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MiR-320c prevents the malignant development of cervical cancer by regulating GABRP level

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Abstract. – OBJECTIVE: This study aims to explore the role of microRNA-320c (miR-320c) in regulating biological behaviors of cervical cancer and the potential mechanism, thus providing experimental references for developing therapeutic target of cervical cancer.

PATIENTS AND METHODS: Differential expressions of miR-320c in cervical cancer samples and normal cervical tissues were determined. Potential association between miR-320c level and clinical characteristics of cervical cancer patients was analyzed. After overexpression of miR-320c, migratory potential changes in HeLa, and C33-A cells were examined. At last, target gene binding to miR-320c was predicted online and its involvement in the malignant development of cervical cancer was finally explored.

RESULTS: It was found that miR-320c was lowly expressed in cervical cancer tissues. Compared with cervical cancer patients with high expression of miR-320c, those with low expression had higher rates of lymphatic metastasis and distant metastasis. Besides, the overexpression of miR-320c markedly inhibited migratory potential in HeLa and C33-A cells. GABRP was verified to be the target gene binding to miR-320c. Notably, GABRP was able to reverse the role of miR-320c in regulating migratory potential in cervical cancer.

CONCLUSIONS: MiR-320c is capable of inhibiting migratory potential in cervical cancer by targeting GABRP, which may be utilized as a therapeutic target of cervical cancer.

Key Words:
MiR-320c, GABRP, Cervical cancer, Migration.

Introduction

Cervical cancer ranks second in the incidence of female tumors, which is one of the leading causes of female deaths, especially in developing countries. Nearly 500,000 women are diagnosed as cervical cancer every year worldwide, and half of them die because of this cancer[1-4]. Due to the application of cervical smear screening and anti-cancer vaccine in developed countries, the incidence of cervical cancer has greatly decreased[2,5]. However, anti-cancer vaccine is expensive and has yet to be validated by clinical trials in China. Cervical cancer seriously affects health and lives of Chinese women[3]. Surgical resection combined with postoperative radiotherapy and radiotherapy is the major strategy in the treatment of cervical cancer[6,7]. Advanced stage patients lose the optimal surgery opportunity, and palliative therapy is applied for these patients as an adjuvant therapy[8,9]. It is urgent to seek for novel therapeutic targets of cervical cancer to enhance clinical outcomes[9,10].

With the in-depth research, tumorigenesis is believed to be resulted from dysfunctional oncogenes and tumor suppressors, as well as subsequent uncontrolled malignant proliferation[11,12]. MicroRNAs (miRNAs) are single-stranded, non-coding RNAs with 19-25 bp long. By recognizing and binding target mRNAs, miRNAs degrade them or inhibit their translation and eventually display post-transcriptional regulation on life activities[13,14]. It is reported that abnormally expressed miRNAs are vital regulators in tumor development[13,15,16]. MiR-320c is downregulated in several types of tumors, serving as a tumor-suppressor gene[17,18].

MiRNAs exert their important roles by mediating downstream gene expressions and functions[20-22]. In this study, potential targets of miR-320c were searched through online bioin-

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formatics websites, and its involvement in the malignant development of cervical cancer was explored.

Patients and Methods

Cervical Cancer Patients and Samples

A total of 64 cervical cancer patients aging (42±9.2) years old were included, and their matched tumor tissues and paracancerous ones were surgically resected. Their clinical data were recorded. Tumor staging was conducted based on the guideline proposed by the Union for International Cancer Control (UICC). This study got approval by Ethics Committee of The Affiliated Changzhou No. 2 People's Hospital of Nanjing Medical University and conducted after informed consent was obtained from each subject.

Cell Culture

Cervical cancer cell lines (HeLa and C33-A) and normal cervical epithelial cell line were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 μg/mL streptomycin in a 5% CO_2 incubator at 37°C. Cell passage was conducted every 2-3 days.

Transfection

Cells were cultured to 60-70% confluence in 6-well plates and transfected with plasmids constructed by GenePharma (Shanghai, China), using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). 48 hours later, the cells were collected for the following use.

Transwell Migration Assay

A total of 200 μL of suspension (2×10^5 cells/mL) was inoculated in the upper transwell chamber (Millipore, Billerica, MA, USA) inserted in a 24-well plate, with 500 μL of medium containing 10% FBS in the bottom. After 48-h incubation, bottom cells were subjected to 15-min methanol fixation and 20-min crystal violet staining. Finally, the cells were captured using a microscope, and migratory cells were counted in 5 random fields per sample.

Wound Healing Assay

Cells were inoculated in 6-well plates and grown to 90% confluence. After creation of an artificial wound in cell monolayer, the original medium was replaced with medium containing 1% FBS. 24 hours later, wound closure was captured.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Extracted RNAs by TRIzol reagent (Invitrogen, Carlsbad, CA, USA) were purified by DNase I treatment, and reversely transcribed into complementary deoxyribose nucleic acids (cDNAs) using PrimeScript RT Reagent (TaKaRa, Otsu, Shiga, Japan). The obtained cDNAs underwent qRT-PCR using SYBR® Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6 were the internal references. Each sample was performed in triplicate, and relative level was calculated by $2^{-\Delta\Delta Ct}$. MiR-320c: forward: 5'-AAAAGCTGGGTTGAGAGGGT-3' and reverse: 5'-GCTGATCCACATCTGCTGGAA-3', U6: forward: 5'-CTCGCTTCGGCAGCACA-3' and reverse: 5'-AACGCTTCACGAATTTGCGT-3', GABRP: forward: 5'-CTCGATTCAGTCCCTGCAAGA-3' and reverse: 5'-GTGCGGGACCCGATCAT-3', and GAPDH: forward: 5'-GCACCGTCAAGGCTGAGAAC-3' and reverse: 5'-ATGGTGGTGAAGACGCCAGT-3'.

Western Blot

Cells were lysed for isolating cellular protein and electrophoresed. Total protein concentration was calculated by bicinchoninic acid (BCA) Protein Assay Kit (Pierce, Rockford, IL, USA). Protein samples were loaded on polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Subsequently, non-specific antigens were blocked in 5% skim milk for 2 h. Then, the membranes reacted with primary and secondary antibodies for indicated time. Band exposure and analyses were finally conducted. Immuno-reactive bands were visualized by enhanced chemiluminescence (ECL) detection kit (Amersham Biosciences, Foster City, CA, USA). The gray value was analyzed using Image J software (Version 1.38; National Institutes of Health, Bethesda, MA, USA).

Luciferase Assay

HeLa and C33-A cells pre-inoculated in 24-well plates were co-transfected with NC mimic/miR-320c mimic and GABRP-WT/GABRP-MUT, re-

spectively, and were lysed for determining the relative Luciferase activity 48 h later (Promega, Madison, WI, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 (IBM Corp., Armonk, NY, USA) was used for data analyses. Data were expressed as mean ± standard deviation (SD). The differences between groups were analyzed by the *t*-test. Chi-square test was performed for analyzing the relationship between miR-320c level and clinical characteristics of cervical cancer patients. Pearson correlation test was applied for evaluating the relationship between expression levels of miR-320c and GABRP in cervical cancer tissues. Kaplan-Meier curves were depicted for survival analysis, followed by Log-rank test. $p<0.05$ suggested that the difference was statistically significant.

Results

MiR-320c Was Lowly Expressed In Cervical Cancer

A total of 64 paired cervical cancer tissues and paracancerous ones were collected. As shown in qRT-PCR data, miR-320c was lowly expressed in cervical cancer tissues (Figure 1A, B). Meanwhile, miR-320c level was lower in cervical cancer cell lines than that in controls (Figure 1C).

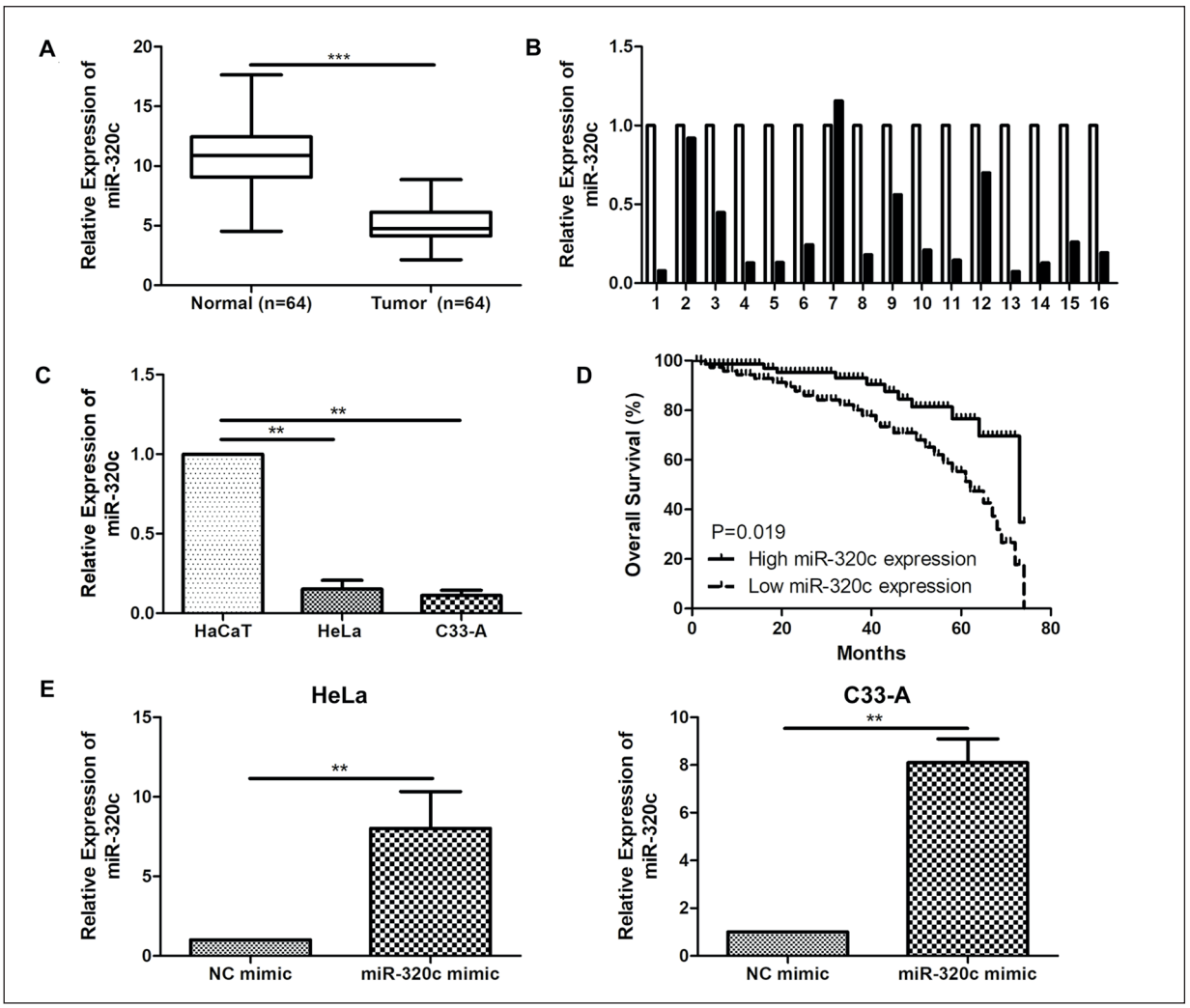

Figure 1. MiR-320c is lowly expressed in cervical cancer. **A, B,** Differential expressions of miR-320c in paracancerous tissues (n=64) and cervical cancer tissues (n=64). **C,** MiR-320c levels in cervical cancer cell lines. **D,** Overall survival in cervical cancer patients expressing high or low level of miR-320c. **E,** Transfection efficacy of miR-320c mimic in HeLa and C33-A cells. Data are expressed as mean ± SD, *$p<0.05$, **$p<0.01$, ***$p<0.001$.

MiR-320c Level Was Linked to Metastasis and Prognosis In Cervical Cancer Patients

According to the median level of miR-320c calculated in included cervical cancer patients, these patients were assigned into high and low miR-320c expression groups. It was analyzed that miR-320c level was negatively correlated with rates of lymphatic and distant metastases, while it was unrelated to age, tumor size, and tumor stage in cervical cancer patients (Table I). Moreover, Kaplan-Meier curves illustrated that lowly expressed miR-320c was unfavorable to overall survival in cervical cancer (Figure 1D). Therefore, it is believed that miR-320c may be a hallmark predicting the prognosis in cervical cancer.

Overexpression of MiR-320c Suppressed Migratory Potential In Cervical Cancer

To further explore the biological role of miR-320c in cervical cancer, miR-320c mimic was constructed. Transfection with miR-320c mimic in HeLa and C33-A cells remarkably upregulated miR-320c, verifying its satisfactory transfection efficacy (Figure 1E). Transwell assay results uncovered that the overexpression of miR-320c decreased migratory cell number in HeLa and C33-A cells compared with those in controls (Figure 2A). In the same way, wound closure percentage was decreased in cervical cancer cells overexpressing miR-320c (Figure 2B). It is concluded that miR-320c is able to suppress migratory potential in cervical cancer.

GABRP Was the Target Gene of MiR-320c

Next, potential target genes of miR-320c were analyzed using TargetScan, MiRDB, and StarBase. It was found that there were three shared targets analyzed in the three databases, namely GABRP, YOD1, and GPBP1 (Figure 3A). The former two genes were differentially expressed in cervical cancer tissues and paracancerous ones, and GABRP was the most pronounced one (Figure 3B). Subsequently, Luciferase assay was conducted based on the binding sequences in the 3'UTR of miR-320c and GABRP. As the data uncovered, the overexpression of miR-320c markedly decreased the Luciferase activity in wild-type GABRP vector, rather than the mutant one (Figure 3C). Hence, it was proved that GABRP did bind to miR-320c, serving as its downstream gene. Western blotting showed that the protein level of GABRP was downregulated in cervical cancer cells overexpressing miR-320c (Figure 3D). Notably, a negative correlation was identified between expression levels of miR-320c and GABRP in cervical cancer species (Figure 3E). Compared with the controls, GABRP was highly expressed in cervical cancer cell lines (Figure 3F).

Overexpression GABRP Abolished the Regulatory Effect of MiR-320c on Migratory Potential In Cervical Cancer

The results of Western blotting showed that the protein level of GABRP was upregulated in co-transfection of miR-320c mimic and pcD-

Table I. Association of miR-320c expression with clinicopathologic characteristics of cervical carcinoma.

Parameters	No. of cases	MiR-320c expression		*p*-value
		High (%)	Low (%)	
Age (years)				0.184
< 60	26	18	8	
≥ 60	38	20	18	
Tumor size (cm)				0.836
≤ 5	31	18	13	
> 5	33	20	13	
T stage				0.987
T1-T2	37	22	15	
T3-T4	27	16	11	
Lymph node metastasis				0.038
No	39	26	11	
Yes	25	12	15	
Distance metastasis				0.008
No	50	34	16	
Yes	14	4	10	

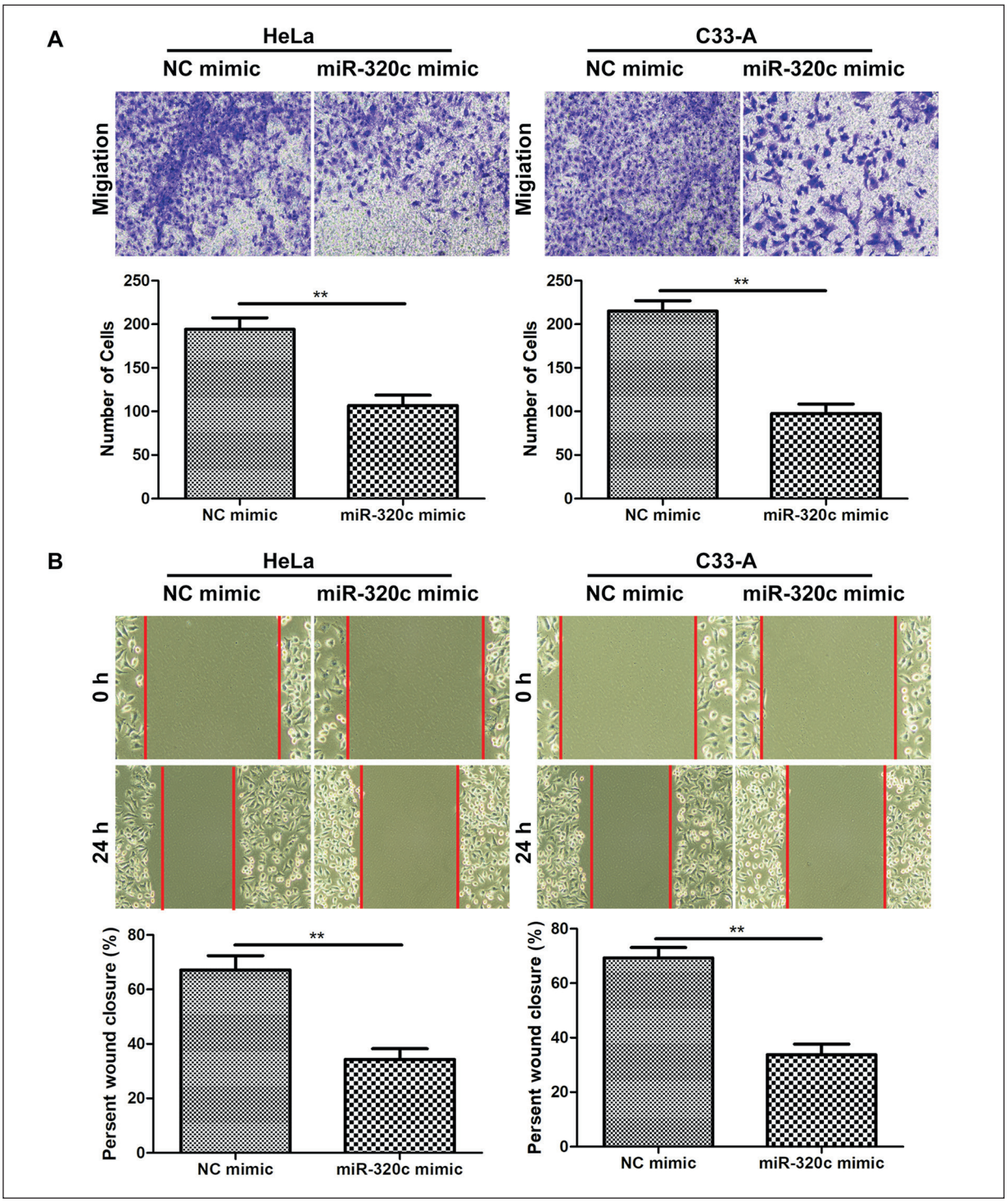

Figure 2. Overexpression of miR-320c suppresses migratory potential in cervical cancer. **A,** Migration in HeLa and C33-A cells transfected with NC mimic or miR-320c mimic (magnification: 40×). **B,** Wound closure percentage in HeLa and C33-A cells transfected with NC mimic or miR-320c mimic (magnification: 40×). Data are expressed as mean ± SD, $^{**}p<0.01$.

NA3.1-GABRP, compared with miR-320c mimic (Figure 4A). Besides, qRT-PCR suggested that co-transfection with miR-320c mimic and pcDNA3.1-GABRP downregulated miR-320c in cervical cancer cells compared with those solely transfected with miR-320c mimic (Figure 4B).

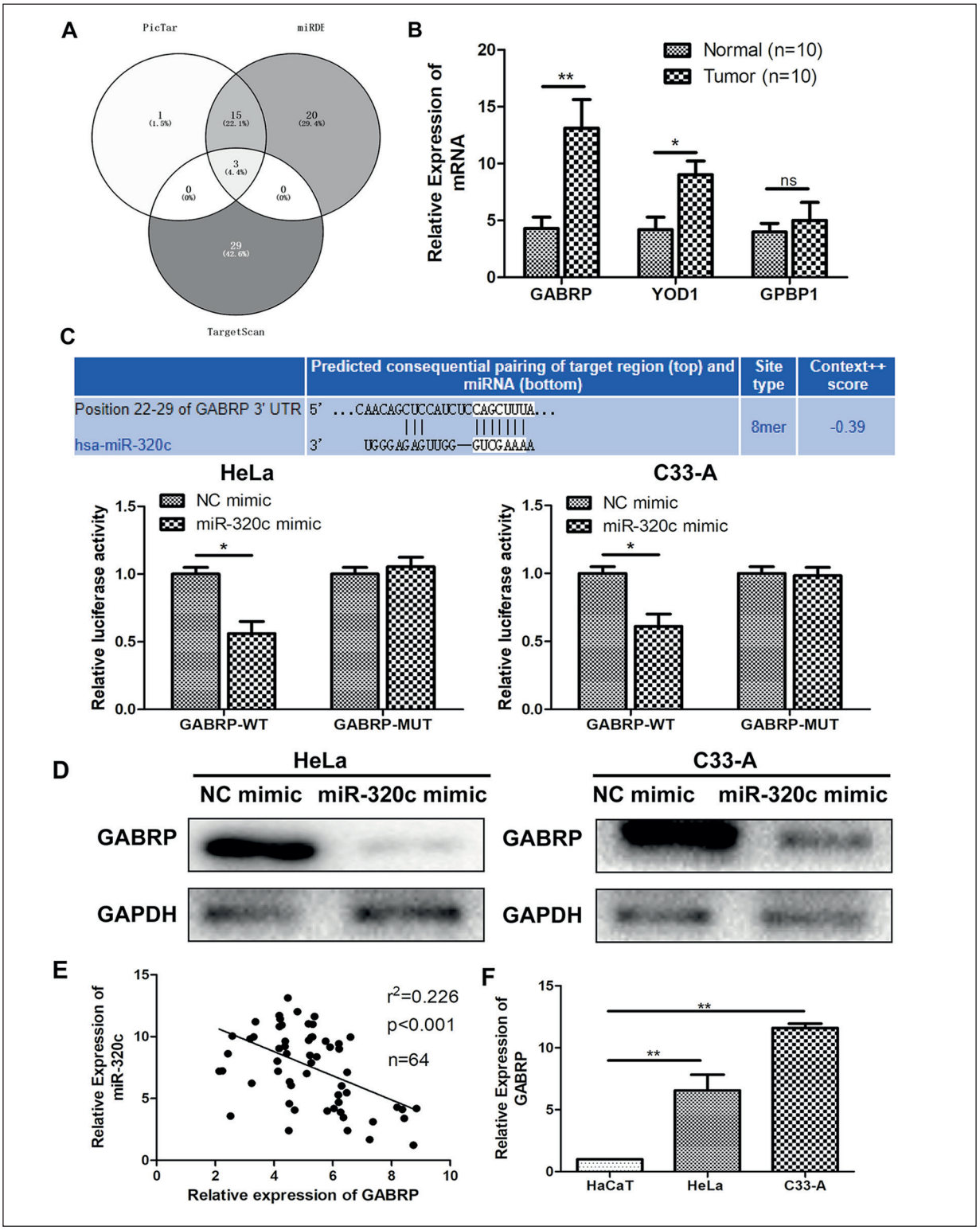

Figure 3. GABRP is the target gene of miR-320c. **A,** Potential targets of miR-320c predicted using TargetScan, MiRDB and Starbase. **B,** Differential expressions of GABRP, YOD1 and GRBP1 between paracancerous tissues and cervical cancer tissues. **C,** Luciferase activity in HeLa and C33-A cells co-transfected with NC mimic/miR-320c mimic and GABRP-WT/GABRP-MUT, respectively. **D,** Protein level of GABRP in HeLa and C33-A cells transfected with NC mimic or miR-320c mimic. **E,** A negative correlation between expression levels of miR-320c and GABRP in cervical cancer tissues. **F,** GABRP levels in cervical cancer cell lines. Data are expressed as mean ± SD, *p<0.05, **p<0.01.

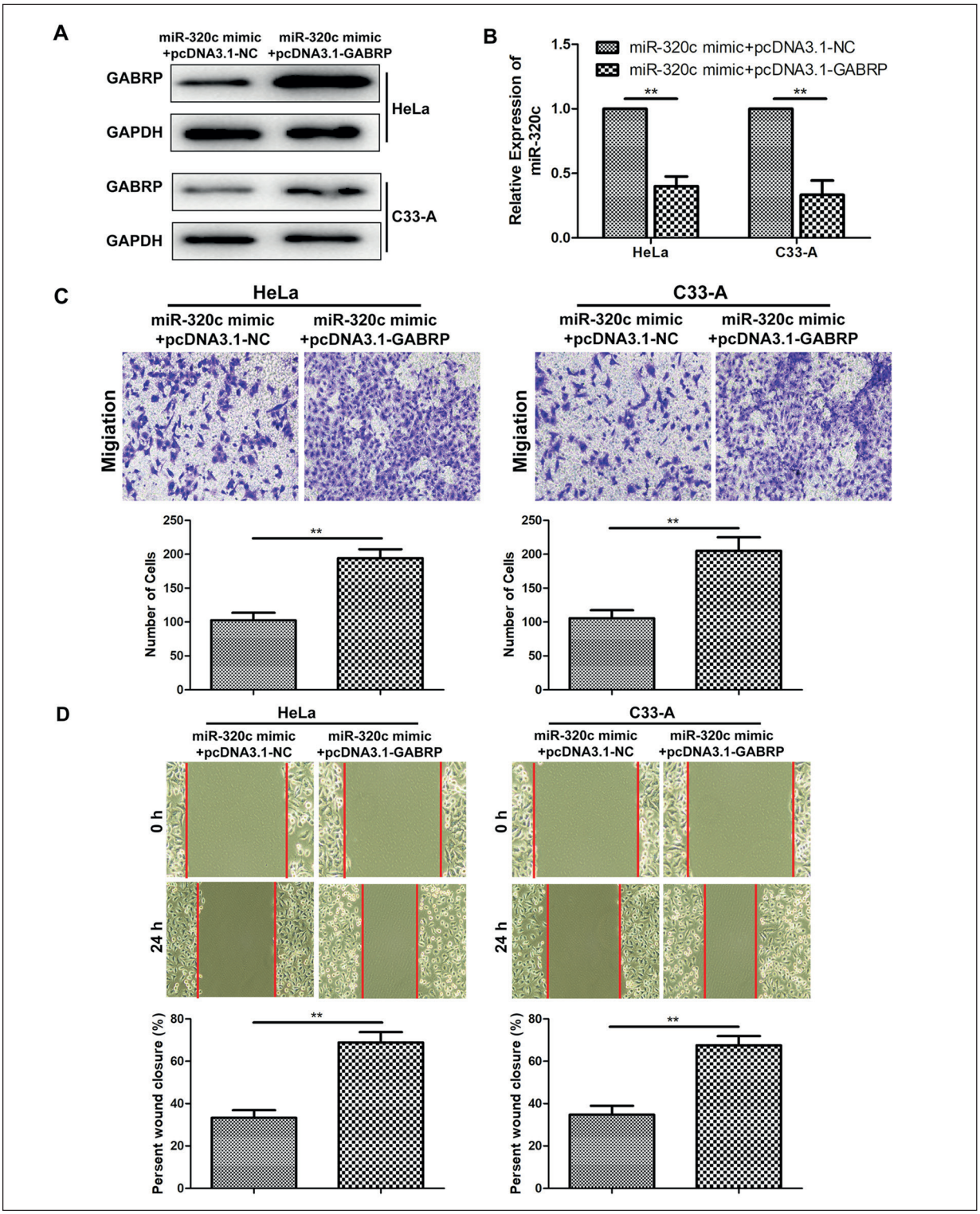

Figure 4. Overexpression GABRP abolishes the regulatory effect of miR-320c on migratory potential in cervical cancer. **A,** Protein level of GABRP in HeLa and C33-A cells transfected with miR-320c mimic + pcDNA3.1-NC or miR-320c mimic + pcDNA3.1-GABRP. **B,** MiR-320c level in HeLa and C33-A cells transfected with miR-320c mimic + pcDNA3.1-NC or miR-320c mimic + pcDNA3.1-GABRP. **C,** Migration in HeLa and C33-A cells transfected with miR-320c mimic + pcDNA3.1-NC or miR-320c mimic + pcDNA3.1-GABRP (magnification: 40×). **D,** Wound closure percentage in HeLa and C33-A cells transfected with miR-320c mimic + pcDNA3.1-NC or miR-320c mimic + pcDNA3.1-GABRP (magnification: 40×). Data are expressed as mean ± SD, $^{**}p<0.01$.

Notably, both migratory cell number and wound closure percentage were higher in cervical cancer cells co-overexpressing miR-320c and GABRP than those solely overexpressing miR-320c (Figure 4C, 4D). It is indicated that GABRP is responsible for migratory potential suppression regulated by miR-320c in cervical cancer.

Discussion

Cervical cancer is common in female malignancies[1-3]. Clinical reports[4-6] have shown that persistent infection of high-risk human papillomavirus (HPV) is the leading cause of cervical cancer. The development from HPV infection into tumorigenesis of cervical cancer is a gradual process involving oncogenes and tumor-suppressor genes[7-10]. Currently, high-throughput sequencing has been greatly advanced, which provides a new method for tumor biology investigations.

MiRNAs are vital regulators in cell activities and human diseases[12-14]. Several abnormally expressed miRNAs in cervical cancer tissues have been identified[19,20]. Based on the expression level in tumor species, miRNAs display an anti-tumor or carcinogenic role in tumor development[14-17]. Scholars[17,18] have reported the involvement of miR-320c in the malignant development of prostate cancer and colorectal cancer. This study indicated that miR-320c was downregulated in cervical cancer species. By analyzing the relationship between miR-320c level and clinical characteristics of included cervical cancer patients, miR-320c level was negatively linked to rates of lymphatic metastasis and distant metastasis. Lowly expressed miR-320c was unfavorable to overall survival in cervical cancer. Subsequently, *in vitro* researches demonstrated the inhibitory effect of miR-320c on migratory potential in cervical cancer cells.

By binding to 3'UTR of target genes, miRNAs mediate downstream genes to exert their biological functions[17,18]. Here, the target gene of miR-320c was predicted using bioinformatics method. GABRP is a type A receptor subunit of GABA, which is involved in the development of some tumors[23,24]. GABA is a non-protein amino acid, which is formed by the decarboxylation of glutamic acid. It is widely distributed in mammals' central nervous system and peripheral tissues and is involved in the signal delivery between cells and cell differentiation[23]. It was detected that GABRP was the target gene binding to miR-320c. Compared with paracancerous tissues, GABRP was upregulated in cervical cancer species. In addition, its level was negatively regulated by miR-320c. To further illustrate the involvement of GABRP in the malignant development of cervical cancer influenced by miR-320c, a series of rescue experiments were designed. Of note, the overexpression of GABRP greatly increased migratory potential in cervical cancer cells overexpressing miR-320c. It is believed that miR-320c suppresses malignant development of cervical cancer by negatively regulating GABRP level.

Conclusions

Altogether, miR-320c is capable of inhibiting migratory potential in cervical cancer by targeting GABRP, which may be utilized as a therapeutic target of cervical cancer.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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