LncRNA MEG3 inhibits the development of nasopharyngeal carcinoma by sponging miR-543 targeting KLF4

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Background: Emerging evidence shows that long non-coding RNAs (lncRNAs) play a crucial role in tumor development by regulating biological behavior in various cancer cells. Several lncRNAs act as miRNA sponges by binding miRNA sequences and thus regulating mRNA expression. The lncRNA maternally expressed gene 3 (MEG3) has decreased expression levels in many cancer cells and acts as a tumor suppressor in different cancers. MEG3 also showed decreased expression in nasopharyngeal carcinoma (NPC) and plays a role in tumor suppression; however, the detailed mechanism of tumor suppression in NPC cells has not been reported. This paper aimed to explore the function and molecular mechanisms of MEG3 in the development of NPC.

Methods: MEG3 and miR-543 levels in NPC cells were detected by quantitative real-time PCR (qRT-PCR). The regulatory role of MEG3 in NPC cells was examined using knockdown and overexpression of MEG3 in C666-1 cells. Cell proliferation was analyzed by the cell counting kit-8 (CCK-8) assay, cell migration and invasion capacities were evaluated using Transwell assay, and cell apoptosis was assessed using flow cytometry. The relationship between MEG3 and miR-543 was investigated by luciferase reporter assay. MEG3- and Krüppel like factor 4 (KLF4)-mediated changes in NPC cell proliferation and apoptosis were analyzed, and KLF4, Bcl-2 and Bax protein expression levels were measured by western blotting.

Results: The results showed that MEG3 was decreased and miR-543 was increased in NPC cell lines, and upregulated MEG3 inhibited cell proliferation, migration, and invasion and promoted apoptosis, suggesting that MEG3 acts as a tumor suppressor in NPC cells. Furthermore, a luciferase reporter assay and western blotting indicated that MEG3 regulated KLF4 expression by sponging miR-543. Functionally, overexpression of MEG3 suppressed cell proliferation, promoted cell apoptosis and affected Bcl-2 and Bax protein levels via regulation of KLF4 expression mediated by sponging miR-543.

Conclusions: These findings show that lncRNA MEG3 inhibits the development of NPC by sponging miR-543 targeting KLF4 and that MEG3 can serve as a new novel target for NPC therapeutics.

Keywords: Nasopharyngeal carcinoma cells (NPC cells); maternally expressed gene 3 (MEG3); miR-543; Krüppel like factor 4 (KLF4)

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Introduction

Nasopharyngeal carcinoma (NPC) is a cancer of the epithelial cells of the pharynx (1). NPC epidemiology research showed a specific geographic distribution in southern China with a high incidence and prevalence rates (2). In addition, population-based analysis revealed a strong familial aggregation tendency among NPC patients, suggesting that genetic susceptibility can be critical in NPC tumorigenesis (3).

Cancer development is a complicated process of accumulated genetic and epigenetic changes over time that include activation of oncogenes or inhibition of tumor suppressor genes (4-6). The activation of oncogenes (activated from proto-oncogenes by chromosomal mutations) enhances abnormal cell biological behavior and results in cancerous cell changes and carcinogenesis (7). Tumor suppressor genes encode proteins and control cancer initiation and progression by cell division inhibition, apoptosis induction, damaged DNA repair and metastasis inhibition (8). However, the Human Genome Project (HPG) has revealed that only approximately 1.5% of the genome encodes protein (9). Most of the human genome can be transcribed but not translated, and since the first identification of regulatory non-coding RNAs (ncRNAs), molecular biology scientists have discovered that regulatory ncRNAs are involved in cell development and pathogenesis (10).

ncRNAs can be subcategorized into two classes by their functions: housekeeping (infrastructural) ncRNAs and regulatory ncRNAs (11). Housekeeping ncRNAs include ribosomal RNA (rRNA), transfer RNA (tRNA), small nuclear RNA (snRNA) and small nucleolar RNA (snoRNA), play pivotal roles in mediating vital cell functions (12). Regulatory ncRNAs can be divided into two subtypes based on their length: small ncRNAs (sncRNAs) with less than 200 nucleotides, which include miRNAs and snoRNAs, and long non-coding RNAs (lncRNAs), which are longer than 200 nucleotides (13,14). To date, most of the studies have investigated sncRNAs, especially miRNAs, as these molecules regulate target gene expression and function as oncogenes or tumor suppressor genes through their different targets (15). However, an increasing number of studies have shown evidence that lncRNAs targets are related to the development and prognosis of different types of cancer, similar to miRNAs (16-21). Some lncRNAs, including LED, maternally expressed gene 3 (MEG3), GUARDIN and PTENP1, have been identified as tumor suppressors, and other lncRNAs, including MALAT1, HOTAIR, NORAD and PVT1, have been identified as tumor promoters (22).

LncRNAs have been shown to act as miRNA sponges to competitively bind miRNA sequences and suppress the interaction between miRNAs and mRNAs (23). For example, lncRNA MEG3 functions as a competitive endogenous RNA (ceRNA) of many different miRNAs, whereby downregulated MEG3 expression causes a change in miRNAs, subsequently affecting the expression levels of their target mRNAs (24). MEG3 can be found in various normal tissues, but gene expression is lost in multiple cancer cells and tissues (25,26). MEG3 is located at chromosome 14q32.3 in humans, and multiple mechanisms lead to alterations in MEG3 expression in tumors (27). Most of the studies have identified MEG3 as a tumor suppressor gene in many different cancers, such as glioma, gastric cancer, bladder cancer, ovarian cancer, cervical cancer, and lung cancer (28-33). MEG3 functions as a tumor suppressor by regulating tumor suppressor genes, inhibiting angiogenesis and miRNA control (34). Chak and colleagues found that MEG3 expression is decreased in NPC cells and plays a role in tumor suppression (35), but its detailed mechanism of tumor suppression in NPC cells is still unclear.

The bioinformatic software starBase 3.0 predicted that there are miRNA response elements (MRE) complementary to miR-543 in the MEG3 sequence, and Krüppel-like factor 4 (KLF4) was identified as a target gene of miR-543. There have been conflicting results regarding miR-543 expression levels in different types of cancers. MiR-543 is upregulated in colorectal cancer, gastric cancer, hepatocellular carcinoma, and prostate cancer, whereas it is decreased in ovarian cancer and endometrial cancer (36-42). Although miR-543 is expressed differently in cancers, it is clear that miR-543 is strongly involved in the development and progression of cancers. However, the role of miR-543 in nasopharyngeal cancer remains unknown.

In the present study, we determined MEG3 expression in nasopharyngeal cancer cells and its functional regulation in tumor development. In addition, we revealed the interactions among MEG3, miR-543 and KLF4 in nasopharyngeal cancer for the first time. This study evaluated MEG3 as a tumor suppressor and its underlying mechanisms in nasopharyngeal cancer.
Methods

Cell culture

Two NPC cell lines (C666-1 and TW03) and the human nasopharyngeal epithelial cell line NP69 were obtained from the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI-1640 medium (Invitrogen, USA) supplemented with 10% fetal bovine serum in a CO₂ incubator (MCO-5AC, Sanyo, Japan) at 37 °C.

Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from cells using NucleoZOL reagent (Machery-Nagel GmbH, Germany). The integrity of the RNA was assessed, and qRT-PCR was performed with an ABI 7500 system to detect the expression levels of MEG3, miR-543 and KLF4. The primer sequences were as follows: MEG3-F: 5'-GGGAAGGGACCTCGAATGTG-3', MEG3-R: 5'-CTGTCCCGTGGGAATAGGTG-3'; miR-543-F: 5'-CGAAACATTCGCGGTGCA-3'; miR-543-R: 5'-AGTGCAGGGTCCGAGGTATT-3', and miR-543-RT: 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAGAAG-3'. GAPDH and U6 were used as internal controls, and the specific primers were as follows: GAPDH-F: 5'-GTCAAGGCTGAGAACGGGAA-3', GAPDH-R: 5'-AAATGAGCCCCAGCCTTCTC-3'; U6-F: 5'-CTCCTTGGCCGACGACATAT-3', U6-R: 5'-ACCGTTCAGAATTTCGCTGTC-3', and RT primer, 5'-AAATATGGAACGCTTCACGAATTTG-3'. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

Cell transfection

si-MEG3, pcDNA-MEG3, miR-543 mimic, miR-543 inhibitor, si-KLF4, and pcDNA-KLF4 were obtained from GenePharma Company (Shanghai, China). Transfection was performed using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's protocols.

Cell proliferation assay

Cell proliferation was measured with the cell counting kit-8 (CCK-8) following the manufacturer's instructions. After 48 hours of transfection, C666-1 cells were seeded into a 96-well plate. Then, 100 μL of 10% CCK-8 solution (CK04, Dojindo) was added into each well at 0, 24, 48, 72 and 96 h after transfection and incubated for 2 h at 37 °C. The absorbance at OD 450 nm was measured by a microplate reader.

Cell apoptosis assay

Cell apoptosis was assessed by flow cytometry analysis. Cells were cultured overnight and transfected when they reached 80–90% confluence. Cells were cultured in a 37 °C incubator supplemented with 5% CO₂ and digested with trypsin without EDTA. The cells were harvested after centrifugation and washed with PBS. The collected cells were mixed with binding buffer and then incubated with Annexin V-FITC and PI staining solutions for approximately 15 min in the dark at room temperature. The apoptosis rate was counted using flow cytometry.

Cell migration and invasion assays

Each group of transfected cells ($1\times10^5$ cells/mL) were diluted in RPMI-1640 medium without serum and seeded into the upper chamber. The lower chamber was filled with RPMI-1640 medium containing 20% FBS. After incubation at 37 °C for 24 h, the cells in the upper chambers were removed. Cells were fixed and stained with crystal violet. Then, randomly selected fields were imaged, and migrated or invaded cells were counted.

Dual luciferase assay

The target genes of miR-543 were predicted using a miRNA database (TargetScan: http://www.targetscan.org/). The putative binding sequences of MEG3-WT (5’GAGUAAUG-GUAGUGAAUGUUU3’) and MEG3-MUT (5’GAGUAAUG-GUAGUCAGAUCAU3’), were cloned into the pcDNA3.1 vector and then cotransfected with miR-543 mimic or negative control in C666-1 cells using Lipofectamine 2000 (Invitrogen, USA). After 36 h of transfection, firefly and Renilla signals were analyzed with a Dual-Luciferase Reporter Assay System (Promega, USA). Luciferase activity was quantified by a microplate reader.

Western blotting

Total protein was isolated using RIPA buffer (Beyotime, Shanghai, China), separated by 10% SDS-PAGE and transferred onto a polyvinylidene difluoride (PVDF)
membrane. Quantification of protein was analyzed using the Bradford method. Visualization of target protein bands on the PVDF membranes were performed with a Pierce ECL kit. The following antibodies were used and were diluted as follows: anti-KLF (1:1,000, Proteintech, USA), anti-Bcl-2 (1:1,000, Cell Signaling Technology, USA) and anti-Bax (1:1,000, Cell Signaling Technology). Anti-β-actin (1:1,000; Cell Signaling Technology) was used as an internal control.

Statistical analysis

The data are expressed as the mean ± standard deviation (SD) and were analyzed by the statistical software SPSS 16.0 (SPSS, Inc., Chicago, IL, USA). Statistical significance was determined using analysis of variance (ANOVA). P value <0.05 was considered to indicate a statistically significant difference, and P value <0.001 was considered to indicate a statistically highly significant difference.

Results

**MEG3 was decreased and miR-543 was increased in human NPC cell lines**

MEG3 and miR-543 expression levels in NPC cell lines (C666-1 and TW03) and in the normal nasopharyngeal epithelial cell line NP69 were determined by qRT-PCR. Compared with those in the normal control, the MEG3 levels in NPC cell lines were decreased (Figure 1A), whereas miR-543 levels were increased (Figure 1B). Ultimately, the C666-1 cell line was selected for the following investigations.

**MEG3 affected NPC cell biological behavior**

To explore the effects of MEG3 on NPC cell biological behavior, pcDNA-MEG3 or si-MEG3 was transfected into C666-1 cells. qRT-PCR results showed that MEG3 expression was markedly increased by pcDNA-MEG3 (Figure 2A, P<0.01) and reduced by si-MEG3 (Figure 2B, P<0.01). Cell proliferation was assessed by the CCK-8 assay. The si-MEG3 group showed gradually increased OD values compared to the control, whereas the OD values in the pcDNA-MEG3 group decreased in a time-dependent manner (Figure 3A). In addition, apoptotic cells were measured by flow cytometry assay, and the results revealed that pcDNA-MEG3 increased the number of cells in the early and late stages of apoptosis, whereas si-MEG3 decreased apoptosis and the number of dead cells (Figure 3B). These findings confirmed that si-MEG3 enhanced proliferation and suppressed apoptosis in C666-1 cells, whereas pcDNA-MEG3 decreased cell proliferation and enhanced cell apoptosis.

Migration and invasion assays were performed in Transwells. Si-MEG3 significantly increased cell migration, whereas pcDNA-MEG3 significantly decreased cell migration (Figure 4, P<0.001). Similarly, invaded cells
MEG3 inhibited NPC by sponging miR-543 targeting KLF4

MEG3 directly sponged miR-543 in C666-1 cells

The preliminary investigation predicted a correlation between miRNA-543 and MEG3. Figure 1A, B shows a significant upregulation of miR-543 expression and significant downregulation of MEG3 in NPC cell lines. Bioinformatics prediction revealed that MEG targeted miR-543. A luciferase assay was used to assess the relationship between miRNA-543 and MEG3. Thus, MEG3 wild-type and mutant luciferase reporter vectors were constructed and transfected into cells. The luciferase activity results indicated that the miR-543 mimic markedly decreased the luciferase activity in the MEG3 wild-type reporter group compared with the NC group. In contrast, the miR-543 mimic had no detectable effect on the luciferase activity in the MEG3 mutant group (Figure 6). These data suggest that MEG3 is a miR-543 target in NPC cells and that there is negative regulation between miR-543 and MEG3 in NPC cells.

MEG3 increased KLF4 expression by sponging miR-543

Some known lncRNAs act as ceRNAs (also called ‘miRNA sponges’) by directly binding to miRNAs to inhibit miRNA expression (43). Downregulation of MEG3 greatly elevated miR-543 levels, but the cotransfection of si-MEG3 markedly inhibited the promotion effect of si-MEG3 on miR-543 expression (Figure 7A). The overexpression of MEG3 dramatically decreased the miR-543 expression level, but cotransfection of pcDNA-MEG3 and miR-543 mimic reversed the inhibitory effect of pcDNA-MEG3 on the expression of miR-543 (Figure 7B). Upregulation of miR-543 inhibited KLF4 expression in C666-1 cells, whereas the cotransfection of miR-543 mimic with pcDNA-MEG3 attenuated the suppressor function of miR-543 on KLF4 expression in C666-1 cells (Figure 7C). The overexpression of MEG3 markedly promoted the KLF4 protein level, whereas the cotransfection of miR-543 mimic with pcDNA-MEG3 reversed the promotion effect of pcDNA-MEG3 on KLF4 expression (Figure 7D). Downregulation of MEG3 significantly decreased KLF4 protein levels, whereas cotransfection of miR-543 inhibitor with si-MEG3 markedly relieved the suppressive effect of si-MEG3 on KLF4 expression (Figure 7E).

MEG3 acted as a tumor suppressor by regulating KLF4 expression in NPC

To further study whether KLF4 mediated the function of MEG3 in the progression of NPC, functional rescue experiments were performed. We found that si-MEG3 promoted cell proliferation and that pcDNA-MEG3 reduced cell proliferation; however, the cotransfection of pcDNA-KLF4 reversed the promoting effect of si-MEG3.
Figure 3 The effects of MEG3 on the proliferation and apoptosis of C666-1 cells. (A) The effect of MEG3 on cell proliferation; (B) the effect of MEG3 on cell apoptosis. *, P<0.05; **, P<0.01; ***, P<0.001. MEG3, maternally expressed gene 3; NC, normal control.
Figure 4 The effect of MEG3 on the migration of C666-1 cells. The cell migration activity of difference groups were measured by transwell assay (×400 magnification). ***, P<0.001. MEG3, maternally expressed gene 3; NC, normal control.
Figure 5 The effect of MEG3 on the invasion of C666-1 cells. The cell invasion activity of difference groups were measured by transwell assay (×400 magnification). ***, P<0.001. MEG3, maternally expressed gene 3; NC, normal control.
on C666-1 cell proliferation, and the cotransfection of si-KLF4 relieved the inhibitory effect of pcDNA-MEG3 on cell proliferation (Figure 8A). Si-MEG3 suppressed cell apoptosis, whereas si-MEG3 + pcDNA-KLF4 alleviated the inhibitory function of si-MEG3 in C666-1 cell apoptosis (Figure 8B). Conversely, we found that pcDNA-MEG3 induced cell apoptosis, whereas pcDNA-MEG3 and si-KLF4 cotransfection inhibited the promoting effect of pcDNA-MEG3 on cell apoptosis (Figure 8B). In addition, the apoptosis-related Bcl-2 and Bax proteins were analyzed by western blotting. The Bcl-2 family includes Bcl-2, which induces apoptosis, and Bax, which promotes apoptosis, is known to inhibit or enhance apoptosis and has become a novel cancer therapeutic to control apoptosis (44-46). In particular, high Bax mRNA expression can be used as a good prognostic indicator of survival rate in NPC patients (47). Knockdown of MEG3 increased Bcl-2 expression and reduced Bax expression; however, forced expression of KLF4 reversed these effects in C666-1 cells (Figure 9A). Overexpression of MEG3 decreased Bcl-2 expression and elevated Bax expression; however, reduced expression of KLF4 reversed these effects in C666-1 cells (Figure 9B). Consequently, the role of MEG3 in C666-1 cell proliferation and apoptosis was regulated, at least in part, by KLF4.

**Discussion**

NPC has a high prevalence and mortality rate in Southeast Asia, and the main reason for the high mortality is the late detection because of the lack of specific symptoms (48). Patients at an advanced stage of NPC have poor prognosis and a high recurrence rate, even with aggressive treatment (49). Thus, recent studies have been conducted to elucidate the molecular basis of NPC carcinogenesis, discover reliable biomarkers of NPC and develop effective molecularly targeted therapies.

After the genome project showed that only approximately 1.5% of the genome encoded protein, Encyclopedia of DNA Elements (ENCODE) project researchers revealed that 80.4% of the human genome is engaged in biochemical events such as binding to protein or chromatin structures, and discovered that regulatory regions can act differently according to the cell type (50). lncRNAs are thought to be involved in various disease etiologies and regulate target gene expression (51). A growing number of studies have shown that lncRNAs can be oncogenes or tumor suppressors in carcinogenesis (52). The first identification of MEG3 involvement in carcinogenesis suppression was conducted by Zhang et al. in 2003 (53), and subsequent investigations identified MEG3 as a tumor suppressor in multiplicate cancer cells. MEG3 physiological and pathological processes in cancer cells have been under continuous investigation, but the role of MEG3 in NPC progression remains unknown.

This research aimed to analyze the MEG3, miR-543 and KLF4 expression and interactions in NPC cells and the effects on NPC cell progression and carcinogenesis. MiR-543 can have oncogenic or tumor suppressive effects depending on the cancer cell type. KLF4 is a known target gene of miR-543 that is involved in cell proliferation and functions as a tumor suppressor and prognostic indicator in cancer (36,54,55). In the present study, the MEG3 expression level was decreased in NPC cells, and pleiotropic regulation of NPC cell biological behavior was observed. MEG3 overexpression decreased cell proliferation, migration and invasion but promoted cell apoptosis. MEG3 exerted a tumor suppressive role by targeting miR-543 and acting as a ceRNA. Furthermore, KLF4 was confirmed as a targeted gene of miR-543 in NPC cells, and this was consistent with previous studies. KLF4 was downregulated by miR-543, and KLF4 expression inhibited C666-1 cell proliferation and induced cell apoptosis. KLF4 in NPC cells regulated Bcl-2 and Bax protein expression, indicating that KLF4 may be a prognostic factor for NPC.

In conclusion, our findings suggest a tumor suppressive role of MEG3 through the MEG3-miR-543-KLF4 axis in NPC cells. Decreased MEG3 was negatively correlated with miR-543 expression and could reverse the decrease in
Figure 7 MEG3 regulated KLF4 by sponging miR-543 in C666-1 cells. (A,B) The effect of MEG3 on miR-543 expression; (C) overexpression of MEG3 increased KLF4 expression, which was downregulated by miR-543 mimic; (D) miR-543 mimic decreased KLF4 expression, which was elevated by overexpression of MEG3; (E) miR-543 inhibitor increased KLF4 expression, which was decreased by knockdown of MEG3. *, P<0.05; **, P<0.01; ***, P<0.001. MEG3, maternally expressed gene 3; KLF4, Krüppel like factor 4; NC, normal control.
Figure 8 MEG3 decreased proliferation and promoted apoptosis in C666-1 cells by affecting KLF4 expression. (A) Overexpression of KLF4 inhibited cell proliferation, which was promoted by si-MEG3; downregulation of KLF4 promoted cell proliferation, which was inhibited by pcDNA-MEG3; (B) overexpression of KLF4 induced cell apoptosis, which was inhibited by si-MEG3; downregulation of KLF4 inhibited cell apoptosis, which was enhanced by pcDNA-MEG3. *, P<0.05; ***, P<0.001. MEG3, maternally expressed gene 3; KLF4, Krüppel like factor 4; NC, normal control.
KLF4 expression caused by miR-543 overexpression, which was associated with NPC progression. MEG3 may represent a new therapeutic biomarker for NPC targeted treatment.

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Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/tcr.2019.12.41). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. An ethics statement was not required for this research as we did the experiment only using cells from the Chinese Academy of Sciences (Shanghai, China).

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Figure 9: The effects of MEG3 on Bcl-2 and Bax protein expression. (A) Knockdown of MEG3 increased Bcl-2 protein expression levels and decreased Bax expression; however, forced expression of KLF4 reversed these effects in C666-1 cells; (B) overexpression of MEG3 decreased Bcl-2 expression and increased Bax expression; however, reduced expression of KLF4 reversed these effects in C666-1 cells. *, P<0.05; ***, P<0.001. MEG3, maternally expressed gene 3; KLF4, Krüppel like factor 4; NC, normal control.
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