Pathology - Research and Practice 215 (2019) 676–682

Contents lists available at ScienceDirect

Pathology - Research and Practice

journal homepage: www.elsevier.com/locate/prp

miR-129-5p attenuates cell proliferation and epithelial mesenchymal transition via HMGB1 in gastric cancer

Shaocheng Wang, Yanyan Chen, Xiongfei Yu, Yimin Lu, Haoao Wang, Fusheng Wu, Lisong Teng*

Department of Surgical Oncology, The First Affiliated Hospital, Zhejiang University, Hangzhou 310003, Zhejiang, PR China

ARTICLE INFO

Keywords:
miR-129-5p
Gastric cancer
Epithelial mesenchymal transition
HMGB1
Proliferation

ABSTRACT

Background: The miR-129-5p has been reported to be aberrant expression and exert vital roles in tumor progression of various malignancies. However, the effects on EMT in gastric cancer and its precise molecular mechanism in gastric cancer remain unclear.

Methods and materials: RT-qPCR was performed to evaluate the expression level of miR-129-5p and HMGB1 in cell lines. Cell proliferation was detected via CCK-8. The epithelial mesenchymal transition (EMT) related proteins and the expression of HMGB1 were detected by western blot analysis. Luciferase assays were used to validate binding seeds between miR-129-5p and HMGB1.

Results: miR-129-5p was downregulated in gastric cancer cells compared with GES-1. At the same time EMT was promoted in gastric cancer cells compared to GES-1. Overexpression of miR-129-5p inhibited EMT and proliferation. MiR-129-5p negatively and directly targeted HMGB1. HMGB1 was upregulated in gastric cancer cells and HMGB1 knocked-down inhibited EMT and cell proliferation.

Conclusion: Taken together, upregulation of miR-129-5p associated with gastric cancer proliferation and EMT, and serves as a potential diagnostic and therapeutic target via miR-129-5p/HMGB1 pathway in gastric cancer.

1. Introduction

Gastric cancer (GC) is one of the most common cancer and the third leading cause of cancer death in the world [1]. Most patients occur high rate of recurrence even they have had surgery [2]. Although there are advances in targeted therapy and pathophysiological processes, the limitation of GC treatment still remain. Metastasis is not only a sigh of deterioration but is also a major cause for failure of GC treatment [3].

MicroRNAs (miRNAs) are endogenous small RNA molecules consisting of 21–23 nucleotides which can bind the region of 3′ UTR of mRNA and subsequently inhibit the transcription and translation of target gene [4,5]. Researches verified that miRNA participates in initiation, progression and metastasis in cancers [6,7]. miR-129-5p is a mature form of miR-129-1 and miR-129-2 which has been demonstrated to be participated in various neoplastic progresses [8]. miR-129-5p is a subunit of miR-129 family which has been discovered in different cancer like prostate cancer, breast cancer and lung cancer [9–11]. miR-129-5p has been validated as a tumor suppressor in lots of cancers including GC. For example, downregulation of miR-129-5p expression inhibited cell proliferation and invasion by targeting ADAM9 [12].

Epithelial-mesenchymal transition (EMT) is considered as a key step of metastasis. Cells lose epithelial characteristics and turn to mesenchymal phenotype which is invasive and migratory during EMT [13]. Moreover, the expression of N-cadherin induced invasion in GC thought to override the E-cadherin function [14]. Many researches indicate that cancer stem cells within primary tumor is metastasized resulting from generation of EMT [15]. Therefore, enhancing better understanding of the molecular mechanisms underlying EMT of gastric cancer may significantly promote the improvement of therapeutic efficiency. Previous study revealed that miRNAs such as miR-646, miR-2392 AND miR-551b inhibit cell proliferation and EMT-induced metastasis [16–18]. However, the detailed roles of miR-129-5p in EMT of GC have not been validated and underlying mechanism need further confirm.

High mobility group box 1 (HMGB1) plays as a vital member of HMG family. Recent studies have indicated that HMGB1 is a chromatin binding nuclear protein which is expressed highly in different types of cancer and participates in cancer development [19,20]. Protein levels

* Corresponding author at: Department of Surgical Oncology, The First Affiliated Hospital, Zhejiang University, No. 79 Qingchun Road, Hangzhou 310003, Zhejiang, PR China.
E-mail address: lst_zhejiang@163.com (L. Teng).

https://doi.org/10.1016/j.prp.2018.12.024
Received 14 November 2018; Received in revised form 30 November 2018; Accepted 24 December 2018

Table 1
Primer sequences for reverse transcription.

Gene	Primer sequences (5'-3')
miR-129-5p	Forward: CGGCGGTTTTTTGCGGTCTGGGCT Reverse: AGCCCAGACCGCAAAAAACCGCCG
HMGB1	forward: AGGATCCCAATGCACCCAAG reverse: CGCAACATCACCAATGGACAG
GAPDH	forward: CCTGCCTCTACTGGCGCTGC reverse: GCAGTGGGGACACGGAAGGC
U6	forward: CTCGCTTCGGCAGAACA reverse: ACGCTTCACGAATTTGCGT

of HMGB1 are reported to be dramatically upregulated in most of gastric cancer tissues [21].

In this study, we explored expression levels of miR-129-5p in GC cells. Downregulation of miR-129-5p expression inhibited cell proliferation and EMT in vitro. In addition, we demonstrated that miR-129-5p attenuated cell proliferation and EMT via targeting HMGB1. Thus, these findings revealed that miR-129-5p as a potential target of GC treatment.

2. Materials and methods

2.1. Cell lines

GC cell lines, including SGC-7901, BGC-823, AGS were purchased from Cell Bank of Chinese Academy of Sciences (Shanghai), and MKN-45 was purchased form

Transfection assay Institute of Basic Medical Science, Chinese Academy of Medical Sciences (Beijing). Gastric mucosa epithelial, GES-1, was purchased from Cobioer, Ltd. (https://cobioer.biomart.cn, Shanghai). All of the cells were cultured in Dulbecco's modified Eagle medium (DMEM; Gibco, ThermoFisher) supplemented with 10% fetal bovine serum (FBS; Gibco, ThermoFisher) at 37 ℃, 5% CO_2.

2.2. Cell transfection

SGC-7901 were seeded in six well plates with the density of 35%. 24 h later, miR-129-5p mimics, mimics control (NC), siRNA-HMGB1(inhibitor 1, inhibitor 2), siRNA-control (NC) (GenePharma Co. Ltd., Shanghai) were transfected into cells using Lipofectamine® 2000 (Invitrogen) according to manual and cultured for 48 h.

2.3. RT-qPCR

Total RNA was isolated from cultured cells using TRIzol (Invitrogen) referring to manual. For detection the level of miR-129-5p and HMGB1, Reverse-transcription was operated by High Capacity RNA-to-cDNA Kit (Applied Biosystems, ThermoFisher) according to the manual. Subsequently, cDNA of miR-129-5p and HMGB1 were amplified through Power SYBR Green (Applied Biosystem, ThermoFisher). U6 and GAPDH were performed as internal control for miRNA and mRNA, respectively. Relative expression level was measured by $2^{-\Delta\Delta CT}$. Primers was performed in Table 1.

2.4. Cell proliferation assay

SGC-7901 cell proliferation was determined via CCK8 assay. Cells were cultured in 96-well plate with the concentration of 5.5×10^6 cells/well. Transfection was performed after the cells attached the wall. Then cell proliferation was measured at 24, 48, 72 h via CCK-8 kit (http://www.medchemexpress.cn, MedChemExpress) assay according to manufacture instructions. Absorbance results were detected at 450 nm.

2.5. Western blotting

Cells were lysed by Pierce IP Lysis Buffer (ThermoFisher) and concentration of protein was measured via Micro BCA Protein Assay Kit (ThermoFisher). SDS-PAGE was performed to separate total proteins and transferred to PVDF membrane. Membrane was blocked via 5% Bovine Serum Albumin (BSA) for 2 h. Then primary antibodies of E-cadherin, N-cadherin, Vimentin, GAPDH and HMGB1(All 1:1000; Cell signaling technology) were incubated over night at 4 ℃. HRP-conjugated secondary antibodies were incubated for 2 h. Enhanced chemiluminescence (ECL) kit was used to detect signals. Image J was used to semi-quantify the expression of proteins.

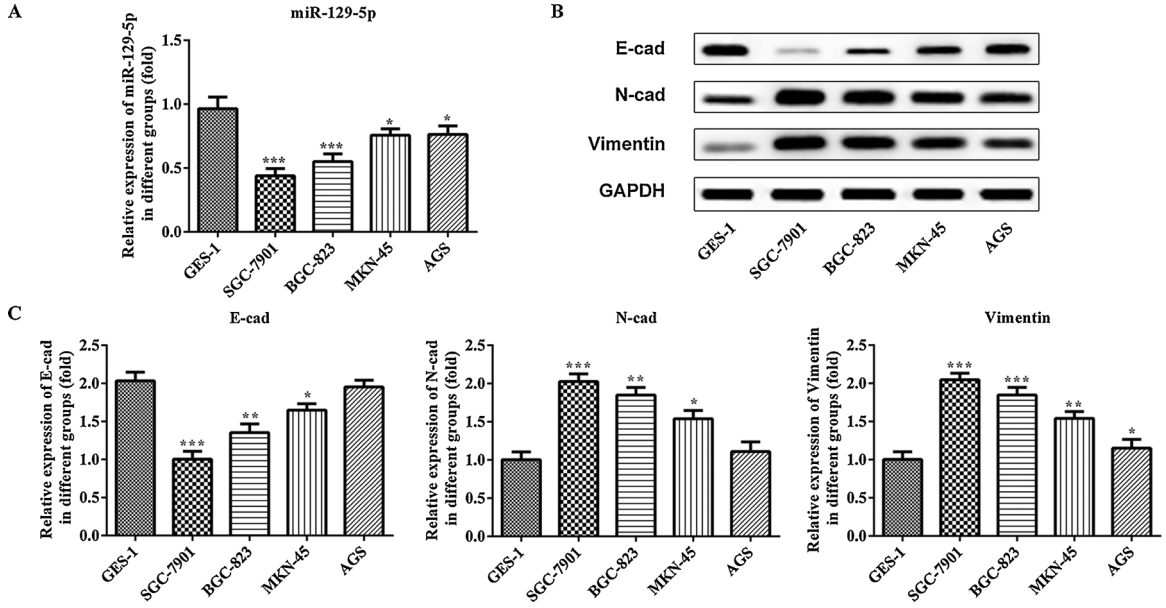

Fig. 1. The expression of miR-129-5p and EMT biomarker in gastric cancer. A. MiR-129-5p was downregulated via RT-qPCR in different gastric cancer cell lines (SGC-7901, BGC-823, MKN-45 and AGS) compared with normal gastric cell (GES-1). B. Expression of EMT biomarker in gastric cancer and normal gastric cell. Data was represented with mean ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus GES-1.

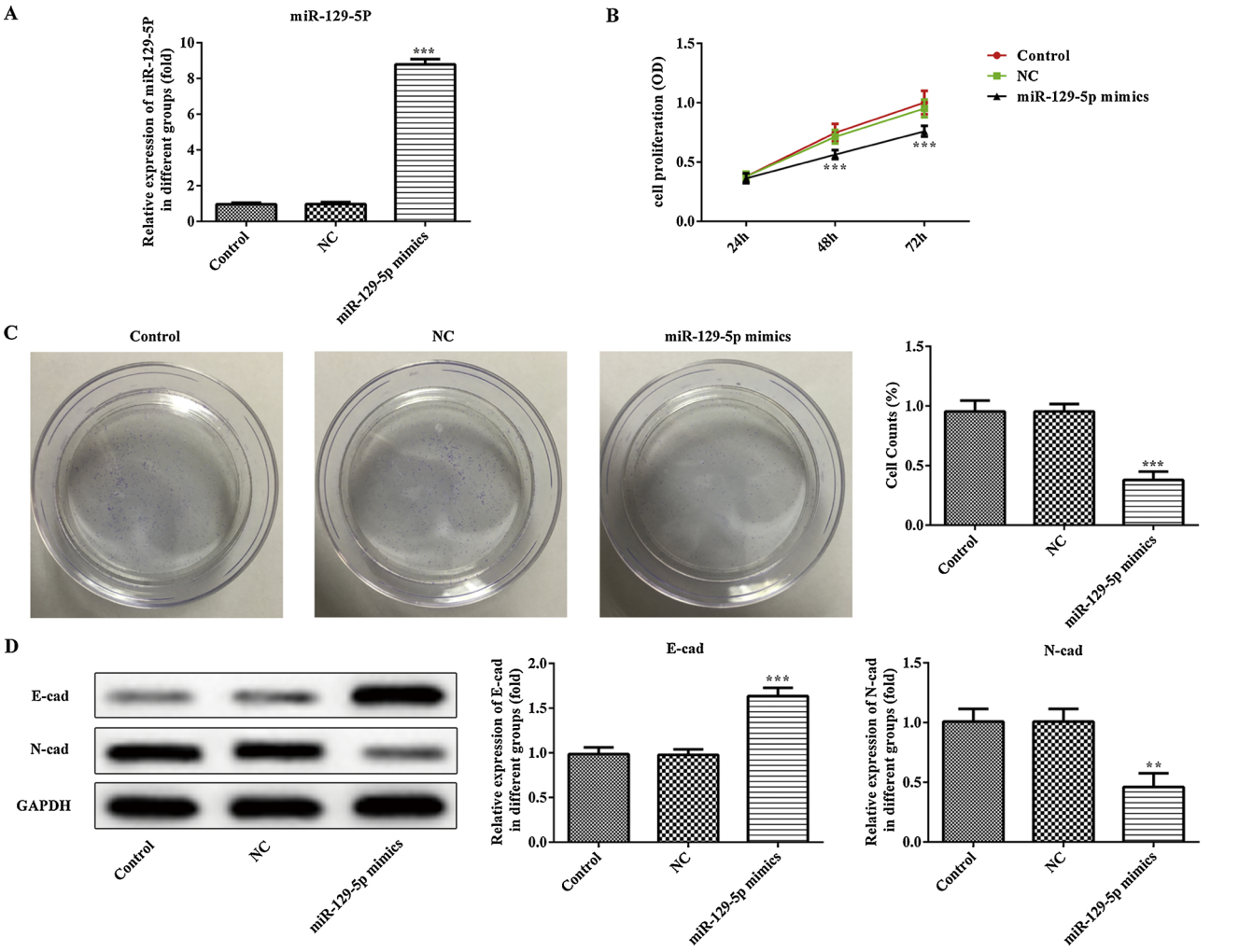

Fig. 2. Effects of miR-129-5p on Cell proliferation and EMT. A. Transfection efficiency of miR-129-5p mimics group in SGC-7901. B. Cell proliferation was evaluated via CCK8. C. Colony formation assay was analyzed via IPP 6.0. D. Western Blotting and gray scale scanning was used to detect and analyzed EMT alterations in SGC-7901 transfected with miR-129-5p mimics. Control, SGC-7901 without any treatment; NC, SGC-7901 was transfected with mimics control; SGC-7901 transfected with miR-129-5p mimics. Data was represented with mean ± SD. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ versus NC.

2.6. Luciferase reporter assay

The potential miR-129-5p binding site in the 3′ untranslated region (UTR) of the HMGB1 gene was predicted through TargetScan (http://www.targetscan.org). SGC-7901 cells were plated into 24-well plates (1×10^5 per well), then co-transfected with a pmir-GLO Dual-Luciferase miRNA Target Expression Vector (Promega Corp., Madison, WI, USA) (containing a wild-type or mutant HMGB1 3′ UTR) and miR-129-5p mimics or NC using Lipofectamine 2000 (Invitrogen). 48 h later, luciferase activity was detected by a Dual Luciferase Reporter Gene Assay Kit (Beyotime, China) according to the manufacturer's instructions.

2.7. Statistical analysis

Data in this investigation were calculated as the means ± standard deviation (mean ± SD). Dramatically differences were evaluated using one-way ANOVA followed by the Tukey's test. Data were analyzed using Graphpad Prism 6.0. Statistical significance is displayed as $P < 0.05$.

3. Results

3.1. miR-129-5p was reduced in GC cells

Expression level of miR-129-5p was evaluated via RT-qPCR analysis and the results showed that miR-129-5p was significantly down-regulated in four GC cell lines (SGC-7901, BGC-823, MKN-45, AGS) compared with normal gastric cells (GES^{-1}), especially in SGC-7901 (Fig. 1A).

3.2. miR-129-5p inhibited cell proliferation and EMT in GC

The expression of E-cadherin in GC cell lines was significantly lower than in normal gastric cell, but the expression of N-cadherin and Vimentin were significantly increased compared with normal gastric

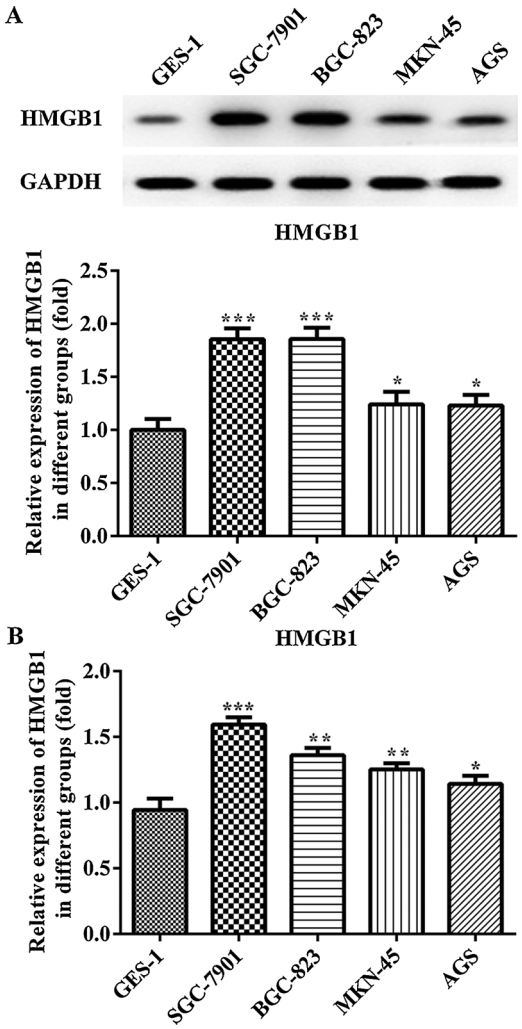

Fig. 3. Expression of HMGB1 in gastric cell lines and normal gastric cell. A & B. HMGB1 was upregulated in gastric cancer cell lines (SGC-7901, BGC-823, MKN-45 and AGS) compared to GES-1 via western blotting and RT-qPCR. $^{**}p < 0.01$, $^{***}p < 0.001$ versus GES-1.

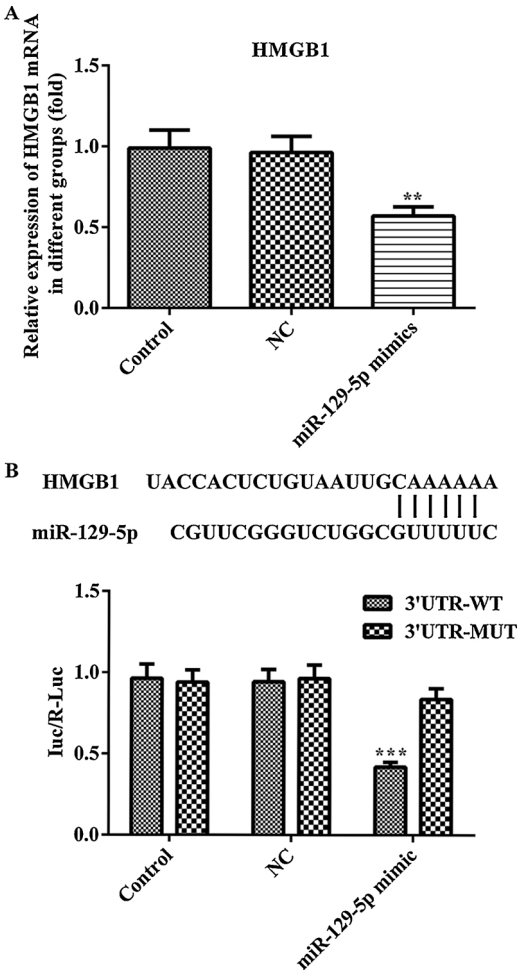

Fig. 4. miR-129-5p directly targeted HMGB1. A. mRNA expression of HMGB1 was detected after miR-129-5p mimics transfected in SGC-7901. B. Dual luciferase reporter assay was performed to evaluate that miR-129-5p negatively targeted HMGB1. Control, SGC-7901 without any treatment; NC, SGC-7901 was transfected with mimics control; SGC-7901 transfected with miR-129-5p mimics. Data was represented with mean ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$ versus NC.

cell, GES-1 (Fig. 1B & C). To investigate the effects of miR-129-5p in GC, miR-129-5p mimics was transfected in SGC-7901. As shown in Fig. 2A, miR-129-5p was upregulated significantly in miR-129-5p mimic group in SGC-7901 which means the transfection is successful. There is no obvious difference between control and transfection control (NC).

CCK-8 and colony formation assays were performed to investigate the cell proliferation. Here, overexpression of miR-129-5p dramatically inhibited cell proliferation of SGC-7901, and there were no significant differences between control and NC (Fig. 2B & C). EMT biomarkers were evaluated by western blotting. E-cadherin was significantly increased, while N-cadherin was significantly decreased after SGC-7901 transfected with miR-129-5p mimics (Fig. 2D). These data show that miR-129-5p inhibited cell proliferation and EMT in GC.

3.3. Downregulation of miR-129-5p directly promoted the expression of HMGB1

HMGB1 was significantly higher in four GC cell lines than normal gastric cells detected by western blotting and RT-qPCR (Fig. 3A & B). As shown in Fig. 4A, upregulation of miR-129-5p inhibited expression of HMGB1. Luciferase reporter assay was used to evaluate that miR-129-5p directly and negatively targeted HMGB1 (Fig. 4B). These results indicate that HMGB1 is a directly target for miR-129-5p.

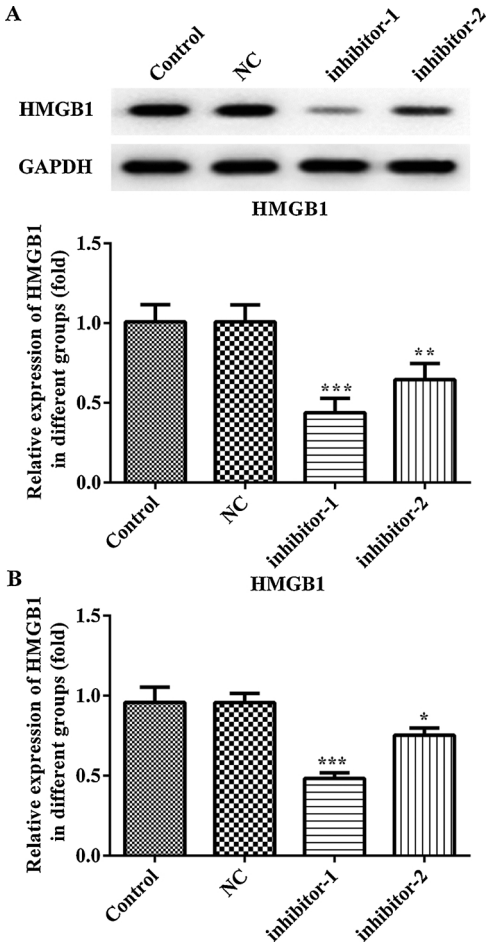

Fig. 5. Transfection efficacy of HMGB1 in SGC-7901. A & B. Western blotting and RT-qPCR were performed to detect the transfection efficacy via transfected with inhibitor-1 and inhibitor-2. Control, SGC-7901 without treatment; NC, SGC-7901 was transfected with inhibitor-control; inhibitor-1, SGC-7901 was transfected with HMGB1-inhibitor-1; inhibitor-2, SGC-7901 was transfected with HMGB1-inhibitor-2. Data was represented with mean ± SD. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ versus NC.

3.4. miR-129-5p attenuated cell proliferation and EMT via HMGB1

To investigate whether HMGB1 affected the effects of cell proliferation and EMT, HMGB1 inhibitor was transfected in SGC-7901. Both RT-qPCR and western blotting results showed that transfection with inhibitor-1 was more efficient than inhibitor-2 (Fig. 5A & B). CCK-8 assay indicated that inhibition of HMGB1 significantly decreased the cell proliferation in SGC-7901 (Fig. 6A). Western blotting results showed that inhibition of HMGB1 relieved the mesenchymal phenotype N-cadherin, and increased the epithelial marker E-cadherin (Fig. 6 B & C). In short, downregulation of HMGB1 in SGC-7901 attenuates cell proliferation and EMT.

4. Discussion

GC is one of the leading causes of cancer related mortality worldwide and the most prevalent cancer in eastern Asia [22]. EMT is associated malignant tumor progression and invasive properties, and promotes stem cell properties and prevents apoptosis. In the gastric mucosa, epithelial cells were closely contacted with neighboring cells through the adherence junction which relies on cadherin [23]. E-cadherin is a major protein of adherence junction. Abundant evidences have revealed that the initiation of GC and its malignant behaviors are closely associated to E-cadherin alterations [24]. In this study, E-cadherin was upregulated in gastric cancer which verified that EMT might promote the initiation of GC. Here we also found miR-129-5p was downregulated in gastric cancer at the same time. Further research will investigate whether knocking out miR-129-5p in normal gastric cell causes tumor-like changes.

miRNA is an important regulator for gene expression. It has been promoted that miR-129-5p was downregulated both in GC tissues and cells [25], and this also confirmed in our research. Increasing number of researches indicate that disorder expression of miRNAs participates in tumorigenesis and tumor development by regulating the target genes and downstream signaling pathway in different type of cancer. Previously, miR-129-5p has been recognized that affects the cell proliferation, radio resistance and cell growth in colon cancer, breast cancer and osteosarcoma, respectively [26–28]. In this study, we proved that overexpression of miR-129-5p significantly inhibited cell viability in GC. Moreover, upregulation of miR-129-5p promoted E-cadherin and reduced N-cadherin in GC, indicating the suppression effect of miR-129-5p on EMT in GC.

miRNAs play their roles mainly through targeting mRNA and inhibiting protein translation. In our study, we identified the expression level of HMGB1 was inversely related with miR-129-5p and HMGB1 was a direct target of miR-129-5p. HMGB1, a non-histone DNA-binding nuclear protein, is involved in both the nuclear and extracellular activities. HMGB1 can work as a positive factor to protect cells from injury like mice will be sensitive to liver ischemia with absent expression of HMGB1 [29]. HMGB1 also has been evaluated as a potential target in various cancer cells to affect EMT, such as miR-200c inhibits EMT via downregulating HMGB1 [30,31]. Hye Won Chung, et al. report that overexpression of extracellular HMGB1 promoted EMT in gastric cancer [32]. In this investigation, we discovered that HMGB1 was greatly expressed in gastric cancer cells. Overexpression of miR-129-5p suppressed the expression of HMGB1. Inhibition of HMGB1 inhibit EMT in gastric cancer. A negative correlation between miR-129-5p and HMGB1 is indicated and it is concluded that miR-129-5p can regulate EMT via modulation of HMGB1 expression.

In summarize, we demonstrate a novel molecular mechanism through which overexpression of miR-129-5p inhibits EMT and cell viability through inhibiting HMGB1 in GC. Our observations suggest that miR-129-5p is as a promising diagnostic and therapeutic target for GC.

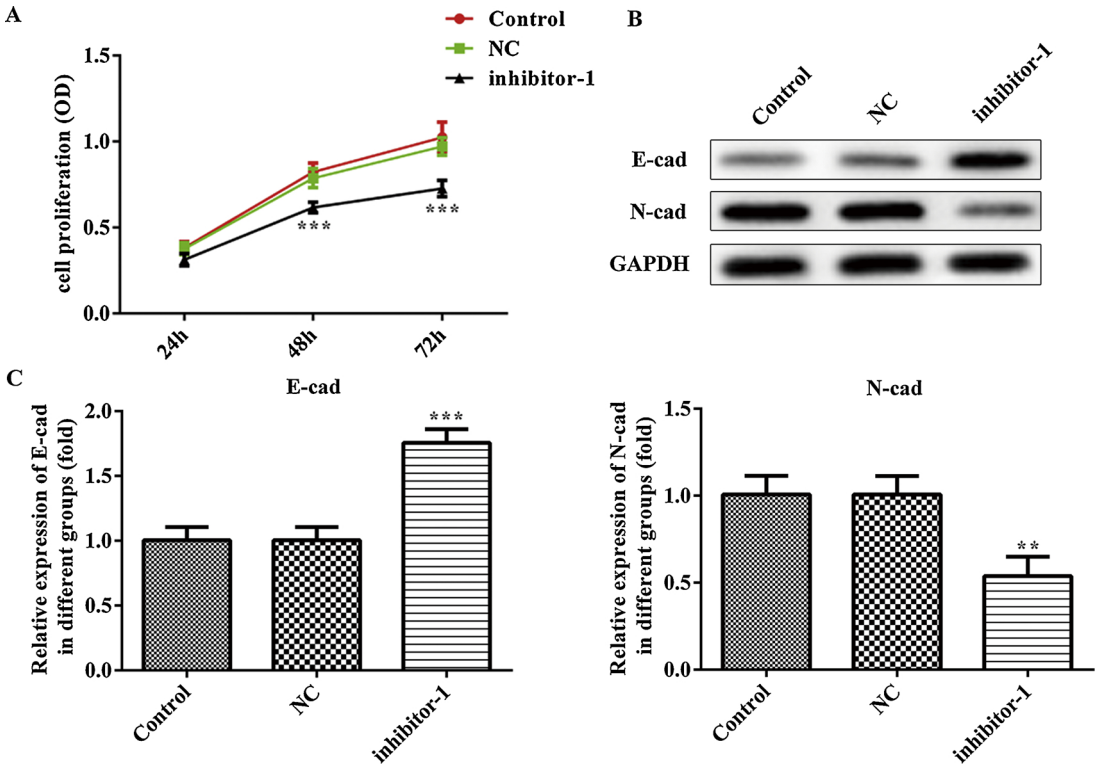

Fig. 6. Downregulation of HMGB1 inhibited cell proliferation and EMT. A. Cell proliferation was detected via CCK8. $^{**}p < 0.01$, $^{***}p < 0.001$ versus 24 h. B. EMT biomarkers were detected via western blotting and analyzed via gray scale scanning. Control, SGC-7901 without treatment; NC, SGC-7901 was transfected with inhibitor-control; inhibitor-1, SGC-7901 was transfected with HMGB1-inhibitor-1. Data was represented with mean ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{**}p < 0.001$ versus NC.

Conflict of interest

There is no conflict of interest in this article. All authors have read and approved of the manuscript being submitted.

Acknowledgement

Not applicable.

References

[1] L.A. Torre, et al., Global cancer statistics, CA Cancer J. Clin. 65 (2) (2012) 87–108 2015.
[2] V. Pasechnikov, et al., Gastric cancer: prevention, screening and early diagnosis, World J. Gastroenterol. 20 (38) (2014) 13842–13862.
[3] Z.Y. Zhang, H.Y. Ge, Micrometastasis in gastric cancer, Cancer Lett. 336 (1) (2013) 34–45.
[4] L. He, G.J. Hannon, MicroRNAs: small RNAs with a big role in gene regulation, Nat. Rev. Genet. 5 (7) (2004) 522–531.
[5] D.P. Bartel, MicroRNAs: target recognition and regulatory functions, Cell 136 (2) (2009) 215–233.
[6] W. Zhang, et al., MicroRNA-199 suppresses cell proliferation, migration and invasion by downregulating RGS17 in hepatocellular carcinoma, Gene 659 (2018) 22–28.
[7] F. Piard, et al., Genetic pathways in colorectal cancer: interest for the pathologist, Ann. Pathol. 22 (4) (2002) 277–288.
[8] X. Yu, et al., Growth inhibitory effects of three miR-129 family members on gastric cancer, Gene 532 (1) (2013) 87–93.
[9] S. Xu, et al., miR-129 predicts prognosis and inhibits cell growth in human prostate carcinoma, Mol. Med. Rep. 14 (6) (2016) 5025–5032.
[10] X. Tang, et al., Downregulation of miR-129-2 by promoter hypermethylation regulates breast cancer cell proliferation and apoptosis, Oncol. Rep. 35 (5) (2016) 2963–2969.
[11] L. Zheng, et al., miR-129b suppresses cell proliferation in the human lung cancer cell lines A549 and H1299, Genet. Mol. Res. 15 (4) (2016).
[12] Q. Liu, et al., MiR-129-5p functions as a tumor suppressor in gastric cancer progression through targeting ADAM9, Biomed. Pharmacother. 105 (2018) 420–427.
[13] A. Nawshad, et al., Transforming growth factor-beta signaling during epithelial-mesenchymal transformation: implications for embryogenesis and tumor metastasis, Cells Tissues Organs 179 (1–2) (2005) 11–23.
[14] R.B. Hazan, et al., Cadherin switch in tumor progression, Ann. N. Y. Acad. Sci. 1014 (2004) 155–163.
[15] D.M. Gonzalez, D. Medici, Signaling mechanisms of the epithelial-mesenchymal transition, Sci. Signal. 7 (344) (2014) re8.
[16] P. Zhang, et al., MiR-646 inhibited cell proliferation and EMT-induced metastasis by targeting FOXK1 in gastric cancer, Br. J. Cancer 117 (4) (2017) 525–534.
[17] J. Li, et al., MiR-2392 suppresses metastasis and epithelial-mesenchymal transition by targeting MAML3 and WHSC1 in gastric cancer, FASEB J. 31 (9) (2017) 3774–3786.
[18] G. Song, et al., miR-551b regulates epithelial-mesenchymal transition and metastasis of gastric cancer by inhibiting ERBB4 expression, Oncotarget 8 (28) (2017) 45725–45735.
[19] J. Zhang, et al., Knockdown of HMGB1 inhibits growth and invasion of gastric cancer cells through the NF-kappaB pathway in vitro and in vivo, Int. J. Oncol. 44 (4) (2014) 1268–1276.
[20] B. Liu, et al., Med19 is involved in chemoresistance by mediating autophagy through HMGB1 in breast cancer, J. Cell. Biochem. 120 (1) (2019) 507–518.
[21] Y. Yue, et al., High mobility group box 1/toll-like receptor 4/myeloid differentiation factor 88 signaling promotes progression of gastric cancer, Tumour Biol. 39 (3) (2017) p. 1010428317694312.
[22] F. Bray, et al., Global estimates of cancer prevalence for 27 sites in the adult population in 2008, Int. J. Cancer 132 (5) (2013) 1133–1145.
[23] M. Furue, Epithelial tumor, invasion and stroma, Ann. Dermatol. 23 (2) (2011) 125–131.
[24] C. Cai, et al., CD133 promotes the invasion and metastasis of gastric cancer via epithelial-mesenchymal transition, Zhonghua Wei Chang Wai Ke Za Zhi 16 (7) (2013) 662–667.
[25] L. Yan, et al., MiR-129-5p influences the progression of gastric cancer cells through interacting with SPOCK1, Tumour Biol. 39 (6) (2017) p. 1010428317706916.
[26] Q. Wu, et al., LncRNA MALAT1 induces colon cancer development by regulating miR-129-5p/HMGB1 axis, J. Cell. Physiol. 233 (9) (2018) 6750–6757.
[27] J. Luo, J. Chen, L. He, mir-129-5p attenuates irradiation-induced autophagy and decreases radioresistance of breast cancer cells by targeting HMGB1, Med. Sci. Monit. 21 (2015) 4122–4129.

[28] K. Liu, et al., MALAT1 promotes osteosarcoma development by regulation of HMGB1 via miR-142-3p and miR-129-5p, Cell Cycle 16 (6) (2017) 578–587.
[29] H. Huang, et al., Hepatocyte-specific high-mobility group box 1 deletion worsens the injury in liver ischemia/reperfusion: a role for intracellular high-mobility group box 1 in cellular protection, Hepatology 59 (5) (2014) 1984–1997.
[30] P.L. Liu, et al., MicroRNA-200c inhibits epithelial-mesenchymal transition, invasion, and migration of lung cancer by targeting HMGB1, PLoS One 12 (7) (2017) e0180844.
[31] L. Zhu, et al., High-mobility group box 1: a novel inducer of the epithelial-mesenchymal transition in colorectal carcinoma, Cancer Lett. 357 (2) (2015) 527–534.
[32] H.W. Chung, et al., Combined targeting of high-mobility group box-1 and interleukin-8 to control micrometastasis potential in gastric cancer, Int. J. Cancer 137 (7) (2015) 1598–1609.