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Original Article

The impact of lncRNA MG3 on laryngeal cancer cell growth, cycle, and apoptosis related factors

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Abstract: Laryngeal cancer is a common head and neck malignant tumor. Long non-coding RNA (lncRNA) is a kind of RNA at the length more than 200 nucleotides that cannot code protein. They widely exist in the human genome. Maternally expressed gene 3 (MG3) is a kind of lncRNA that is associated with various malignant tumors development. This study explores the influence of MG3 on laryngeal cancer Hep-2 cell growth, cycle, and apoptosis related factors. Hep2 cells transfected by MG3 were treated as experimental group, while untransfected Hep2 cells were treated as control. MG3 expression was tested by real time PCR. Cell proliferation was evaluated by MTT assay. Cell cycle was determined by flow cytometry. Bcl-2, Bax, and survivin protein levels were detected by Western blot. MG3 expression significantly increased in Hep2 cells compared with control ($P < 0.05$). Hep2 cell OD value and cell percentage in S phase were obviously declined, while cell apoptosis were markedly enhanced ($P < 0.05$). OD value and cell percentage in S phase apparently reduced in 12 h, 24 h, and 48 h from experimental group ($P < 0.05$). Bcl-2 and survivin protein downregulated, while Bax protein elevated in experimental group following time extension ($P < 0.05$). MG3 overexpression inhibited laryngeal cancer Hep2 cell proliferation and arrested cell cycle with time dependence, which may achieve by suppressing Bcl-2 and survivin protein, and facilitating Bax protein expression.

Keywords: MG3, laryngeal cancer, proliferation, cell cycle, Bcl-2, surviving, Bax

Introduction

As a common type of head and neck malignant tumor in medical oncology, laryngeal cancer accounts for about 5.7-7.6% among various malignant tumors. Laryngeal cancer can be divided into three main types according to pathogenic site, such as supraglottic type, glottis type, and subglottic type. Its major pathologic type is squamous cell carcinoma [1]. Surgery, radiotherapy, chemotherapy, and gene therapy are the main forms of treatment for laryngeal cancer. Laryngeal cancer in early stage can be cured by surgery or radiochemotherapy. On the contrary, the curative effect and quality of life in patients with advanced laryngeal cancer is significantly reduced [2]. At the genetic level, there are numerous modes of human genome transcription. Only 2% of genes transcribe into proteins, whereas 98% of genes may become noncoding RNA (ncRNA) [3]. Most of ncRNAs are long non-coding RNAs (lncRNAs) at the length of longer than 200 amino acids, which mainly exist in the nucleus and cytoplasm of eukaryotes. Instead of protein coding, lncRNAs can only participate in biological processes at genetic, transcription, and posttranscription levels [4, 5]. Maternally expressed gene 3 (MG3) is a kind of lncRNA expressed in the brain, bone marrow, breast, uterus, lung, and gastrointestinal tract. MG3 is found expression deletion in multiple malignant tumors [6]. MG3 downregulation may involve in gastric cancer growth, proliferation, and apoptosis [7]. However, MG3 expression in laryngeal cancer and impact on cell proliferation and cell cycle are still unclear. This study aimed to investigate the impact of lncRNA MG3 on laryngeal cancer cell growth, cycle, and apoptosis related factors by transfecting MG3 to Hep2 cells.

Table 1. Primer sequences

Gene		Sequences (5'-3')
MG3	Forward	5'-AGCCGTCAAGAGCAATAACGAA-3'
	Reverse	5'-GTGCAGGGTCCGAGGT-3'
U6	Forward	5'-CTCGCTTCGGCAGCACA-3'
	Reverse	5'-AACGCTTCACGAATTTGCGT-3'

Materials and methods

Cells and reagents

Laryngeal cancer Hep-2 cells were obtained from Chinese Academy of Medical Sciences Institute of Basic Medical Sciences. MTT was purchased from Sigma (USA). Fetal bovine serum was bought from Sijiqing (Hangzhou, China). Annexin V-PE apoptosis detection kit was derived from Sigma (USA). Rabbit anti human Bcl-2, Bax, and survivin polyclonal antibodies, and HRP labeled goat anti rabbit IgG secondary antibody were got from ZSbio (Beijing, China). Rabbit anti human β-actin monoclonal antibody was purchased from Sigma (USA).

Routine cell culture

Hep2 cells were cultured in DMEM medium containing 10% FBS and maintained at 5% CO_2 and 37°C. The cells were digested by 0.25% trypsin and seeded in 24-well plate at 10^5/L.

Grouping

Experimental group: Hep-2 cells in logarithmic phase were seeded in 6-well plate at 1×10^4/ml. MG3 vector was transfected to Hep-2 cells using lipofectamine 2000 when the cell fusion reached 60%. The cells were further cultured for 48 h after fluid change.

Control group: Hep-2 cells cultured in RPMI-1640 medium containing 10% FBS.

Real-time PCR

Trizol was used to extract total RNA from Hep2 cells. The cells were then added with 0.2 ml chloroform at room temperature for 3 min. Next, the supernatant was added with 0.5 ml isopropanol at room temperature for 10 min. After centrifuged to remove the supernatant, the RNA was washed by 75% ethanol and dissolved in 40 μl DEPC water. Total RNA was reverse transcribed to cDNA by synthesizing poly A tail. cDNA was used as template for PCR amplification. The primer sequences were listed **Table 1**. The PCR reaction was composed of 95°C predegeneration for 5 min, followed by 30 cycles of 95°C degeneration for 30 s, 58°C annealing for 30 s, and 72°C elongation for 30 s, and 72°C for 10 min at last.

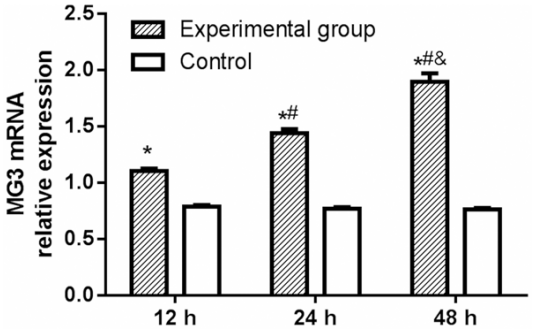


Figure 1. MG3 expression in Hep-2 cells. RNA was isolated from Hep-2 cells transfected with MG3 vector after culture for 12 h, 24 h and 48 h followed by measuring MG3 mRNA expression by RT-PCR. *P < 0.05, compared with control. #P < 0.05, compared with 12 h. &P < 0.05, compared with 24 h.

MTT assay

Hep-2 cells in logarithmic phase were seeded at 8×10^4/well for 12 h, 24 h, and 48 h, respectively. Then the cells were added with 20 μl MTT solution at 5 mg/ml for 4 h. Next, the cells were treated by 150 μl DMSO for 10 min and measured at 570 nm on microplate reader. The growth curve was drawn upon OD value and time.

Annexin V/PI detection

Hep-2 cells were collected at 48 h after MG3 transfection and added with FITC labeled Annexin V at 4°C for 10 min. Then the cells were added with 5 μg/ml PI for 2 min. At last, the cells were resuspended in buffer and analyzed on flow cytometry.

Cell cycle analysis

Hep-2 cells were digested and centrifuged at 1000 r/min for 5 min. Next, the cells were collected in centrifuge tube at 1×10^9/L and fixed in 75% ice ethanol for 12 h. At last, the cells were stained by PI avoid of light at 4°C for 30 min and analyzed on flow cytometry.

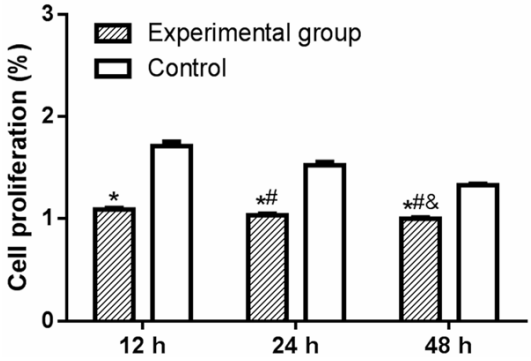


Figure 2. Hep-2 cell proliferation. After transfected with MG3 vector, proliferation of Hep-2 cell was measured by MTT assay. *P < 0.05, compared with control. #P < 0.05, compared with 12 h. &P < 0.05, compared with 24 h.

Western blot

The cells were lysed by RIPA containing 0.1 mg/ml PMSF, aprotinin, and phosphatase inhibitor and centrifuged at 4°C and 12000 rpm for 30 min. The protein was added with loading buffer and boiled at 100°C for 5 min. Total protein was separated by 10% SDS-PAGE and transferred to PVDF membrane. After washed by TBST, the membrane was blocked at 4°C overnight. Next, the membrane was incubated in primary antibody (1:100) at 37°C for 2 h and secondary antibody at room temperature for 1 h. At last, the membrane was scanned and analyzed by Alphamager TM 2200 software to calculate the relative expression.

Statistical analysis

All data analyses were performed on SPSS 17.0 software. Enumeration data were compared by chi-square test, while measurement data were depicted as mean ± standard deviation and evaluated by ANOVA. P < 0.05 was considered as statistical significance.

Results

MG3 expression in Hep-2 cells

We applied RT-PCR to detect MG3 expression in Hep-2 cells. Compared with control, MG3 expression significantly increased in Hep-2 cells from experimental group (P < 0.05). MG3 level gradually elevated in Hep-2 cells following time extension (P < 0.05) (**Figure 1**).

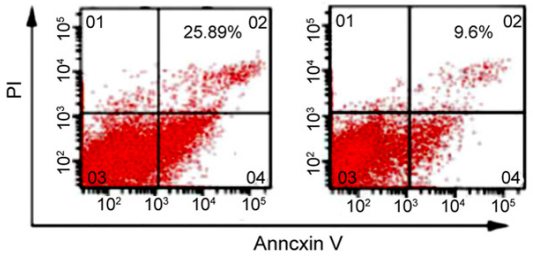


Figure 3. Hep-2 cell apoptosis after 48 h. Apoptosis of Hep-2 cells was measured by flow cytometry using PI and Annexin-V staining.

Hep-2 cell proliferation comparison

We adopted MTT assay to compare Hep-2 cell proliferation. It was showed that Hep-2 cell OD value significantly declined in experimental group compared with control following time extension (P < 0.05) (**Figure 2**).

Hep-2 cell apoptosis comparison

Hep-2 cell apoptosis obviously enhanced in experimental group compared with control (**Figure 3**).

Hep-2 cell cycle comparison

It was found that cell percentage in S phase markedly reduced in 12 h, 24 h, and 48 h from experimental group compared with control (P < 0.05). Its proportion in S phase kept decreasing following time expansion (P < 0.05) (**Figure 4**).

Bcl-2, Bax, survivin protein expression in Hep-2 cells

Bcl-2 and survivin protein downregulated, while Bax protein elevated in experimental group following time extension (P < 0.05) (**Figures 5**, **6**).

Discussion

LncRNA is a kind of RNA with transcript longer than 200 nt. It locates in the nucleus or cytoplasm without protein coding function [8]. In recent years, it was showed that lncRNAs participate in regulating gene expression at multiple levels, such as X chromosome silence, genomic imprinting, and chromatin modification, transcription activation, nuclear transport [9]. LncRNAs participate in the process of cell apoptosis and cell cycle. Their abnormal expressions may induce various diseases, especially

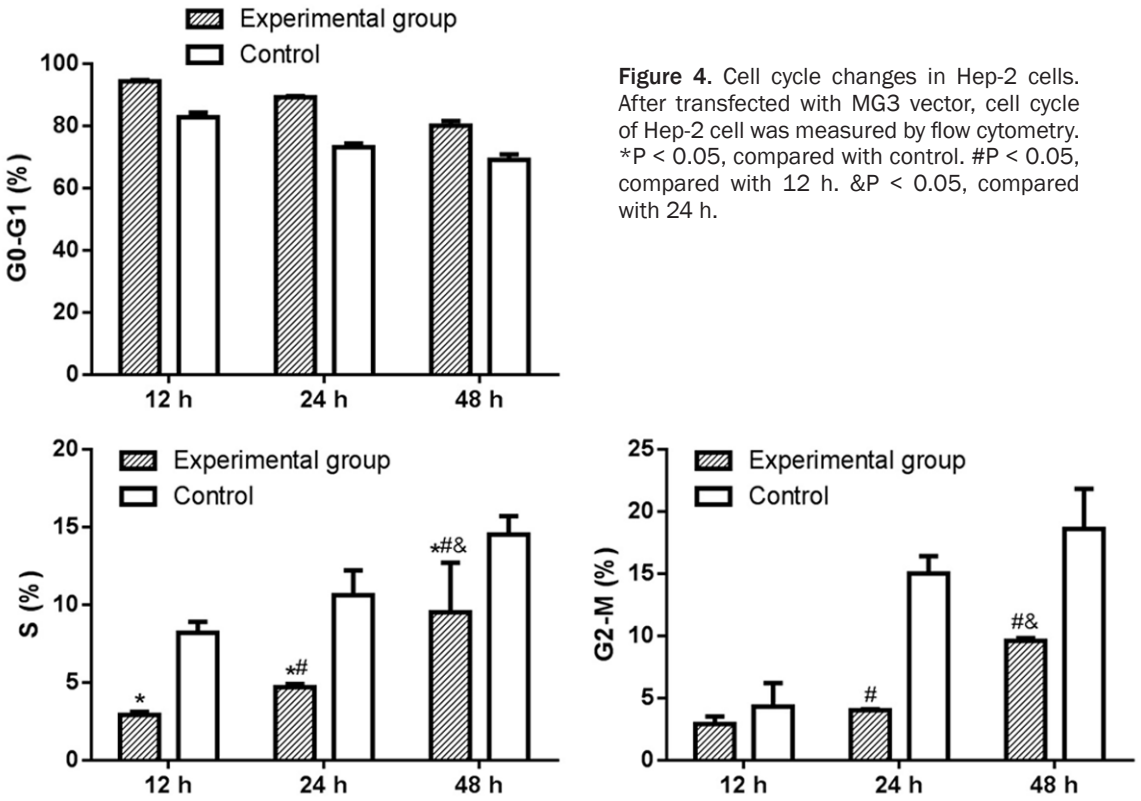


Figure 4. Cell cycle changes in Hep-2 cells. After transfected with MG3 vector, cell cycle of Hep-2 cell was measured by flow cytometry. *P < 0.05, compared with control. #P < 0.05, compared with 12 h. &P < 0.05, compared with 24 h.

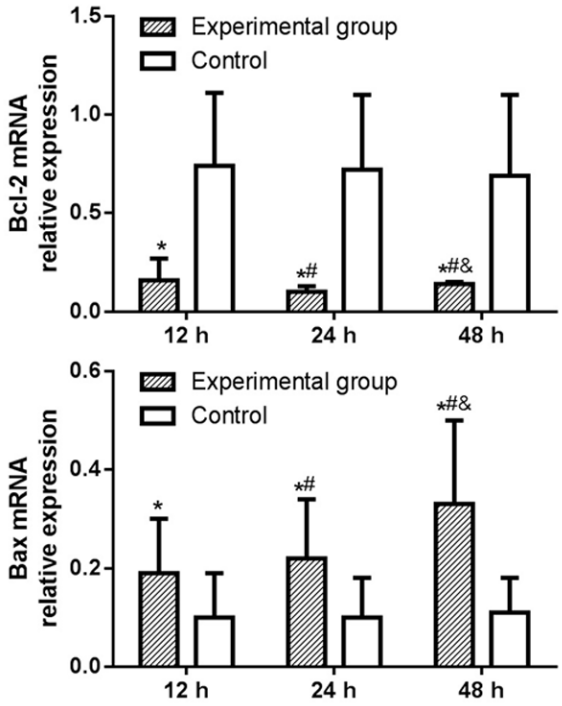
cancer [10]. MG3 is a kind of lncRNA that inhibits cell proliferation and promotes apoptosis [11]. This study selected laryngeal cancer Hep-2 cells to explore the influence of MG3 on laryngeal cancer cell growth, cycle, and apoptosis related factors.

This study transfected MG3 to Hep-2 cells and observed MG3 level increased in experimental group following time extension. It suggested successful MG3 transfection. Previous study revealed that as an lncRNA coded by imprinted gene, MG3 was obviously expressed in embryonic development and a variety of normal cells. However, it was found declined or deleted in multiple tumors, such as hypophysoma, hepatocellular carcinoma, and ovarian cancer [12, 13].

Hep2 cell OD value was obviously declined in experimental group following time expansion, while cell apoptosis was markedly enhanced after 48 h. It indicated that MG3 overexpression suppressed Hep-2 cell proliferation. Regulation of p53 can affect non-small cell lung cancer cell proliferation and apoptosis [14]. MG3 downregulation was found correlated with poor prognosis of gastric cancer [15]. This was similar with our results.

The most obvious characteristics of tumor cells are incessant cell division and proliferation. Cell cycle out of control plays a crucial role in malignant tumor, presenting as cell cycle disorders, cell proliferation enhancement, and apoptosis reduced [16]. Dysregulation of checkpoint for G1/S and G2/M phases may lead to abnormal cell proliferation, resulting in the occurrence of malignant tumor [17]. It was found that cell percentage in S phase markedly reduced in 12 h, 24 h, and 48 h from experimental group compared with control. Its proportion in S phase kept decreasing following time expansion. It suggested that MG3 overexpression can increase Hep-2 cell proportion in G1 phase and reduce cell proportion in S phase, leading to G1/S phase arrest. It indicated that MG3 can inhibit tumor growth and proliferation by regulating cell cycle with time dependence.

A variety of studies confirmed that cell apoptosis may cause cell death or lost. It was observed



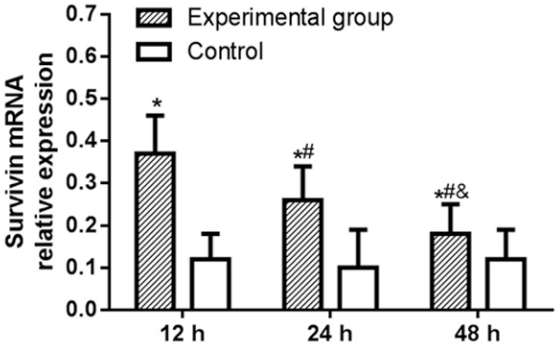


Figure 5. Bcl-2, Bax, survivin mRNA expressions in Hep-2 cells. RNA was isolated from Hep-2 cells followed by measuring mRNA expression of Bcl-2, Bax and Survivin by RT-PCR. *P < 0.05, compared with control. #P < 0.05, compared with 12 h. &P < 0.05, compared with 24 h.

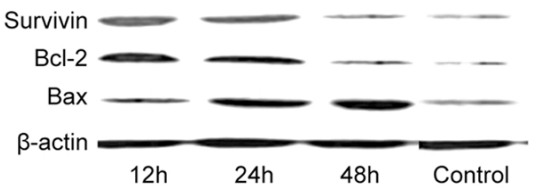


Figure 6. Bcl-2, Bax, survivin protein expressions in Hep-2 cells. Protein was isolated from Hep-2 cells for analysis of the expression of Bcl-2, Bax and Survivin by western blot.

that multiple genes play critical roles in cell apoptosis, including pro-apoptotic genes and anti-apoptotic genes [18]. We used Western blot to test Bcl-2, Bax, and survivin protein expressions in Hep-2 cells. Bcl-2 and survivin protein downregulated, while Bax protein elevated in experimental group following time extension. Caspase-3 participates in cell apoptosis induced by various factors, thus is the most important apoptosis effect protein [19]. Bcl-2 can prolong cell survival, thus plays an inhibitory role on cell apoptosis without affecting cell division [20]. Bax may induce cell apoptosis via downregulating Bcl-2 [21]. It suggested that MG3 promoted cell apoptosis by suppressing Bcl-2 and survivin protein, and facilitating Bax protein expression.

Conclusion

MG3 overexpression inhibited laryngeal cancer Hep2 cell proliferation and arrested cell cycle with time dependence, which may achieve by suppressing Bcl-2 and survivin protein, and facilitating Bax protein expression. Detection of MG3 level in clinic in laryngeal cancer patients may provide valuable evaluation on prognosis from gene and protein aspects [22]. Further in-depth investigation may provide better choice for the laryngeal cancer treatment.

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Disclosure of conflict of interest

None.

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