngeal carcinoma tissues (R=-0.7992, P<0.01) (Fig. 3B). These data indicate that EZH2 is a direct target of let-7a in nasopharyngeal carcinoma.

Let-7a-mediated EZH2 expression affects cell proliferation in nasopharyngeal carcinoma. To determine the EZH2-mediated effects of let-7a on nasopharyngeal carcinoma cell proliferation, we first analyzed which genes associated with cell proliferation correlated with EZH2 expression in nasopharyngeal carcinoma. First, EZH2 was found to be overexpressed in the majority of the nasopharyngeal carcinoma samples as compared to the normal tissues. However, in a few samples the EZH2 mRNA expression was found to be in the same range as in the normal tissues (Fig. 4A). Out of the 1,134 genes that were linked to cell proliferation as determined by AmiGO (7), 57 genes showed a clear correlation (>60%) with EZH2

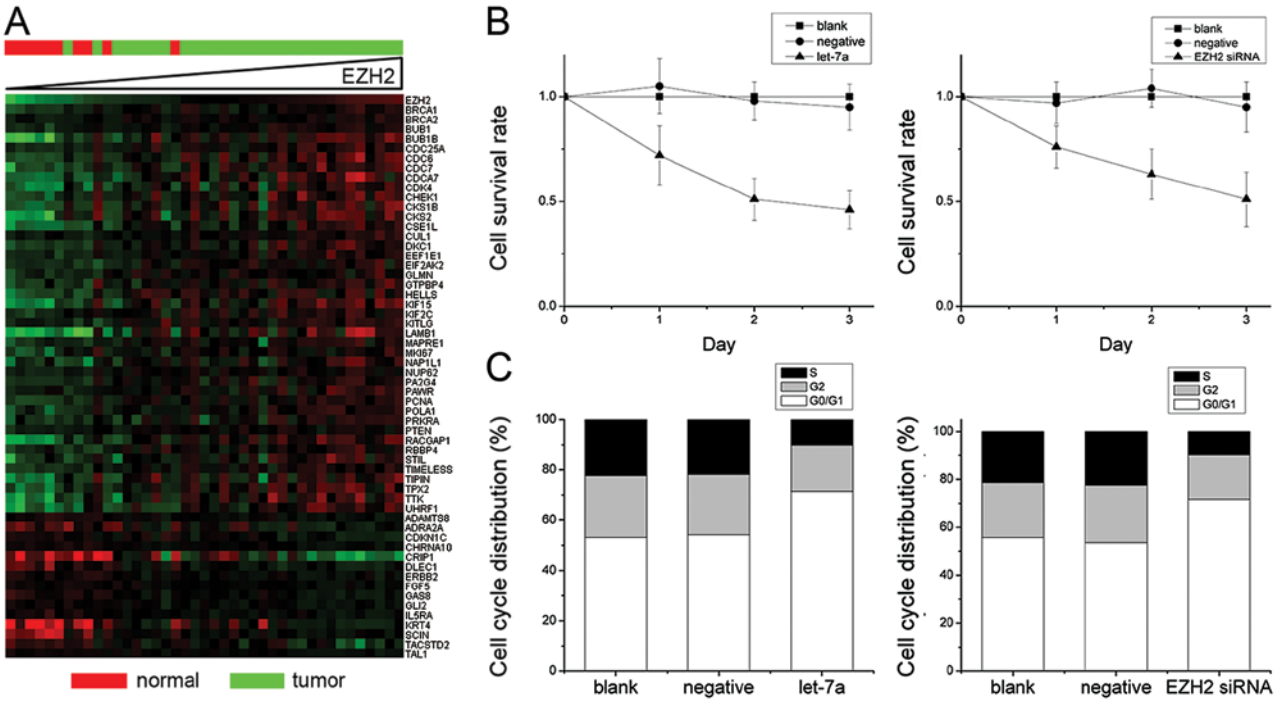

Figure 4. Let-7a EZH2 affects cell proliferation *in vitro*. (A) *In silico* analysis of EZH2 mRNA expression and the correlation to proliferation-related mRNAs. Heatmap of percentile fold-change of gene expression of proliferation-related genes sorted by correlation with EZH2 expression (rows) in patients sorted by level of EZH2 expression (columns). (B) MTT assay displayed that cells treated with let-7a or EZH siRNA proliferated at a significantly lower rate than control groups after transfection. (C) After 48-h treatment, cells were harvested and performed by cell cycle assay. Data are presented as the means of triplicate experiments.

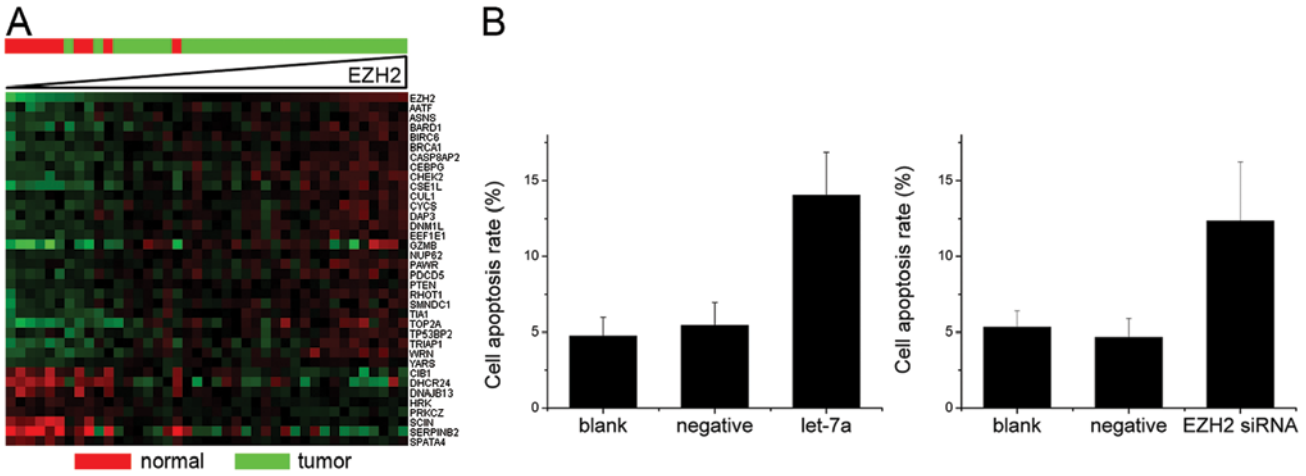

Figure 5. Let-7a EZH2 affects cell apoptosis *in vitro*. (A) *In silico* analysis of EZH2 mRNA expression and the correlation to apoptosis-related mRNAs. Heatmap of percentile fold-change of gene expression of apoptosis-related genes sorted by correlation with EZH2 expression (rows) in patients sorted by level of EZH2 expression (columns). (B) After 48-h treatment of let-7a or EZH2 siRNA, cells were harvested and performed by cell cycle assay. Data are presented as the means of triplicate experiments.

expression in nasopharyngeal carcinoma (Fig. 4A). Of note, the nasopharyngeal carcinoma samples with normal EZH2 expression levels also showed a decreased expression of the genes associated with cell proliferation. Cellular proliferation was then examined in nasopharyngeal carcinoma cell cultures in order to determine whether EZH2 influences the proliferation of nasopharyngeal carcinoma cells. Let-7a induction and EZH2 knockdown by siRNA significantly reduced cellular proliferation in CNE-2 cells (Fig. 4B). Furthermore, the let-7a-treated cells demonstrated a significant increase in the number of cells in the G0/G1 phase in comparison to the untreated CNE-2 cells (Fig. 4C).

Let-7a-mediated EZH2 expression affects cell apoptosis in nasopharyngeal carcinoma. To determine the EZH2-mediated effects of let-7a on nasopharyngeal carcinoma cell apoptosis, we analyzed which genes associated with apoptosis correlated with EZH2 expression in the nasopharyngeal carcinoma and

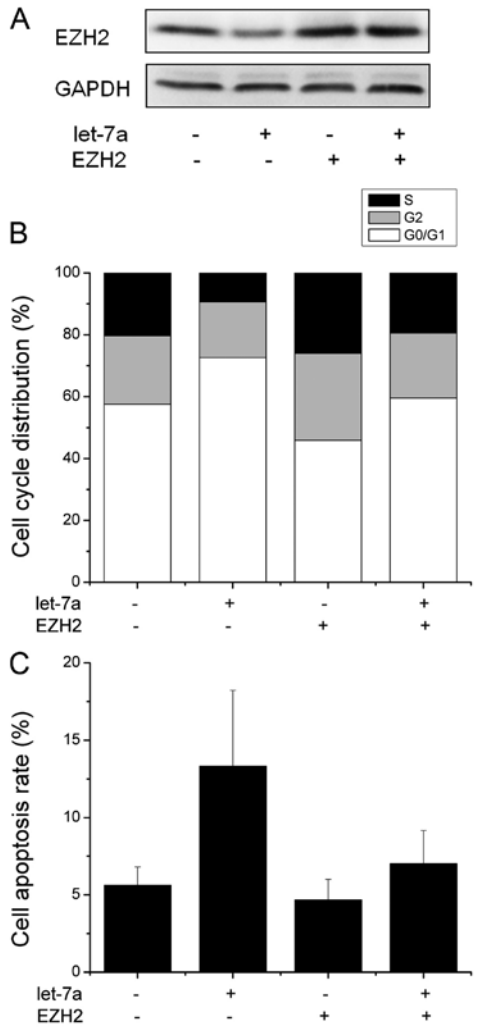

Figure 6. Expression of EZH2 abrogates let-7a-mediated cell proliferation and apoptosis. (A) The cells were transfected with pcDNA-EZH2 (without the 3'UTR) and let-7a, and the EZH2 protein level was detected by western blot analysis. GAPDH protein was regarded as endogenous normalizer. (B) The cells were transfected with pcDNA-EZH2 (without the 3'UTR) and let-7a, and cell cycle assay was then performed. (C) The cells were transfected with pcDNA-EZH2 (without the 3'UTR) and let-7a, and cell apoptosis assay was then performed. Data are expressed as the means ± SD of 3 independent experiments.

normal tissues. A significant correlation between the expression of 35 out of 424 genes associated with cell apoptosis and EZH2 expression was observed (Fig. 5A). In order to determine whether the let-7a upregulation or EZH2 inhibition also affected nasopharyngeal carcinoma cell apoptosis, Annexin V and PI double-staining was performed. The upregulation of let-7a resulted in a significant increase of apoptosis in the CNE-2 cells (Fig. 5B).

Core role of EZH2 in let-7a-mediated cell proliferation and apoptosis. Having demonstrated EZH2 as a direct target of let-7a, we then examined the importance of EZH2 in let-7a-mediated cell proliferation and apoptosis. We first transfected EZH2 without 3'UTR into the CNE-2 cells. Western blot analysis showed that the transfection with EZH2 without 3'UTR overrided EZH2 expression targeted by let-7a (Fig. 6A). As shown in Fig. 6B, when we transfected the cells with EZH2 without 3'UTR and let-7a, the expression of EZH2 significantly abrogated the effect of let-7a on cell cycle distribution and apoptosis (Fig. 6B and C). These findings suggest that EZH2 is a major target of miR-106b involved in nasopharyngeal carcinoma cell proliferation and apoptosis.

Discussion

Recent evidence has indicated that let-7a plays a role in the development and progression of human tumors, such as breast cancer, renal cell carcinoma, gastric and hepatocellular cancer (9-12). In this study, the overexpression of let-7a led to the downregulation of EZH2 expression via targeting the 3'UTR of EZH2, resulting in the inhibition of cell proliferation, cell cycle arrest at the G0/G1 phase and the induction of cell apoptosis in the nasopharyngeal carcinoma cells.

Polycomb repressive complex 2 (PRC2) is recognized as an important epigenetic group of regulators which function as transcriptional repressors that silence specific sets of genes through chromatin modification (13,14). PRC2 includes EZH2, the suppressor of zeste 12 (SUZ12) and embryonic ectoderm development (EED). EZH2 is the catalytically active component of PRC2 and is capable of trimethylating lysine 27 of histone H3 (H3K27) (15-17). Recently, an increasing number of reports have documented the overexpression of EZH2 in a variety of human cancers, including prostate cancer, lymphoblastic leukemia, neuroblastoma and lung cancer (18-21). Compared with normal nasopharyngeal tissue, the expression levels of EZH2 are significantly upregulated in nasopharyngeal carcinoma specimens (22). The increased expression of EZH2 in nasopharyngeal carcinoma is closely associated with an aggressive and poor prognostic phenotype (23). These data suggest the involvement of EZH2 in disease progression and tumor aggressiveness, and also suggest that EZH2 may be used as a prognostic indicator in patients with nasopharyngeal carcinoma. In this study, we show that EZH2 is overexpressed in nasopharyngeal carcinoma tissues, and that the suppression of EZH2 expression inhibits nasopharyngeal carcinoma cell proliferation and induces apoptosis, consistent with previous data.

EZH2 may be directly regulated by several miRNAs, such as miR-124, miR-138 and miR-214 (24-26). In nasopharyngeal carcinoma, miR-26a has been shown to inhibit cell growth and cell cycle progression by targeting EZH2. The introduction of EZH2 cDNA abrogates the suppressive effect of miR-26a (27). In another study of nasopharyngeal carcinoma, EZH2 has been shown to be a direct target for miR-98, miR-26a and miR-101 (28). In this study, we show that let-7a regulates EZH2 expression via targeting the 3'UTR of EZH2, and that the expression of EZH2 significantly abrogates let-7a-mediated cell proliferation and apoptosis in nasopharyngeal carcinoma cells.

In conclusion, our results show that let-7a is a tumor suppressor miRNA in nasopharyngeal carcinoma and that EZH2 is a novel and critical target of let-7a. Our data suggest that let-7a and EZH2 may be useful as potential therapeutic targets for nasopharyngeal carcinoma and warrant further investigation.

Acknowledgements

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