Promoter hypermethylation-mediated downregulation of miR-770 and its host gene MEG3, a long non-coding RNA, in the development of gastric cardia adenocarcinoma

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Maternally expressed gene 3 (MEG3) is an imprinted gene located at 14q32 which encodes an IncRNA and is downregulated in an expanding list of cancer cell lines and primary human cancers. The miR-770 is transcribed from the intronic sequence of MEG3 and MEG3 may be the host gene for miR-770. However, the biological role of MEG3 and miR-770 in gastric cardia adenocarcinoma (GCA) development and prognosis is poorly defined. The present study was to investigate the function and methylation status of MEG3 in GCA, and further to detect the functional association of miR-770 and its host gene MEG3 in GCA carcinogenesis and prognosis. MEG3 and miR-770 was significantly downregulated in GCA patients and cell lines, and their expression was associated with TNM stage and lymph node metastasis. Overexpression of MEG3 and miR-770 inhibited gastric cancer cell proliferation and invasion in vitro. Furthermore, the expression level of MEG3 and miR-770 was significantly increased in cancer cells after treated with 5-Aza-dC. The aberrant hypermethylation of proximal promoter and enhancer region of MEG3 was detected in GCA tissues. In addition, the proximal promoter and enhancer region hypermethylation and dysregulation of MEG3 and miR-770 were associated with poorer GCA patients’ survival. These findings suggest that miR-770 and its host gene MEG3 may play tumor suppressor role and hypermethylation of proximal promoter and enhancer region may be one of the critical mechanisms in inactivation of MEG3 and miR-770 in GCA development. MEG3 and miR-770 may be used as potential biomarkers in predicting GCA patients’ prognosis.

KEYWORDS
expression, gastric cardia adenocarcinoma, MEG3, methylation, miR-770

1 INTRODUCTION

Gastric cardia adenocarcinoma (GCA), as a malignant tumor with poor prognostic outcomes, arising in the transformation zone between the esophagus and the stomach, was formerly registered as gastric cancer or esophageal cancer. However, due to the distinct epidemiological and biological characteristics and improvement in classification, GCA has been diagnosed independently in very recent years. Despite the steady decline in non-cardia gastric cancer incidence and mortality rates worldwide, the incidence rate for GCA continues to show an increase in developed and developing countries during the past few decades.1 In the United Kingdom and United States, GCA accounted for about 50% of gastric cancer in men.2 In China, GCA has unique epidemiological features distinguishing this tumor from the adenocarcinomas of the distal stomach and shares similar geographic distribution with esophageal squamous cell carcinoma (ESCC).3 The incidence rate of GCA increased gradually over the past few years in China and a significant portion of GCA patients strongly point to upper gastrointestinal cancers (UGIC) family history, especially in the highest incidence areas of ESCC in the world, such as in some counties bordering Henan, Hebei, Gansu, and Shanxi Provinces. However, the incidence of adenocarcinoma arising from the distal stomach is relatively low in this area.4 Unlike Helicobacter pylori infection is the strongest identified risk factor for non-cardia gastric cancer,5 the main risk factors, and the exact molecular pathogenesis of GCA still remain unclarified.
Long non-coding RNAs (lncRNAs), ranging in length from 200 nt to 100 kb, constitute a large proportion of non-coding transcripts and have recently emerged as new players in a wide range of biological processes including cancer biology. Although our current understanding of the functional role of lncRNAs is limited, recent studies have suggested that lncRNAs may function as key regulators in various biological processes, such as their involvement in chromatin remodeling, maintaining the integrity of the nuclear structure, and regulation of gene expression. Mounting evidence showed that many lncRNAs have altered expression in various types of human cancer and dysregulated lncRNAs may function as tumor suppressors or oncogenes to contribute to the development and progression of cancer by influencing proliferation, differentiation, migration, metastasis, self-renewal, and apoptosis through either transcriptional or post-transcriptional regulation. Maternally expressed gene 3 (MEG3) is a maternally expressed imprinted gene representing a large non-coding RNA as its transcript lacks a significant open reading frame. MEG3 is expressed in many normal tissues, however is lost or downregulated in an expanding list of human tumor cell lines and primary tumors. Growing evidence suggests that MEG3 has tumor suppressor properties and is capable of interacting with p53, cyclic AMP, murine double minute 2 (MDM2), and growth differentiation factor 15 (GDF15) to regulate proliferation, migration, and invasion of tumor cells. Hypermethylation of promoter or the differentially methylated regions (DMRs) upstream of the MEG3 gene has been found to contribute to the loss of MEG3 expression in several tumors.

Further analysis of the structure of MEG3 gene finds that miR-770 may be transcribed from within intron 6 of MEG3 major transcript (Fig. 1A). Although it is shown that about 26% of the mammalian intronic miRNAs (miRNAs) may be transcribed from their own promoters, 40% of the known mammalian miRNAs are located within the introns of protein coding genes, so called host genes, and are transcriptionally linked to their host gene expression and processed from the same primary transcript. A microarray profiling survey analyzed the expression patterns of 175 human miRNAs across 24 different human organs and found that the expression of intronic miRNAs largely coincides with their host gene mRNA, indicating that the intronic miRNAs and their host genes may be co-regulated and are generated from a common precursor transcript. However, to our best knowledge, the effects and methylation status of MEG3, the association of MEG3 and miR-770, and the biological significance of this association in GCA tumorigenesis have not been clarified so far. In the present study, we examined the function and methylation status of MEG3 in GCA, and further detected the functional association of miR-770 and its host gene MEG3 in GCA carcinogenesis and analyzed the possibility of MEG3 and miR-770 as potential targets for GCA metastasis and prognosis.

2 MATERIALS AND METHODS

2.1 Patients and specimens

Primary GCA tissues and corresponding adjacent normal tissues were obtained from 134 GCA patients undergoing surgery at the Fourth Affiliated Hospital, Hebei Medical University between the years of 2007 and 2010. All study patients were ethnically homogeneous Han nationality and residents of Hebei Province and its surrounding regions and written informed consent was obtained from each case before surgery. The patients consisted of 106 males and 28 females with a median age of 58 years (ranged from 36 to 76 years). Information on clinicopathologic characteristics was available from hospital recordings and pathological diagnosis. All gastric cardia carcinomas were adenocarcinomas with their epiphenets at the gastroesophageal junction, that is, from 1 cm above until 2 cm below the junction between the end of the tubular esophagus and the beginning of the saccular stomach. Tissue samples were divided into two parallel parts, one part were formalin-fixed and paraffin-embedded, the other part were frozen and stored at −80°C to extract genomic DNA and RNA. All subjects were interviewed by professional interviewers for their age, gender, histopathological diagnosis, and upper gastrointestinal cancers (UGIC) family history. Individuals with at least one first-degree relative or at least two second-degree relatives having esophageal/cardia/gastric cancer were defined as having family history of UGIC. Recurrence and survival data were ascertained through the Tumor Registry and Hospital chart review (Supplementary Table S1). The study was approved by the Ethics Committee of the Fourth Affiliated Hospital, Hebei Medical University.

2.2 Cell culture and treatment

A total of four human gastric cancer cell lines MKN45, SGC7901, BGC823, and AGS were examined in this study and were cultured in DMEM (Gibco, Invitrogen, Life Technologies, Germany) medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin in humidified air at 37°C with 5% CO2. GES1 cells, a normal gastric epithelium cell line, were cultured according to the manufacturer’s instructions. Cells were seeded at a low density and incubated for 24 h prior to treatment with DNA methyltransferase inhibitor 5-aza-2′-deoxycytidine (5-Aza-dC). All four gastric cancer cells and normal gastric epithelium cells (2 × 10^5/mL) were treated with 5 µmol/L 5-Aza-dC (Sigma, St. Louis, MO) for 72 h and medium containing 5-Aza-dC was changed every 24 h. Control cells received no drug treatment.

2.3 Cell transfection

For overexpression of MEG3, the sequence of MEG3 was synthesized and subcloned into pcDNA3.1 (Invitrogen, Carlsbad, CA). The MKN45 cells were transfected with MEG3 expression plasmid (pcDNA3.1-MEG3) or the empty vector (pcDNA3.1-EV) as control at a final concentration of 2 µg/µL using FuGENE HD Transfection Reagent (Promega, Madison, WI) according to the manufacturer’s instructions. For overexpression of miR-770, MKN45 cells were transfected with miR-770 mimic or mimic control (Ambion, Austin, TX) at a final concentration of 25 nmol/L.

2.4 RNA isolation and quantitative real-time RT-PCR assay

Total RNA was isolated from cell lines, frozen tumor and corresponding normal tissues using TRIzol reagent (Invitrogen, Carlsbad, CA) according
to the manufacturer’s instructions. For MEG3 expression, 2 µg RNA was used to synthesize single-stranded cDNA using the advantage RT-for-PCR kit (Clontech, Palo Alto, CA) and the cDNA from each sample was used as quantitative real-time RT-PCR template. Power SYBR Green PCR Master Mix (Life Technology, Foster City, CA) was used as amplification reaction mixture in accordance with the manufacturer’s instructions. The primers and reaction conditions for MEG3 are listed in Supplementary Table S2. Human GAPDH gene was used as an internal
control. For miR-770-5p expression, miRcute miRNA First-strand cDNA Synthesis Kit (Tiangen, China) was used to synthesize the first strand of cDNA, and miRcute miRNA qPCR Detection Kit (SYBR Green) (Tiangen, China) was adopted to detect miR-770-5p expression. Human U6 snRNA was used for miR-770-5p normalization. The fold change for the target genes were calculated using the $2^{-\Delta\Delta CT}$ method. All the samples were run in triplicate.

2.5 Cell proliferation assay

The proliferation of MEG3 transfected or miR-770 mimic treated MKN45 cells was measured by cell-counting kit-8 (CCK-8) assay. Before proliferation detected, 10 μL of CCK8 (Dojindo, Japan) was added to the 100 μL cultured cells, and after incubated for 2 h in a humidified incubator containing 5% CO₂ at 37°C, the absorbance of each well was measured at a wavelength of 450 nm. Proliferation rates were determined at 0, 24, 48, 72, and 96 h after transfection. All experiments were performed in triplicate.

2.6 Cell invasion assay

The invasiveness of MEG3 transfected or miR-770 mimic treated MKN45 cells was evaluated in 24-well transwell chambers (Corning, Kennebunk, ME). The transwell chambers were coated with 20 μL Matrigel and incubated at 37°C for 1 h. After 24 h transfection, the 5 × 10⁴ cells/well were seeded in the upper chambers, meanwhile the DMEM medium with 10% FBS was added to the lower chambers. After incubated for 24 h, invasive cells located on the lower chamber were stained with hematoxylin. The number of cells invaded through the membrane to the lower surface was counted in five microscopic fields (at 100× magnification) per filter. The experiments were repeated in triplicate.

2.7 DNA extraction and sodium bisulfite treatment

Genomic DNA was extracted from frozen GCA tumor and corresponding normal tissues using a simplified Proteinase K digestion method. To examine the DNA methylation patterns, 1 μg of genomic DNA was bisulfite modified using Epitect Fast Bisulfite Conversion Kits (Qiagen, Germany) in accordance with the manufacturer’s instructions. After the standard sodium bisulfite DNA modification, unmethylated cytosine residues were converted to thymine, whereas methylated cytosine residues were retained as cytosine at CpG sites.

2.8 Methylation status of four regions of MEG3 promoter via bisulfite genomic sequencing (BGS) method

The methylation status of every CpG site in the four regions of MEG3 promoter was verified by BGS assay in four matched tissue sets showing tumor-specific reduction of transcript levels. For BGS assay, primers were designed to recognize sodium bisulfite converted DNA and encompassing four regions within the human MEG3 promoter region (region 1: From −433 to +4 bp, region 2: From −929 to −454 bp, region 3: From −1483 to −1039 bp, region 4: From −1946 to −1504 bp). A total of 50 ng of bisulfite-modified DNA was subjected to PCR amplification and the PCR products were cloned into pGEM-T vectors (Promega, Madison, WI) and 8-10 clones of each specimen were sequenced by automated fluorescence-based DNA sequencing.

2.9 Methylation analysis of MEG3 via bisulfite conversion-specific and methylation-specific polymerase chain reaction (BS-MSP) method

The methylation status of MEG3 in GCA tissues was then determined by BS-MSP method as previous description using bisulfite treated genomic DNA. According to the BGS analysis of the distribution of the main methylated Cpg sites, four regions located in the promoter region of MEG3 (region 1: From −312 to −193 bp, region 2: From −885 to −601 bp, region 3: From −1385 to −1153 bp, region 4: From −1774 to −1615 bp) were respectively analyzed. The primers and reaction conditions were listed in Supplementary Table S2. Genomic DNA, methylated in vitro by CpG methyltransferase (Sss I) following the manufacturer’s directions (New England BioLabs, Beverly, MA), was used as a positive control and water blank was used as a negative control. BS-MSP products were analyzed on 2% agarose gel with ethidium bromide staining, and were determined to have methylation if a visible band was observed in the methylation reaction. The reactions were performed in duplicate with each of the samples.

2.10 Statistical analysis

Statistical analysis was performed with SPSS19.0 software package (SPSS Company, Chicago, Illinois). The real-time RT-PCR results were expressed as the mean ± S.D. Student’s t-test was used to compare the expression means between different groups. The status of gene methylation between different groups was analyzed using Pearson’s Chi-square test. Survival curves were made by using the Kaplan Meier method and the Log-rank or the Breslow tests were used as needed for the univariate comparison of MEG3 expression and methylation categories. Cox’s multivariate test applied in a stepwise forward method was used to adjust for potentially confounding variables and to evaluate the role of MEG3 as an independent predictor of patients’ prognosis. All statistical tests were two sided; and $P < 0.05$ was considered to be statistically significant.

3 RESULTS

3.1 Decreased expression of MEG3 in human GCA tissues

To determine the expression level of MEG3 in GCA, qRT-PCR method was first used to detect the expression level of MEG3 in 134 pairs of clinic GCA tissues and corresponding normal tissues. As shown in Fig. 1B, the expression level of MEG3 in GCA tumor tissues was significantly decreased compared to corresponding normal tissues ($P < 0.01$). Among them, 95 patients (70.9%) showed less than 50% expression level of MEG3 in tumor tissues compared with their corresponding normal tissues. When stratified for clinicopathologic characteristics, the expression level of MEG3 in GCA tumor tissues...
was associated with TNM stage, depth of invasion, lymph node metastasis, and distant metastasis or recurrence (P < 0.05) (Fig. 1C).

3.2 | Downregulation of miR-770 in human GCA tissues

The miR-770 is transcribed from within intron 6 of MEG3, we hypothesized that MEG3 may be the host gene for miR-770 and the transcriptional regulation of miR-770 may follow that of its host gene MEG3. To test our hypothesis, we then detected the expression level of miR-770 in GCA tumor tissues was significantly decreased compared to corresponding normal tissues. As shown in Fig. 1D, the expression level of miR-770 in GCA tumor tissues was significantly decreased compared to corresponding normal tissues (P < 0.01). Among the GCA cases, 84 cases (62.7%) demonstrated less than 50% expression level of miR-770 in tumor tissues compared with their corresponding normal tissues. When stratified for clinicopathologic characteristics, the expression level of miR-770 in GCA tumor tissues was associated with TNM stage, depth of invasion, lymph node metastasis, and distant metastasis or recurrence (P < 0.05) (Fig. 1E). Correlation analysis further suggested a strong positive correlation between the expression of MEG3 and miR-770 in GCA tissues (Table 1).

3.3 | Downregulation of MEG3 and upregulation of the gene by 5-Aza-dC treatment in gastric cancer cell lines

We then performed qRT-PCR analysis to examine the expression level of MEG3 in four gastric cancer cell lines. As shown in Fig. 2A, the expression level of MEG3 was remarkably downregulated in gastric cancer cell lines when compared with normal gastric epithelium cell line GES1. To examine the role of aberrant methylation in deregulation of MEG3 in gastric cancer cells, we further evaluated the effect of DNA methyltransferase inhibitor 5-Aza-dC on MEG3 expression. After treatment with 5-Aza-dC, the expression level of MEG3 was significantly increased in the four gastric cancer cell lines, indicating the important role of aberrant hypermethylation of MEG3-DMRs in the downregulation of MEG3 in gastric cancer cell lines.

3.4 | MEG3 inhibits gastric cancer cell proliferation and invasion in vitro

The MKN45 cell line was selected to study the function of MEG3. A construct containing MEG3 transcripts (pcDNA3.1-MEG3) was transfected into MKN45 cells. As shown in Fig. 2B, the expression level of MEG3 was significantly up-regulated in pcDNA3.1-MEG3 transfected MKN45 cells compared with the empty vector pcDNA3.1-EV transfected MKN45 cells. We further analyzed the proliferation and invasion of the MEG3 transfected MKN45 cells. As shown in Fig. 2C, transfection of MEG3 led to a significant inhibition of MKN45 cells proliferation. MEG3 transfection also resulted in a significant inhibition of invasiveness in MKN45 cells (Fig. 2D). Taken together, our data suggested that MEG3 may act as a tumor suppressor through inhibiting cell proliferation and invasion in gastric cancer cells.

3.5 | The miR-770 is epigenetically regulated in the gastric cancer cell lines

To detect whether MEG3 and miR-770 share the same epigenetic regulation mechanism, we further detected the expression level of miR-770 in 5-Aza-dC treated and untreated gastric cancer cell lines. As shown in Fig. 2E, the expression level of miR-770 was remarkably downregulated in gastric cancer cell lines and the expression level of miR-770 was significantly increased in the four gastric cancer cell lines after treatment with 5-Aza-dC. These results clearly demonstrated that both miR-770 and its host gene MEG3 may share the common epigenetic regulation mechanism in gastric cancer cells.

3.6 | Overexpression of miR-770 inhibits gastric cancer cell proliferation and invasion in vitro

We then used MKN45 cells to analyze the function of miR-770. As shown in Fig. 2F, the expression level of miR-770 was significantly increased in miR-770 mimic transfected MKN45 cells. The CCK-8 assay in the same cells showed a significant inhibition of MKN45 cells proliferation after transfection with miR-770 mimic, whereas an mimic control had no inhibitory effect (Fig. 2G). Furthermore, transwell invasion assay showed a significant reduction in cell invasiveness in miR-770 mimic transfected MKN45 cells compared to the mimic control transfected cells (Fig. 2H). These results indicated the same anti-oncogenic role of miR-770 as the tumor suppressor role of MEG3 in gastric cancer cells.

3.7 | Aberrant methylation of MEG3 in GCA tumor tissues

Sequence analyses find that the promoter region of MEG3 is rich in CpG dinucleotides; the MethPrimer program was further used to determine the distribution of CpG islands of MEG3. As shown in Fig. 3A, several CpG islands are found to be located in the promoter region of MEG3. In order to have a basic understanding of the methylation pattern of the CpG sites within the MEG3 promoter region, the methylation status of 98 CpG sites spanning 5′-flanking region (from −1946 to +4 bp), which was divided into four regions, was verified by BGS assay in four GCA tissues with loss expression of MEG3 and their corresponding normal tissues. As shown in Fig. 3B, the large majority of CpG sites were unmethylated in normal tissues;

<table>
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<th>MiR-770-5p expression</th>
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<tr>
<td>High</td>
<td>34</td>
<td>16</td>
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<tr>
<td>Low</td>
<td>5</td>
<td>79</td>
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</table>

TABLE 1 Correlation between the expression of MEG3 and miR-770-5p in GCA tumor tissues
frequent hypermethylation of the CpG sites within regions 1 and 4 was detected in the four tumor tissues, while hypermethylation of the CpG sites within regions 2 and 3 was rarely detected in the four tumor tissues.

The four pairs of primers of BS-MSP method, which were respectively located in the four regions of BGS assay, were then designed according to the distribution of methylated CpG sites to detect the methylation status of MEG3 within these regions in all tissue specimens (Fig. 3C). As shown in Table 2, of primary tumor tissues and corresponding normal tissues, hypermethylation was observed in 36.6% (49/134) and 3.0% (4/134) at region 1, 2.2% (3/134) and 0.0% (0/134) at region 2, 3.7% (5/134) and 0.0% (0/134) at region 3, 68.6% (92/134) and 10.4% (14/134) at region 4, respectively. Among the four regions, the methylation frequency of...
FIGURE 3  Methylation status of CpG dinucleotides in the MEG3 promoter region. (A) Schematic structure of MEG3 CpG islands. The four MSP regions analyzed are indicated. (B) High-resolution mapping of the methylation status of every CpG site in the four MEG3 promoter regions by BGS assay in four GCA tissues (with loss expression of MEG3) and corresponding normal tissues. Each CpG site is shown at the top row as an individual number. Percentage methylation was determined as percentage of methylated cytosines from 8 to 10 sequenced colonies. The color of circles for each CpG site represents the percentage of methylation. (C) The methylation status of four regions of MEG3 determined by BS-MSP analysis in GCA tumor tissues. m: methylated; u: unmethylated. (D) Relative expression of MEG3 in the tumor tissues with and without methylation of the four regions. *P < 0.05. (E) Relative expression of miR-770-5p in the tumor tissues with and without methylation of the four regions. *P < 0.05.
regions 1 and 4 was significantly higher than that of regions 2 and 3 in tumor tissues and the methylation status of regions 1 and 4 was more tumor-specific. When stratified for clinicopathologic characteristics, the methylation status of region 1 was associated with TNM stage, and distant metastasis or recurrence; the methylation status of region 4 was associated with TNM stage, depth of invasion, LN metastasis, and distant metastasis or recurrence (Table 3).

3.8 | Association between MEG3 and miR-770 expression and promoter methylation status

The association between MEG3 and miR-770 expression and promoter methylation was further detected in GCA tissues. As shown in Fig. 3D,E, the expression level of MEG3 and miR-770 in GCA tissues with hypermethylation of regions 1 and 4 was significantly lower than that with unmethylation of both regions, and the effect was more significant at region 4 (P < 0.05). These data further demonstrated that regions 1 and 4 may be more functionally important on MEG3 gene and miR-770 regulation.

3.9 | Survival analysis of MEG3 and miR-770 in ESCC

We further analyzed the relationship between the promoter methylation and expression of MEG3 and miR-770 and the prognosis of ESCC patients. Kaplan-Meier analysis indicated that downregulation of MEG3 was significantly associated with poorer ESCC patients’ overall survival (log-rank test, P < 0.05) (Fig. 4A). ESCC cases with hypermethylation of regions 1 or 4 showed worse survival rates compared to the ESCC cases with unmethylation of both regions, and the effect was more obvious for region 4 (Fig. 4B,C). As shown in Fig. 4D, downregulation of miR-770 was significantly associated with poorer ESCC patients’ survival.

Cox multivariate analysis was done using MEG3 expression, regions 1 and 4 methylation, miR-770 expression, tumor stage, pathological differentiation, as well as other confounding variables such as patient age, gender, and UGIC family history. As shown in Table 4, TNM stages, pathological differentiation, and MEG3 expression were independently associated with GCA patients’ survival.

4 | DISCUSSION

Maternally expressed gene 3 (MEG3) is located at 14q32, a region in which chromosomal abnormalities are associated with the pathogenesis and progression of several tumor types. Gene structure analysis reveals that miR-770 is transcribed from within intron 6 of MEG3, and MEG3 may be the host gene for miR-770 and the transcriptional regulation of miR-770 may follow that of its host gene MEG3. However, the role and inactivation mechanisms of MEG3 and miR-770 have not been thoroughly investigated in GCA. In the present study, significant downregulation of MEG3 and miR-770 was detected in GCA tumor tissues compared with normal tissues, and the expression level of MEG3 and miR-770 in GCA tumor tissues was associated with TNM stage, depth of invasion, lymph node metastasis, and distant metastasis or recurrence. Furthermore, a strong positive correlation was found between the expression of MEG3 and miR-770 in GCA tissues. The expression level of MEG3 and miR-770 was remarkably downregulated in gastric cancer cell lines and the expression level was significantly reversed by treatment with 5-Aza-dC. Overexpression of MEG3 and miR-770 demonstrated the inhibition effects on gastric cancer cell proliferation and invasion in vitro. Further methylation analysis showed frequent hypermethylation of the CpG sites within regions 1 and 4 in tumor tissues, while hypermethylation of the CpG sites within regions 2 and 3 was rarely detected in tumor tissues. The expression level of MEG3 and miR-770 was significantly associated with hypermethylation of regions 1 and 4, and the effect was more significant at region 4. In addition, MEG3 and miR-770 expression and regions 1 and 4 methylation status may be associated with GCA patients’ survival. Therefore, MEG3 and miR-770 may act as tumor suppressors in GCA progression and prognosis.

Downregulation of MEG3 has been found in many types of cancer cell lines and primary human tumors.11-20 It has been reported that aberrant methylation of DMRs of MEG3 may contribute to the downregulation of the gene.14-20 MEG3 gene expression may be tightly controlled by at least two differentially methylated regions (DMRs): The intergenic DMR (IG-DMR) and the MEG3-DMR. The two DMRs are located upstream of the MEG3 gene. The investigation about the epigenetic mechanism underlying the absence of MEG3 expression in human clinically non-functioning pituitary tumors demonstrated no genomic abnormality in the tumors examined. However, two 5′-flanking regions, immediately in front of and approximately 1.6-2.1 kb upstream of the first exon (overlaps with the MEG3-DMR), respectively, were hypermethylated in pituitary tumors without MEG3 expression compared with the normal pituitary. These two regions clearly demonstrated strong promoter activities in activation of reporter gene expression.19 Further analysis demonstrated

| TABLE 2 | Methylation status of MEG3 in GCA tumor tissues and corresponding normal tissues |
|----------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
| Group    | Region 1                          | Region 2                          | Region 3                          | Region 4                          |
|          | n (%) P                           | n (%) P                           | n (%) P                           | n (%) P                           |
| Normal tissues | 134 4(3.0) 0.001 | 0(0.0) 0.001 | 0(0.0) 0.001 | 14(10.4) 0.001 |
| Tumor tissues | 134 49(36.6) <0.001 | 3(2.2) 0.247 | 5(3.7) 0.060 | 92(68.6) <0.001 |
that 1.6-2.1 kb upstream of the first exon of MEG3 was located in the enhancer region, and the hypermethylation of this region was also observed in epithelial ovarian cancer, menigoia, acute myeloid leukemia and myelodysplastic syndromes, myeloma, and hepatocellular cancer. In the present study, we detected the methylation status of four regions spanning the 5′-flanking region of MEG3, consistent with the studies in pituitary tumors, we also found frequent hypermethylation of proximal promoter (region 1) and enhancer region (region 4) in GCA tissues, suggesting that this two regions may be more functionally important on MEG3 gene regulation.

Most of the intronic miRNAs show correlated expression and similar regulation mechanisms with their host genes in human. The miR-31 and its host gene IncRNA LOC554202 are regulated by promoter hypermethylation in triple-negative breast cancer, miR-26a/b and their host genes CTDP1/2/L are frequently downregulated in hepatocellular carcinoma tissues and cooperate to inhibit the G1/S transition by activating the pRb protein. In the present study, we first showed that miR-770 was transcribed from the intronic sequence of MEG3, both miR-770 and its host gene MEG3 were expressed abundantly in normal gastric epithelium cell line GES1 and downregulated in gastric cancer cell lines and GCA tissues, together with the positive correlation between MEG3 and miR-770 expression in GCA tissues, clearly suggesting that the transcription regulation of miR-770 might be under the control of MEG3. The increased expression of both MEG3 and miR-770 in the gastric cancer cell lines after treatment with 5-Aza-dC, the intimate association between MEG3 and miR-770 expression and regions 1 and 4 methylation status in GCA tissues further indicated that MEG3 and miR-770 may share common epigenetic regulation mechanism. However, more experimental analyses are required to verify the results.

It has been reported that low expression levels of MEG3 is an independent prognostic factor for poor clinical outcome in tongue squamous cell carcinoma patients, non-small cell lung cancer patients with decreased MEG3 expression levels have significantly shorter survival times than those with high MEG3 expression levels. In order to clarify the relationship between the methylation status and expression of MEG3 and miR-770 and the prognosis of GCA, we further detected the critical role of MEG3 and miR-770 in the prognosis of GCA. We found that the overall survival time of patients with downregulation of MEG3 or miR-770 or regions 1 and 4 hypermethylation was significantly

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<tr>
<td>Pathological differentiation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well/moderate</td>
<td>89</td>
<td>30 (33.7)</td>
<td>0.334</td>
<td>57 (64.0)</td>
<td>0.310</td>
</tr>
<tr>
<td>Poor</td>
<td>45</td>
<td>19 (42.2)</td>
<td>0.334</td>
<td>35 (77.8)</td>
<td>0.106</td>
</tr>
<tr>
<td>Depth of invasion</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1/2</td>
<td>42</td>
<td>12 (28.6)</td>
<td>0.334</td>
<td>23 (54.8)</td>
<td>0.106</td>
</tr>
<tr>
<td>T3/4</td>
<td>92</td>
<td>37 (40.2)</td>
<td>0.194</td>
<td>69 (75.0)</td>
<td>0.019</td>
</tr>
<tr>
<td>LN metastasis</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Negative (N0)</td>
<td>20</td>
<td>4 (20.0)</td>
<td>0.095</td>
<td>9 (45.0)</td>
<td>0.013</td>
</tr>
<tr>
<td>Positive (N1/2/3)</td>
<td>114</td>
<td>45 (39.5)</td>
<td>0.095</td>
<td>83 (72.8)</td>
<td>0.013</td>
</tr>
<tr>
<td>Distant metastasis or recurrence</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>61</td>
<td>16 (26.2)</td>
<td>0.023</td>
<td>35 (57.4)</td>
<td>0.010</td>
</tr>
<tr>
<td>Positive</td>
<td>73</td>
<td>33 (45.2)</td>
<td>0.023</td>
<td>57 (78.1)</td>
<td>0.010</td>
</tr>
<tr>
<td>Family history of UGIC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>78</td>
<td>25 (32.1)</td>
<td>0.200</td>
<td>50 (64.1)</td>
<td>0.180</td>
</tr>
<tr>
<td>Positive</td>
<td>56</td>
<td>24 (42.9)</td>
<td>0.200</td>
<td>42 (57.0)</td>
<td>0.180</td>
</tr>
</tbody>
</table>
shorter than that of patients with higher expression levels of MEG3 or miR-770 or unmethylation of regions 1 and 4. Therefore, the proximal promoter and enhancer region hypermethylation and dysregulation of MEG3 and miR-770 may be useful markers for GCA tumor progression and prognosis.

In summary, decreased expression of miR-770 and its host gene MEG3 is a common event underlying GCA development, indicating the tumor suppressor role of both genes in GCA progression. Furthermore, aberrant hypermethylation of proximal promoter and enhancer region of MEG3 may be one of the critical mechanisms on MEG3 and miR-770 silencing in GCA. In addition, MEG3 and miR-770 may be potential useful biomarkers in predicting GCA patients’ prognosis and act as key regulators in human GCA carcinogenesis.

<table>
<thead>
<tr>
<th>Variable</th>
<th>B</th>
<th>SE</th>
<th>P</th>
<th>Odds ratio (95%CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNM stage</td>
<td>1.312</td>
<td>0.301</td>
<td>0.000</td>
<td>3.714 (2.059-6.699)</td>
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<tr>
<td>Pathological differentiation</td>
<td>0.399</td>
<td>0.153</td>
<td>0.009</td>
<td>1.490 (1.105-2.010)</td>
</tr>
<tr>
<td>Family history of UGIC</td>
<td>0.195</td>
<td>0.231</td>
<td>0.400</td>
<td>1.215 (0.772-1.912)</td>
</tr>
<tr>
<td>MEG3 expression</td>
<td>1.583</td>
<td>0.597</td>
<td>0.008</td>
<td>4.870 (1.510-15.704)</td>
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<tr>
<td>MEG3 region 1 methylation</td>
<td>−0.136</td>
<td>0.252</td>
<td>0.590</td>
<td>0.873 (0.533-1.431)</td>
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<tr>
<td>MEG3 region 4 methylation</td>
<td>−0.721</td>
<td>0.609</td>
<td>0.236</td>
<td>0.486 (0.147-1.603)</td>
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<tr>
<td>MiR-770-5p expression</td>
<td>0.269</td>
<td>0.380</td>
<td>0.478</td>
<td>1.309 (0.622-2.755)</td>
</tr>
</tbody>
</table>
ACKNOWLEDGMENTS

We thank the cases for taking part in this study. This study was supported by Grants from the National Natural Science Foundation (Nos. 81472335 and 81572441), Natural Science Foundation of Hebei Province (Nos. H2015206196 and H2015206420).

REFERENCES


SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.