Original Article

MEG3 regulates cell cycle progression via control of P53 expression in gastric cancer

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Abstract: Gastric cancer is the second leading cause of cancer deaths worldwide. Recently IncRNA has been reported playing important role in many diseases especially in the progress of tumor formation. However the roles of many IncRNAs in gastric cancer remain largely unclear. IncRNA-MEG3 has been invested playing key role in many diseases especially in central nervous system diseases. In our study, we examined the expression of MEG3 and P53 mRNA in 59 gastric cancer tissues and their adjacent tissues. In addition we tested the expression level of IncRNA-MEG3 in gastric epithelial cells and gastric cancer cell lines such as AGS, BGC823, SGC7901, N87 using qRT-PCR and found that the expression level of MEG3 in gastric cancer tissues and cell lines were much lower than that of adjacent tissues and GES1 cell line. Transfection of pCDNA-MEG3 could significantly inhibit the cell cycle in BGC823 cell line. What’s more, we also found the expression of protein P53 was upregulated in a great extent in the group of pCDNA-MEG3 compared with the. In conclusion our study suggested that IncRNA-MEG3 is down expression in gastric cancer and regulate the process of carcinogenesis and may serve as a therapeutic target of gastric cancer in future.

Keywords: Gastric cancer, IncRNA-MEG3, P53, cell cycle, therapeutic target

Introduction

Gastric cancer is the fourth most prevalent cancer worldwide and the second leading cause of cancer-related death [1]. In China, gastric cancer is the second leading cause of death from cancer [2, 3]. Although great improvements have been made in the early detection and treatment of gastric cancer, the molecular mechanisms of the development of gastric cancer remain unclear [4, 5].

Therefore, in this study, we explored the mechanisms of gastric cancer. The Human Genome Project (HGP) found that more than 90% of the genome is noncoding RNA. Long noncoding RNA (lncRNA) is a class of endogenous, poorly conserved RNA that lacks protein-coding potential because of the absence of ORF. In contrast to the length of small ncRNAs such as miRNAs, siRNAs, and piRNAs, the length of long noncoding RNA varies from 200 hundred nucleotides to tens of thousands of bases [6-8]. lncRNA studies have reported that noncoding transcripts can, among other effects, change the chromosome structure [9], and modulate gene activity [10] as well as enhance gene transcription [11]. These aforementioned molecular mechanisms have been cited as suggesting that lncRNAs participate in diverse biological processes, especially tumorigenesis. The biological features of cancer influenced by lncRNAs include cell proliferation and cell cycle [12], migration [13], differentiation, moving, apoptosis [14]. In gastric cancer, many lncRNAs can function as either tumor suppressors or oncogenesis with complicated mechanisms. For example, GAS5, an lncRNA, can influence gastric cancer cell proliferation, partly by regulating E2F1 and P21 expression [14].

MEG3 was first identified on mouse distal chromosome 12 while locating on human's chromosome 14q32 [15, 16]. It expressed in many normal tissues such as human pituitary but lost in kinds of human cancers and tumor cell lines including urothelia carcinoma [17], tongue...
squamous cell carcinoma [18], liver fibrogenesis [19] and lung cancer [20]. Studies have suggested that hypermethylation of differentially methylated regions (DMRs) contribute to the silence of MEG3 gene in human tumors [21, 22]. In addition it has been proved that MEG3 can induce the accumulation of P53 and stimulate the expression of the growth differentiation factor 15 (GDF15) [16].

Here, paired gastric cancer tissue samples were collected to detect the relative expression of MEG3 by RT-PCR and the relationship between clinicopathological features and expression of MEG3 was analyzed. We demonstrated that upregulation of IncRNA-MEG3 can inhibit cell proliferation while cells accumulated in G1 phase. To make further, western blot analysis was used to detect whether the expression of P53 changed or not after the upregulation of MEG3. Hence we come to a conclusion that IncRNA-MEG3 can affect the cell cycle via regulating the key player P53.

**Materials and methods**

**Patient and tissue samples**

Matched gastric cancer and adjacent nontumor tissues were obtained from 59 patients who underwent primary surgical resection at Qilu Hospital of Shandong University between February 2015 and September 2015. All tissues were immediately snap-frozen in liquid nitrogen and stored at -80°C until total RNA was isolated. The tumor was staged by Tumor Node Metastasis (TNM) Staging. This project was approved by the Clinical Research Ethics Committee of Qilu Hospital of Shandong University.

**Cell lines and cultures**

Human gastric cancer cell lines (BGC823, SGC7901, AGS and N87) and human gastric epithelium-immortalized cell line (GES1) were gifted from Shandong Province Key Laboratory for Tumor Target Molecules, Medical Research and Laboratory Diagnostic Center. The BGC823, AGC-7901, N87 and GES1 were maintained in an atmosphere of 5% CO₂ in RPMI-1640 medium (Hyclone, Beijing, China) supplemented with 10% fetal bovine serum (Hyclone, Beijing, China) at 37°C while AGS maintained in F12 medium with the same condition.

**Transfection**

Cells (BGC823 cell line) were seeded into six-well plates and the plasmid with pCDNA-MEG3 (Gene Chem, Shanghai) and empty vectors were transfected into the cells by Lipofectamine 2000 reagent (Invitrogen, Shanghai, China) according to the manufacturer's protocols. After 24 h and 72 h, transfected cells were harvested for protein extraction and cell cycle analysis.

**Total RNA extraction and quantitative reverse-transcription polymerase chain reaction analyses**

Total RNA was isolated from frozen tissues and cultured cells using TRIzol reagent (Invitrogen, USA) according to the manufacturer's protocols. The RNA concentration was measured by ultraviolet-visible spectrophotometer at A260/280. Then cDNA was generated using PrimeScriptTM RT reagent Kit (Takara, Dalian, China) with 500 ng total RNA, 2 ul 5×PrimeScript Buffer, 0.5 ul PrimeScript RT Enzyme Mix I, 0.5 ul Oligo dT Primer (50 μM), 0.5 ul Random 6mers (100 μM) and RNase Free dH₂O up to 10 ul incubated at 37°C for 15 min and 85°C for 5 sec. For quantitative real-time polymerase chain reaction (qRT-PCR), we used the SYBR® Premix Ex TaqTM II (Takara, Dalian, China) in the CFX-96 real-time PCR System (BIO-RAD, USA). The PCR mixture solution included 12.5 ul SYBR Premix Ex Taq II, 1 ul forward primer (10 μM), 1 ul reverse primer (10 μM), 2 ul cDNA product and 8.5 ul nuclease-free water and incubated at 95°C for 15 s, 40 cycles of 95°C for 5 s and 60°C for 30 s followed by the melt curve to test the specificity of the PCR product. GAPDH was used to normalize the relative expression of RNA in our experiments. The PCR primers for MEG3, P53 mRNA and GAPDH were as follows: MEG3 sense 5'GCCTGCTGCCCATCTACAC3' and reverse 5'CCTCTTCATCCTTTGC-CATC3'; P53 sense 5'GTTCCGAGAGCTGAATGGG3' and reverse 5'TCTGAGTCAGGCCCTTCTGT3'; GAPDH sense 5'AGCCACATCGCTCAGACGC3' and reverse 5'GCCCCATAGCGAAATTCC3'. The 2-ΔΔCT method was used to calculate the relative expression of P53 mRNA and IncRNA-MEG3 in all samples.

**Western blotting analysis**

For western blotting, BGC823 cells were transfected with pCDNA-MEG3 plasmid and the
MEG3 and P53 in gastric cancer

Figure 1. LncRNA-MEG3 and P53 mRNA expression was tested by qRT-PCR. A. The relative expression of MEG3 in gastric cancer tissues (n=59) were significantly lower than those in paired adjacent tissues (P=0.003); B. The P53 mRNA expression levels in carcinoma lesions was significantly lower compared to those of noncancerous lesions (P=0.004). *P<0.05, **P<0.01.

Table 1. Association between the expression of MEG3 and the clinicopathological factors in human gastric-cancers

<table>
<thead>
<tr>
<th>Clinical parameters</th>
<th>n</th>
<th>Relative expression of MEG3</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>I</td>
<td>II</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td>0.581</td>
</tr>
<tr>
<td>Male</td>
<td>39</td>
<td>0.618 (0.114-1.459)</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>20</td>
<td>0.318 (0.090-1.201)</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td>0.872</td>
</tr>
<tr>
<td>≥60</td>
<td>25</td>
<td>0.277 (0.102-1.392)</td>
<td></td>
</tr>
<tr>
<td>&lt;60</td>
<td>34</td>
<td>0.502 (0.095-1.220)</td>
<td></td>
</tr>
<tr>
<td>Diameter (cm)</td>
<td></td>
<td></td>
<td>0.953</td>
</tr>
<tr>
<td>≥10</td>
<td>7</td>
<td>0.275 (0.149-0.755)</td>
<td></td>
</tr>
<tr>
<td>10&gt;X≥5</td>
<td>24</td>
<td>0.265 (0.101-1.278)</td>
<td></td>
</tr>
<tr>
<td>&lt;5</td>
<td>28</td>
<td>0.558 (0.091-1.467)</td>
<td></td>
</tr>
<tr>
<td>Differentiation</td>
<td></td>
<td></td>
<td>0.677</td>
</tr>
<tr>
<td>Well</td>
<td>5</td>
<td>0.362 (0.108-1.087)</td>
<td></td>
</tr>
<tr>
<td>Moderate</td>
<td>16</td>
<td>0.562 (0.065-1.426)</td>
<td></td>
</tr>
<tr>
<td>Moderate-poor</td>
<td>31</td>
<td>0.498 (0.149-1.586)</td>
<td></td>
</tr>
<tr>
<td>Poor</td>
<td>7</td>
<td>0.176 (0.081-0.249)</td>
<td></td>
</tr>
<tr>
<td>Lymphatic metastasis</td>
<td></td>
<td></td>
<td>0.425</td>
</tr>
<tr>
<td>NO-N1</td>
<td>32</td>
<td>0.633 (0.100-1.320)</td>
<td></td>
</tr>
<tr>
<td>N2-N3</td>
<td>27</td>
<td>0.274 (0.091-1.470)</td>
<td></td>
</tr>
<tr>
<td>Distant metastasis</td>
<td></td>
<td></td>
<td>0.017</td>
</tr>
<tr>
<td>M0</td>
<td>54</td>
<td>0.401 (0.095-1.179)</td>
<td></td>
</tr>
<tr>
<td>M1</td>
<td>5</td>
<td>1.772 (1.108-2.824)</td>
<td></td>
</tr>
</tbody>
</table>

I: Median of relative expression, with 25th-75th percentile in parentheses. II: P<0.05 was considered to indicate a statistical analysis.

with RIPA lysis buffer (Beyotime, Beijing, China) according to the manufacturer’s instructions. Protein (20 ug) for each sample was resolved by 10% SDS-PAGE and then transferred to PVDF membranes. The membranes were blocked using TBST buffer (TBS plus 0.1% Tween-20) with 5% fat-free milk and then incubated with rabbit anti P53 monoclonal antibody (1:1000, Cell Signaling Technology, Danvers, MA, USA). The rabbit anti β-actin monoclonal antibody (1:1000, Cell Signaling Technology, Danvers, MA, USA) as the internal control at 4°C overnight. The specific HRP-conjugated secondary antibody (1:5000, Zhanshan Goldenbridge, Beijing, China) was used as secondary antibodies and ECL chromogenic substrate was used to quantify the expression level of specific protein and signals were quantified by FluorChem E (Protein Simple, USA).

Cell cycle analysis

Cells were harvested by typsinization and washed three times using the cold phosphate-buffered saline (PBS). 70% ethanol was used to fix the cells for cell cycle analysis at 4°C about 16 hours and then cells were washed by cold PBS. 200 ul cold PBS was added to weight the cells and mixed the presidium iodide (PI, BD) 500 ul with the cells for 30 min in the dark. The BD FACScato II was used to analyze the cell cycle.
MEG3 and P53 in gastric cancer

Figure 2. MEG3 and P53 expression in gastric cancer cell lines. $1 \times 10^6$ cells were collected and lysed with TRIzol reagent to extract total RNA and further the relative expression of lncRNA-MEG3 (A) and P53 mRNA (B) was examined by qRT-PCR. The results displayed that not only lncRNA-MEG3 expression in gastric cancer cells was lower compared with GES1 cells but also P53 mRNA was lower significantly; (C) RIPA lysis buffer was used to extract the protein from cells and western blot to determine the protein expression with $\beta$-actin as the internal control.

Figure 3. The lncRNA-MEG3 up regulates P53 expression at the posttranscriptional level. BGC823 cells were transfected with pCDNA-MEG3 plasmid and empty vector by Lip 2000. The expression of MEG3 expression was determined by RT-PCR (A) while P53 was detected using RT-PCR (B) and Western Blotting (C). The data suggested that the lncRNA-MEG3 can up-regulate the P53 expression at the posttranscriptional level.
MEG3 and P53 in gastric cancer

Expression of lncRNA-MEG3 and P53 mRNA in human gastric cancer and non-tumor tissues

The lncRNA-MEG3 and P53 mRNA expression levels of tumor and adjacent noncancerous tissues from the gastric cancer patients were tested by qRT-PCR. Significantly decreased of the expression level of MEG3 was observed in tumor tissues compared with the adjacent tissues ($P=0.003<0.01$, **Figure 1A**). In addition, we found that the expression level of P53 mRNA in cancer tissues from patients with gastric cancer was lower than that in matched non-tumor tissues ($P=0.004<0.01$, **Figure 1B**).

The association of MEG3 expression levels and clinicopathological factors

The relationship between lncRNA-MEG3 expression level and clinicopathological factors of human gastric cancer was also been explored in our study. The expression of MEG3 in patients with distant metastasis was significantly higher than that in other tissues and data was shown in the **Table 1**. However, MEG3 expression level didn’t show significantly association with other clinicopathological factors such as gender ($P=0.581$), age ($P=0.872$), diameters ($P=0.953$).

MEG3 and P53 expression in gastric cancer cell lines

The expression of lncRNA-MEG3 and P53 mRNA was quantified by qRT-PCR in gastric cancer cell lines and normalized by GAPDH. As

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**Figure 4.** The transfection efficiency of the plasmid. As shown in the picture, the transfection efficiency can up to 90% in both pCDNA-MEG3 and empty vector group.
MEG3 and P53 in gastric cancer

The data shown in Figure 2, the expression of MEG3 in gastric cancer lines was significantly lower than that in the GES1 cells (P<0.01) and what’s more, P53 mRNA expression displayed similar to lncRNA-MEG3. In addition, the result of protein P53 determined by western blot showed expression deletion in SGC7901, BGC823 and N87 cell lines while less expression in AGS cells.

The IncRNA-MEG3 regulates P53 expression

P53 has been investigated to be a tumor suppressor in many cancers. In our study, we devoted to clarify the relationship between IncRNA-MEG3 and P53 in gastric cancer. The plasmid with pCDNA-MEG3 and empty vector were transfected into gastric cell line BGC823. We found that compared with the group of empty vector, the expression of MEG3 in the group with pCDNA-MEG3 plasmid was up-expression about 5000 fold or even more (P<0.01). On the other hand, the expression of P53 mRNA in BGC823 cell with pCDNA-MEG3 was 1.21 (24 h), 1.16 (72 h) fold compared to the normal control (P<0.05). At the same time, western blot was also used to detect the changement of the protein expression of P53.

Table 2. Cell cycle distribution of BGC823 cell lines with different treatment

<table>
<thead>
<tr>
<th></th>
<th>G0/G1 (%)</th>
<th>S (%)</th>
<th>G2/M (%)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells untreated</td>
<td>71.42±6.10</td>
<td>20.91±5.36</td>
<td>7.68±0.81</td>
<td></td>
</tr>
<tr>
<td>Empty vector</td>
<td>72.78±3.96</td>
<td>19.15±2.19</td>
<td>8.07±2.11</td>
<td></td>
</tr>
<tr>
<td>pCDNA-MEG3</td>
<td>76.06±2.16</td>
<td>17.92±1.53</td>
<td>6.02±1.47</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

As shown in Figure 3, the protein expression of P53 in BGC823 cells with the pCDNA-MEG3 was much higher than the group.

Low expression of MEG3 delayed cell cycle progression

To explore the role of MEG3 in gastric tumor cell cycle distribution in vivo, flow cytometric analysis was conducted. The plasmid with pCDNA-MEG3 and empty vector were transfected into gastric cell line BGC823. The results displayed that the group with pCDNA-MEG3 showed significantly G1 arrest (76.06±2.16%) compared with the empty plasmid group (72.78±3.96%), while there are no significant difference between non-transfected BGC823 cells and the empty control (Figures 4, 5 and Table 2). The results suggested that IncRNA-MEG3 can delay the cell cycle via G1 arrest and as a result the lower expression of IncRNA-MEG3 cause the tumor growing faster.

Discussion

A commonly definition for IncRNA is a class of RNA that longer than 200 nucleotides and without the function of translation into protein [23]. Many studies have investigated that the IncRNA play key roles in many diseases including central nervous system diseases [24-26] and especially cancers [23] such as bladder cancer [27], breast cancer [28], lung cancer [29]. In gastric cancer, many IncRNAs have been demonstrated to participate in the tumorigenecity...
MEG3 and P53 in gastric cancer

As we all know, P53 functioned as a tumor suppressor by activating or downregulating gene expression. It has been confirmed that P53 can regulate the cell cycle by the p53-p21-DREAM-CDE/CHR pathway [31]. In our study, the IncRNA-MEG3 was significantly downexpressed in both gastric cancer tissues and cell lines. To explore the relationship between MEG3 and P53, the pCDNA-MEG3 plasmid was constructed and transfected into gastric cancer line BGC823. The results showed that up-expression of MEG3 can positively regulate the expression of P53 protein but the P53 mRNA showed negative changement. It can be suspect that IncRNA-MEG3 regulate the P53 expression mainly at posttranscriptional level. Maybe IncRNA-MEG3 activates the P53 expression by interacting with P53 DNA binding domains or activating the P53 promoter or other P53 target genes. Further more studies need to be conducted to clarify the specific molecular mechanism of MEG3 and P53 interactions.

We further demonstrated that IncRNA-MEG3 plays role in the cell cycle distribution by overexpression the P53. Therefore the downexpression of IncRNA-MEG3 contribute to the development of gastric cancer. In addition, the relationship between different expression level of MEG3 and the gastric tumor pathological stage distant metastasis (P<0.05). The results suggested that the gastric cancer with higher expression of IncRNA-MEG3 was limited the cells proliferation compared with the lower group and then the group of higher expression choose the distant metastasis.

Our findings indicated that the IncRNA-MEG3 can regulate the protein P53 expression at posttranscriptional level in the development of gastric cancer. However the specific molecular mechanisms remain unclear. What’s more, it has been demonstrated the IncRNA-MEG3 can enhance the ability of p53 by binding to the growth differentiation factor 15 (GDF15) to stimulate the expression of the GDF15 using HCT116 and U2OS cell lines [16].

Conclusion

In brief, the expression of IncRNA-MEG3 was positively correlated with the distant metastasis of gastric tumor and the cell cycle distribution. The research also has the condition to limit, the size of tissue samples was not enough to do more analysis and the survival time also needs to keep observation. In addition, making the genetically engineered mouse models would be of great method to clarify the physiological functions and specific molecular mechanisms. Maybe IncRNA-MEG3 can be a therapeutic target of gastric cancer in the future and helpful in individualized medication.

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Disclosure of conflict of interest

None.

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