

Original Article

miR-204 regulates HMGA2 to suppress the proliferation and promote the apoptosis of esophageal squamous carcinoma cells

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Abstract: Objective: To investigate the role of miR-204 in esophageal squamous cell carcinoma (ESCC) and to determine the potential molecular mechanisms underlying its action. Methods: miR-204 expression in ESCC tissues and cell lines was detected through qRT-PCR. The targeted regulation of miR-204 on the target gene HMGA2 was verified through dual-luciferase reporter genes. Meanwhile, the effect of the miR-204/HMGA2 axis on ESCC cells was investigated through cell CCK-8 assay and flow cytometry. Results: miR-204 expression in ESCC tissues was markedly lower than that in para-carcinoma tissues and low miR-204 expression was closely correlated with the lymph node metastasis and tumor stage of ESCC patients. Moreover, miR-204 expression in human ESCC cell lines TE-13, Eca-109, KYSE-410 and EC9706 was notably lower than that in normal human esophageal cell line Het-1A. miR-204 could bind with the binding site at the 3' UTR of HMGA2, thus markedly down-regulating the luciferase activity. Up-regulating miR-204 expression in KYSE-410 cells could remarkably down-regulate the HMGA2 mRNA and protein expression, suppress the cell proliferation activity, and promote cell apoptosis. In addition, Up-regulating HMGA2 expression could reverse these effects. Conclusion: miR-204 expression is abnormally down-regulated in ESCC, and miR-204 overexpression can suppress the proliferation and promote the apoptosis of ESCC cells, the mechanism of which may be related to the targeted regulation on HMGA2.

Keywords: Esophageal squamous cell carcinoma, miR-204, proliferation, apoptosis, HMGA2

Introduction

Esophageal carcinoma is a highly malignant digestive system tumor, which is associated with a high mortality and great hazards. With regard to pathological type, esophageal adenocarcinoma is more frequently seen in European and American countries, while esophageal squamous cell carcinoma (ESCC) is more commonly seen in China and Japan [1]. Comprehensive treatments such as aggressive surgery, as well as radiochemotherapy, have been adopted; however, most esophageal carcinoma patients will inevitably die of local tumor recurrence and distant metastasis, and the overall 5-year survival remains low [2]. In recent years, people can learn tumor from a more microscop-

ic angle with the continuous development of disciplines like molecular biology. Besides, progresses have been attained in the pathogenesis study and tumor treatment, which render rapid development of targeted therapy and immune therapy. On the other hand, the formation process of esophageal carcinoma also involves a series of abnormal molecular events, such as genetic and epigenetic changes. However, key gene for the early detection, early diagnosis and specific treatment of esophageal carcinoma has not been discovered so far.

miRNA family exists in all animals and plants, accounting for about 4% of the genome. It plays a wide role in eukaryotic gene expression and regulation and participates in the basic pro-

cess of regulating life. It is believed that miRNA is directly involved in the expression of at least 50% of mammalian protein coding genes [3]. miRNA genes are often located near fragile sites in the genome and chromosomal regions associated with tumors. In recent years, it has been proved that miRNA plays a role of tumor suppressor or oncogene in the development of various malignant tumors [4-6]. miR-204 is one of the important members of the miRNA family, which is located in human chromosome 9. Recent studies suggest that miR-204 is abnormally expressed in numerous malignant tumors, including breast cancer [7], hepatocellular carcinoma (HCC) [8], colon cancer [9], cervical cancer [10] and prostate cancer [11], which is closely correlated with the tumor biological behaviors like tumor cell proliferation and apoptosis, but the mechanism of action remains to be fully elucidated. Therefore, this study analyzed the expression of miR-204 in ESCC, so as to explore the underlying mechanism of action.

Material and methods

Tissue specimens

The tissue specimens were derived from the fresh cancer tissue specimens and the fresh corresponding para-carcinoma tissues (at least 5 cm from the tumor edge) in 140 esophageal carcinoma patients undergoing surgical resection. The necrotic and connective tissues were removed from all tissue specimens immediately after separation, and the tissue specimens were washed with the pre-cooled saline, and preserved in the refrigerator at -80°C . The derived specimens were pathologically verified to be squamous cell carcinoma. All patients had no malignant tumors in other organs, and were naive to chemotherapy or radiotherapy before surgery. This study was approved by the Medical Ethics Committee of our hospital, and all patients had signed the informed consent form to participate in the study.

Cell lines and cell transfection

Human ESCC cell lines, including TE-13, Eca-109, KYSE-410 and EC9706, as well as normal human esophageal cell line Het-1A, were derived from Shanghai Institute of Cellular Biology of Chinese Academy of Sciences. KYSE-410 cells at exponential phase were collected and inoculated in the 6-well culture plates

under conventional conditions, and cell transfection was performed after 60-70% cell convergence was achieved. In experiment one, NC-mimics and miR-204 mimics (Gemma Pharmaceutical, China) were transfected into KYSE-410 cells, respectively, in strict accordance with the Lipofectamine™2000 (Invitrogen, USA) instructions. 24 h after transfection, miR-204 expression was detected through qRT-PCR, and the transfection efficiency was determined prior to subsequent cell experiment. In experiment two, HMGA2-overexpression plasmid pcDNA-HMGA2 (Gemma Pharmaceutical, China) was transfected into KYSE-410 cells with high expression of miR-204. 24 h after transfection, HMGA2 protein expression was detected through Western blotting.

qRT-PCR

The total RNA in cells was extracted by TRIzol reagent (Invitrogen, USA), and reversely transcribed into cDNA after identifying its purity and content in accordance with the reverse transcription kit (Baosheng Biology, China) instructions. Then, the reaction system for PCR reaction was prepared according to the qRT-PCR kit (Baosheng Biology, China), with cDNA as the template. The miR-204, U6, HMGA2 and GAPDH primers were purchased from RiboBio (China). The $2^{-\Delta\Delta\text{Ct}}$ method was used for result analysis.

Dual-luciferase reporter genes

The luciferase genes were constructed by Gemma Pharmaceutical (China), among which, the luciferase vector pmirGLO-HMGA2-WT carrying the wild type HMGA2 3'UTR was named as WT-HMGA2, while the luciferase vector pmirGLO-HMGA2-MT carrying the mutant HMGA2 3'UTR after point mutation was named as MT-HMGA2. Afterwards, the NC-mimics or miR-mimics and WT-HMGA2 or MT-HMGA2 vectors were co-transfected into the KYSE-410 by Lipofectamine™ 2000 (Invitrogen, USA). Cells were collected 24 h after transfection, and the luciferase activity were detected using the dual-luciferase activity detection kit (Promega, USA).

Western blot

The total protein in cells was extracted conventionally. 40 μg sample was extracted from each

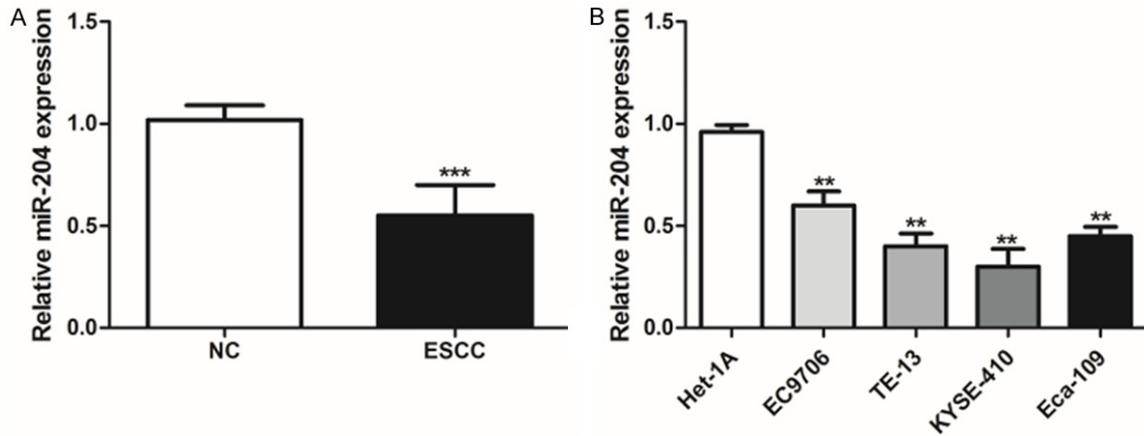


Figure 1. The expression levels of miR-204 in ESCC tissues and cells. A. miR-204 expression in ESCC tissues was remarkably lower than that in para-carcinoma tissues. ***, $P < 0.001$. B. miR-204 expression in human ESCC cell lines (TE-13, Eca-109, KYSE-410 and EC9706) was dramatically lower than that in normal human esophageal cell line (Het-1A). **, $P < 0.01$.

sample and separated through 10% SDS-PAGE, the electrophoresis products were transferred onto the PVDF membranes, sealed with 5% skim milk powder at 4°C for 4 h, and washed with TBS buffer solution for three times. Subsequently, primary antibodies HMGA2 (Santa Cruz, USA) at appropriate concentrations were added to incubate at 4°C overnight. Later, secondary antibody (Santa Cruz, USA) was added to incubate at room temperature for 2 h, followed by ECL developing. The band gray scale was analyzed using the Quality One software, and the ratio of HMGA2 to β -actin gray scale was indicative of the relative expression quantity.

CCK-8

CCK-8 was performed in accordance with the CCK-8 kit (Beyotime, China) instructions. Cells were inoculated into the 96-well plate. Three duplicate wells were set for each time point, and 10 μ L CCK-8 reaction solution was added into the detection wells at 24, 48, 72 and 96 h, respectively, to further incubate for 3 h at 37°C. Then, the optical density (OD) values of each well at different time points were determined at the wavelength of 450 nm on the Spectra Max 190 microplate reader, and the proliferation curve could then be plotted.

Flow cytometry

Cells in each group 48 h after transfection were collected, isolated using trypsin digestive solu-

tion, and re-suspended after centrifugation, then 5 μ L Annexin V-FITC (eBioscience, USA) was added to react in dark for 10 min, and 10 μ L PI (eBioscience, USA) was also added to mix sufficiently using the oscillator. Later, flow cytometry was performed to calculate the cell apoptosis rate.

Statistical method

Data were statistically analyzed using the SPSS 19.0, measurement data were expressed as mean \pm SD, and t -test was used to compare the measurement data between two groups, and one-way analysis of variance (ANOVA) was used to compare the measurement data between multiple groups. The correlation of miR-204 expression with clinic and pathological characteristics was analyzed using the χ^2 -test. All experiments were repeated for at least 3 times. A difference of $P < 0.05$ was deemed as statistically significant.

Results

miR-204 expression was down-regulated in ESCC

As shown in **Figure 1A**, miR-204 expression in ESCC tissues was remarkably lower than that in para-carcinoma tissues ($P < 0.001$), and that in human ESCC cell lines (TE-13, Eca-109, KYSE-410 and EC9706) was also dramatically lower than that in normal human esophageal cell line (Het-1A) ($P < 0.01$, **Figure 1B**).

Table 1. Relationships between miR-204 expression level and the clinicopathological features of ESCC patients

Parameters	Total	Low-miR-204	High-miR-204	P-value
Gender				0.262
Male	100	47	53	
Female	40	23	17	
Age (year)				0.410
≥60	110	57	53	
<60	30	13	17	
Tumor size (cm)				0.172
≥5	80	44	36	
<5	60	26	34	
Tumor differentiation				0.122
High+Moderate	83	37	46	
Poor+Undifferentiation	57	33	24	
TNM stage				0.004
I+II	77	30	47	
III	63	40	23	
Lymphatic metastasis				0.008
Yes	91	53	38	
No	49	17	32	

Relationships of miR-204 expression level with the clinicopathological features of ESCC patients

The median level of miR-204 expression was treated as the threshold to divide the patients into high miR-204 expression group and low miR-204 expression group. Statistical analysis discovered that, low miR-204 expression was closely correlated with the lymph node metastasis and tumor stage of ESCC patients ($P<0.01$), but not with the gender, age, tumor length and differentiation degree ($P>0.05$) (Table 1).

miR-204 binds directly and specifically to the 3'UTR of HMGA2

The potential target gene of miR-204 was predicted using bioinformatics. HMGA2 was predicted as the potential target gene of miR-204 after looking up to miRanda and Targentscan, and there was complementary binding site in terms of seed sequence in the 3'UTR between miR-204 and HMGA2 (Figure 2A). Results of dual-luciferase activity detection indicated that, miR-204 mimics could markedly suppress the luciferase activity of WT-HMGA2 gene reporter vector ($P<0.001$), but it showed no obvious suppression on that of MT-HMGA2 gene

reporter vector ($P>0.05$, Figure 2B), revealing that miR-204 might directly bind to the 3'UTR of HMGA2.

Up-regulating miR-204 expression could specifically regulate HMGA2 gene to suppress the proliferation activity of KYSE-410 cells and promote their apoptosis

qRT-PCR results were presented in Figure 3A, as could be seen, the miR-204 expression level in KYSE-410 cells of miR-204 mimics group was evidently higher than that in NC-mimics group ($P<0.001$), suggesting higher transfection efficiency of miR-204 mimics. The HMGA2 mRNA and protein expression levels in miR-204 mimics group were evidently lower than those in NC-mimics group ($P<0.01$, Figure 3B, 3C), further verifying that miR-204

could directly and specifically regulate HMGA2 gene. The proliferation activity of KYSE-410 cells in miR-204 mimics group was notably lower than that in NC-mimics group ($P<0.01$, Figure 3D, 3E), while the apoptosis rate was markedly higher than that in NC-mimics group ($P<0.001$), demonstrating that up-regulating miR-204 expression could specifically regulate HMGA2 gene to suppress the proliferation activity of KYSE-410 cells and promote their apoptosis.

Up-regulation of HMGA2 expression could reverse the effects of miR-204 on proliferation and apoptosis of KYSE-410 cells

To further verify whether miR-204 could affect the proliferation and apoptosis of KYSE-410 cells through specifically regulating HMGA2 gene, we upregulated the HMGA2 expression of KYSE-410 cells overexpressing miR-204 by transfecting HMGA2-overexpression plasmid pcDNA-HMGA2 ($P<0.01$) (Figure 4A). Up-regulation of HMGA2 expression could reverse the inhibitory effect of miR-204 on KYSE-410 cell proliferation ($P<0.01$) (Figure 4B), and reverse the promoting effect of miR-204 on KYSE-410 cell apoptosis ($P<0.001$) (Figure 4C). Such findings further suggested that, up-regulating miR-204 could suppress the proliferation activity

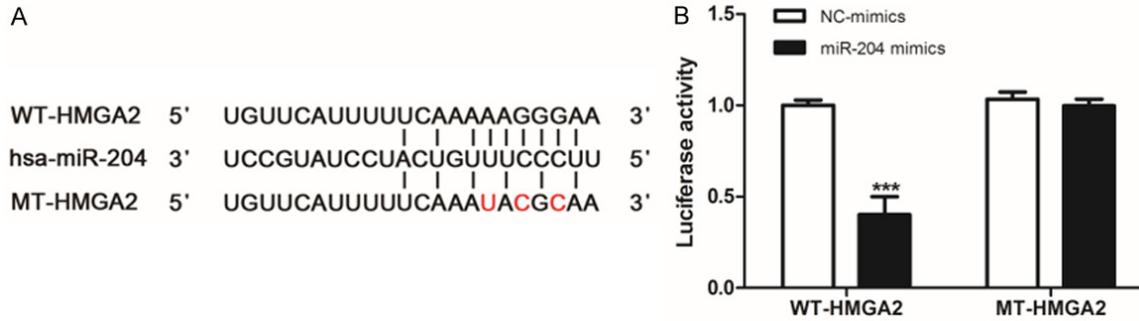


Figure 2. HMGA2 is a direct targeted regulatory gene of miR-204. A. Binding sites of miR-204 and HMGA2. B. Relative activity of luciferase reporters. ***, $P < 0.001$.

and promote the apoptosis of KYSE-410 cells through regulating HMGA2.

Discussions

In the world, esophageal cancer has become a serious threat to human life. In China, the number of new esophageal cancer patients reaches 250000 every year, and the morbidity and mortality rate rank the fourth. Due to the lack of early screening and targeted treatment strategies, the prognosis of esophageal cancer is still poor. As a new tumor treatment method, targeted therapy has attracted much attention. But up to now, there are few biological diagnostic markers and therapeutic targets of esophageal cancer that have practical clinical application value. Therefore, it is of great clinical value to study new target of biotherapy for esophageal cancer. A large number of studies indicate that, miRNAs play critical roles in the genesis and development of malignant tumors. miRNAs are frequently located in the tumor-related genomic region, and their functions as oncogene or tumor suppressor gene depend on the genes that they regulate and the tumor type [12]. The abnormal expression of many miRNAs is closely related to esophageal cancer. Research finds that expression of miR-516b [13], miR-125b [14], and miR-320 [15] is down-regulated in ESCC, which are deemed as the tumor suppressor genes to exert their roles. By contrast, miR-543 [16], miR-765 [17] and miR-141-3p [18] are markedly up-regulated in ESCC, which are considered as the oncogenes to exert their roles. miRNAs can regulate their target genes, thus participating in regulating the biological behaviors of esophageal carcinoma cells (invasion, metastasis and proliferation), as well as the genesis and progression of malignant tumors.

miR-204 is located in human chromosome [9], which is suggested to be down-regulated in numerous malignant tumors, such as breast cancer [7], HCC [8, 19], colon cancer [9], cervical cancer [10, 20], prostate cancer [11], glioblastoma multiforme [21], and gastric cancer [22], thus playing a role as a tumor suppressor gene. These findings indicate that miR-204 is involved in the development and progression of malignant tumors, but few related studies on the role of miR-204 in ESCC are available at present, and its function and mechanism in ESCC remain unclear yet. Therefore, in this study, it was found that miR-204 expression in ESCC decreased abnormally. Further statistical analysis revealed that, low miR-204 expression was closely correlated with the lymph node metastasis and tumor stage of patients, and miR-204 might also play a role as tumor suppressor gene in the genesis and development of ESCC. These studies demonstrate that miR-204 participates in the genesis and development of ESCC, which is promising to serve as the potential molecular target for the diagnosis and treatment of ESCC.

Infinite cell proliferation and blocked apoptosis are the basic biological characteristics of malignant tumor, and developing the endogenous miRNAs as the tumor therapeutic targets is a well-recognized promising research field at present. Targeting miRNAs can almost involve each feature of tumor cells. Therefore, targeting miRNAs is promising to become a crucial strategy for the targeted therapy of tumor [23]. In this study, miR-204 mimics was transfected into KYSE-410 cells through lipofection method. Results of CCK-8 assay indicated that, up-regulating miR-204 could markedly inhibit the proliferation activity of KYSE-410 cells, while flow cytometry indicated that up-regulating

miR-204 and HMGA2 in ESCC

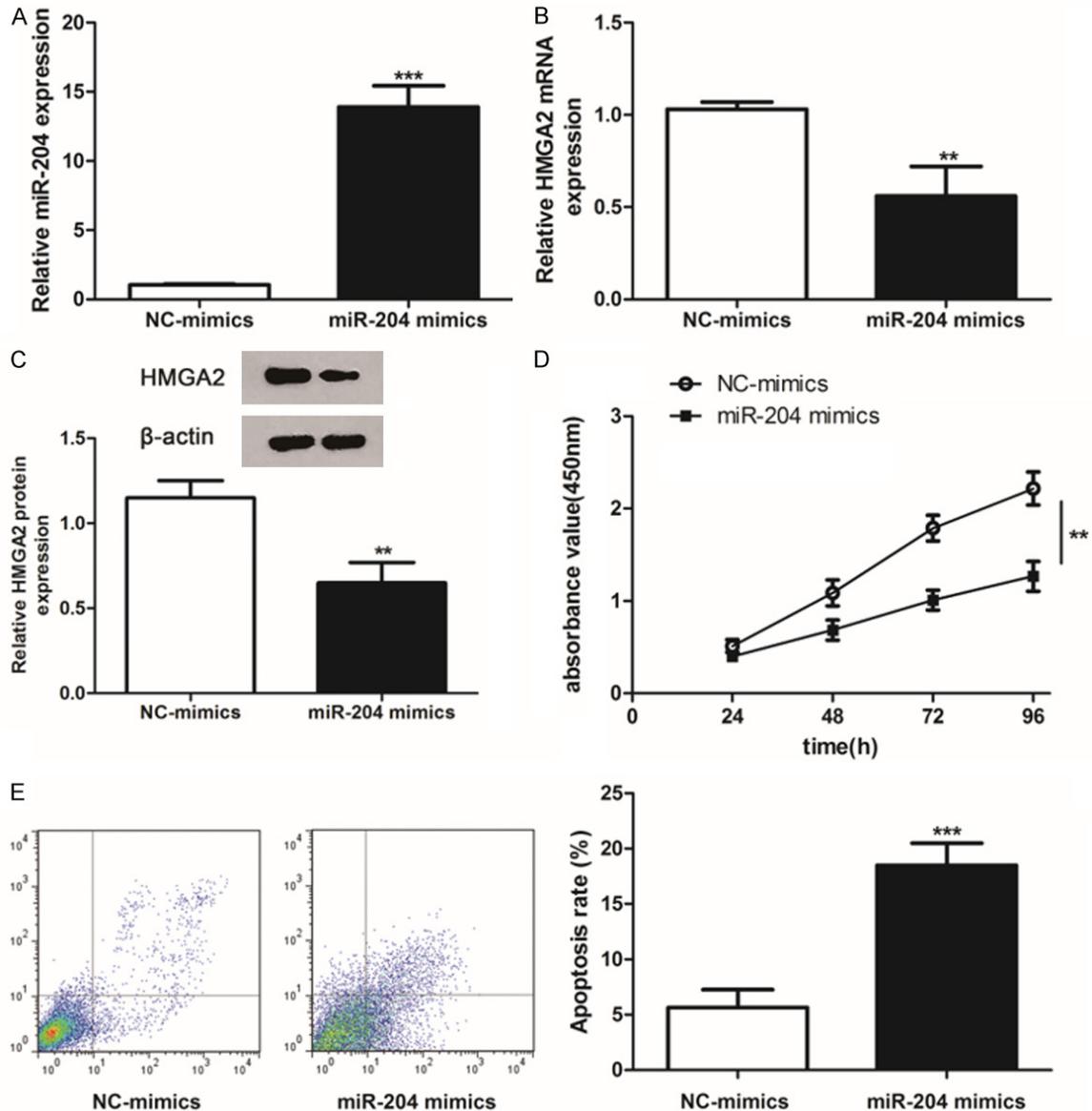


Figure 3. Up-regulating miR-204 expression could specifically regulate HMGA2 gene to suppress the proliferation activity of KYSE-410 cells and promote their apoptosis. A. miR-204 expression was detected through qRT-PCR. ***, $P < 0.001$. B and C. After overexpression of miR-204, HMGA2 mRNA and protein of KYSE-410 cells were decreased. **, $P < 0.01$. D. After overexpression of miR-204, the proliferative activity of KYSE-410 cells was decreased. **, $P < 0.01$. E. After overexpression of miR-204, the apoptotic rate of KYSE-410 cells was increased. ***, $P < 0.001$.

miR-204 could dramatically promote the apoptosis of KYSE-410 cells. These results were similar to those from studies on the role of miR-204 in other tumors [7-11, 19-22].

At present, an increasing number of studies indicate that, miRNAs exert different biological functions, which is closely correlated with the downstream target genes that it regulates [24]. The downstream molecular pathway by which miR-204 suppresses tumor cell proliferation

and promote apoptosis remains to be fully understood. To explore the downstream target gene of miR-204 in regulating ESCC cells, the most commonly used bioinformatics softwares miRanda and Targetscan were employed, which predicted that HMGA2 might be the target gene of miR-204, since there was seed sequence complementary binding site in the 3'UTR between miR-204 and HMGA2. Moreover, WU et al. [25] had recently discovered in their study that, miR-204 could suppress thymo-

miR-204 and HMGA2 in ESCC

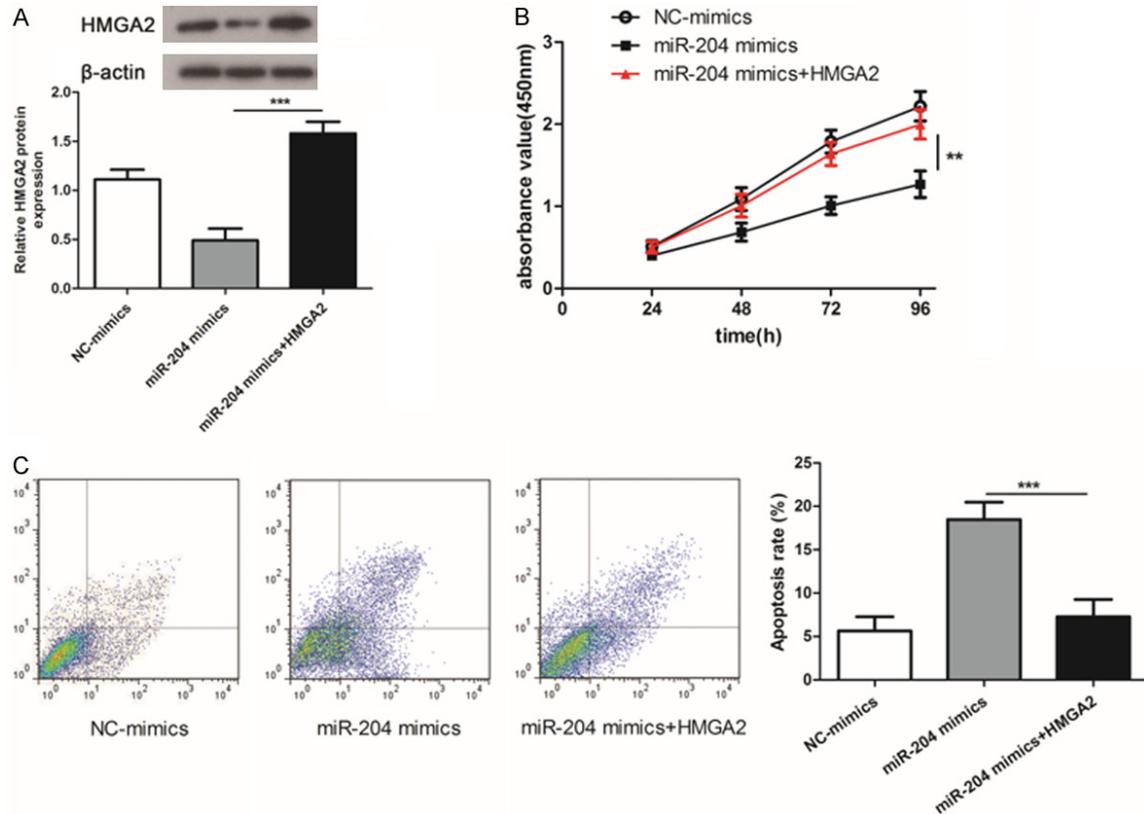


Figure 4. Up-regulation of HMGA2 expression could reverse the effects of miR-204 on proliferation and apoptosis of KYSE-410 cells. A. after transfected with HMGA2-overexpression plasmid, HMGA2 protein of KYSE-410 cells was increased. ***, $P < 0.001$. B. Up-regulation of HMGA2 expression could reverse the inhibitory effect of miR-204 on KYSE-410 cell proliferation. **, $P < 0.01$. C. Up-regulation of HMGA2 expression could reverse the promoting effect of miR-204 on KYSE-410 cell apoptosis. ***, $P < 0.001$.

cyte proliferation through specifically regulating HMGA2. In addition, it is also verified that, in oral carcinoma [26] and colon cancer [27], miR-204 can regulate the biological behaviors of tumor cells through specifically regulating HMGA2. To examine whether miR-204 could regulate HMGA2 to affect the biological behaviors of tumor cells in ESCC, dual-luciferase reporter gene assay was applied, which discovered that, miR-204 could directly and specifically bind with the 3'UTR of HMGA2 gene, thus decreasing the luciferase activity. Furthermore, it was discovered that, the HMGA2 mRNA and protein expression levels in ESCC KYSE-410 cells were markedly down-regulated after up-regulating miR-204 expression in cells. These studies have verified that, HMGA2 is a direct targeted regulatory gene of miR-204 in ESCC.

HMGA2 is a non-histone chromosomal protein, which is also one of the members of the high mobility group. In the early stage of embryonic

development, HMGA2 is expressed in almost all tissues except brain, and plays an important role in embryonic development. After 15 days of embryonic development, HMGA2 gene expression was shut down, which was almost undetectable in normal mature tissues except lungs and kidneys. However, there is abnormal activation of HMGA2 expression in many malignant tumors, including ESCC. HMGA2 is highly expressed in malignant tumors and is considered to be an important oncogene [28]. In recent years, the relationship between HMGA2 and ESCC has been reported. Many studies have confirmed that HMGA2 is abnormally high in ESCC, and the high expression of HMGA2 is related to the low differentiation, lymph node metastasis, recurrence, distant metastasis and poor prognosis of ESCC [29-31]. Recent studies indicate that, HMGA2 can up-regulate the expression of cyclin, accelerate the transformation of G2/M [32], promote cell proliferation [33], suppress cell apoptosis [34], and

thus promote tumor genesis and development. To further verify whether miR-204 could specifically regulate HMGA2 gene to affect the proliferation and apoptosis of KYSE-410 cells, we upregulated the HMGA2 expression of KYSE-410 cells with high expression of miR-204 by transfecting HMGA2-overexpression plasmid. The results showed that up-regulation of HMGA2 expression could reverse the inhibitory effect of miR-204 on KYSE-410 cell proliferation, and reverse the promoting effect of miR-204 on KYSE-410 cell apoptosis, further proving that miR-204 could restrain the proliferation activity and promote the apoptosis of KYSE-410 cells through regulating HMGA2.

In conclusion, miR-204 expression in ESCC decreased abnormally. Up-regulating miR-204 expression can markedly suppress the proliferation and promote the apoptosis of ESCC cells, and its mechanism may be related to the effect of miR-204 on specifically down-regulating HMGA2 gene expression.

Disclosure of conflict of interest

None.

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