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Abnormal expression of Rap2A as a prognostic marker for human breast cancer

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Abstract. – OBJECTIVE: The aim of this study was to explore the expression of GTPase protein Ras-related protein Rap-2a (Rap2A) in breast cancer (BC). Furthermore, the associations of Rap2A with clinicopathological parameters of BC patients were investigated.

PATIENTS AND METHODS: quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was used to examine Rap2A expression in BC tissues and cells. The association between Rap2A expression and clinicopathological characteristics was analyzed by Chi-square test. Low expression of Rap2A in BC cells was conducted by transfection of small interfering RNA (siRNA). Subsequently, colony formation assay and transwell assay were used to detect the proliferation and invasion abilities of BC cells, respectively.

RESULTS: Rap2A was highly expressed in both BC tissues and cells ($p<0.05$). Further analysis showed that tumor size, clinical stage, and distant metastasis were correlated with Rap2A expression ($p<0.05$). Besides, inhibition of Rap2A significantly decreased the proliferation and invasion abilities of BC cells ($p<0.05$).

CONCLUSIONS: Rap2A acted as a promotor in the development of BC. Our findings suggested that Rap2A might be a new potential therapeutic target marker for BC treatment.

Key Words:
Breast cancer (BC), GTPase protein Ras-related protein Rap-2a (Rap2A), Clinicopathological parameters.

Introduction

Breast cancer (BC) is one of the most common tumors in females, whose morbidity rate ranks highest among all malignant tumors in women in Western developed countries. Statistics[1,2] have reported that about 400,000 people die of BC every year in the world. The morbidity rate of BC is rising year by year, and patients become younger and younger in many countries, especially for economically developed regions[3]. The occurrence and development of BC is a complex process, involving multiple factors and genes. Currently, there are diversified therapeutic methods for BC, dominated by surgical procedures, chemotherapy, radiotherapy, and endocrine therapy[4-6]. However, the long-term prognosis of BC patients remains unsatisfactory[7,8]. Despite constant exploration of pathogenic factors, the pathogenesis of BC has not been clarified yet. In recent years, with the emergence of studies on molecules, genes and immune-diagnosis, the investigation in the incidence and progression of BC has been deepened continuously. These findings are of extremely important significance for the diagnosis, treatment, and prevention of BC[9-11].

Rap2A is located in chromosome 13q34[12]. Similar to other proteins of the Rap family, Rap2A acquires its activity when binding to guanosine triphosphate (GTP) and loses its activity when binding to guanosine diphosphate (GDP)[13,14]. It plays vital roles in various cell processes, such as cytoskeleton reconstruction, boundary bristle generation, and cell migration[15]. Rap2A can also conjugate with the citron-homology domains of TNIK, MINK, and MAP4K4 and initiate a variety of downstream signaling pathways to perform its biological function[16]. Moreover, it is highly expressed in a large number of human tumors, including lung cancer[17], follicular thyroid carcinoma[18], and nasopharyngeal carcinoma[19].

However, the exact role of Rap2A in the development of BC has not been fully elucidated. In this study, we first detected Rap2A expression in BC tissues. The association between Rap2A expression and clinicopathological characteristics of BC patients was explored. Furthermore, we in-

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vestigated the effects of Rap2A dys-regulation on the proliferation and invasion abilities of BC cells.

Patients and Materials

Tissue Collection

A total of 156 young patients definitely diagnosed with BC *via* postoperative pathology in Linyi Cancer Hospital from May 2016 to July 2018 were enrolled in this study. The selection of patients was based on the guideline proposed by the Union for International Cancer Control (UICC). Normal tissues located ≥2 cm and ≤5 cm away from the margin of BC tissues were collected. Informed consent was obtained from each patient before the study. This study was approved by the Ethics Committee of Linyi Cancer Hospital.

Cell Culture

BC cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) medium (Gibco, Rockville, MD, USA) containing 10% inactivated fetal bovine serum (FBS; Gibco, Rockville, MD, USA) in an incubator at 37°C with 5% CO_2. Culture medium was replaced every 2-3 days. When the density reached 80-90%, the cells were first digested with 0.25% trypsin at 37°C. Next, they were gently pipetted into cell suspension using a proper amount of medium, followed by cell passage. After centrifugation at 1,000 rpm for 5 min, an appropriate amount of cells were re-suspended in fresh medium and seeded onto a new culture dish for subsequent experiments.

Cell Transfection

Cells in the logarithmic growth phase were trypsinized, sufficiently pipetted, and mixed evenly. Then, the cells were seeded into 6-well plates at a density of 4.0×10^5 cells/well, followed by culture overnight in an incubator at 37°C with 5% CO_2. After that, two Eppendorf (EP; Hamburg, Germany) tubes were added with 200 μL of serum-free DMEM, 3 μL of siRNA-NC (si-NC group) or siRNA-Rap2A (si-Rap2A group) and 6 μL of Lipofectamine 2000 transfection reagent. After standing for 5 min, they were mixed, and followed by incubation at room temperature for 20 min. The mixture was added into a 6-well plate which was shaken to distribute the cells evenly. Finally, the cells were cultured in an incubator at 37°C with 5% CO_2 and collected for subsequent experiments.

Colony Formation Assay

A total of 1×10^3 BC cells were collected and seeded into 6-well plates after si-NC and si-Rap2A transfection. After 7 days, the formed colonies were stained with 0.5% Crystal Violet (Sigma-Aldrich, St. Louis, MO, USA), followed by washing with phosphate-buffered saline (PBS) for 3 times. The number of surviving cell colonies with per colony ≥ 50 cells was finally counted.

Transwell Assay

Transfection cells were collected and plated into the upper chamber of a transwell chamber in 24-well plate. Subsequently, the plate was gently moved to a cell incubator for 24 h of culture. Next, the plate was fixed with 500 μL of 4% paraformaldehyde and stained with 100 μL of Giemsa stain (Sigma-Aldrich, St. Louis, MO, USA) at room temperature. Then, a cotton swab was used to wiped off the cells not passing through the membrane. After drying, the cells were observed under a microscope. 5 fields were randomly selected for each sample, and the number of migrating cells was finally counted.

Real Time-quantitative Polymerase Chain Reaction (RT-qPCR) Analysis

Firstly, the cells were washed twice with pre-cooled PBS at 4°C and broken in a proper amount of ribonucleic acid (RNA) extracting solution (TRIzol; Invitrogen, Carlsbad, CA, USA). The concentration and purity of RNA were determined using a NanoDrop 2000 nucleic acid detector. Secondly, extracted total RNA was reversely transcribed into complementary deoxyribonucleic acid (cDNA), 1 μL of which was subjected to qRT-PCR reaction system. 3 replicates were set for each sample. The reaction conditions were as follows: 95°C for 1 min, 95°C for 15 s, and 60°C for 1 min, for a total of 40 cycles. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the internal reference in the quantitative analysis of the Rap2A expression. The primers used in this study were: Rap2A: F: 5'-AATACGACCCCACCATCGAG-3', R: 5'-ACCTTCTCATACCGCTTCACG-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Western Blot Analysis

Cells in each group were collected and washed with pre-cooled PBS twice. Then, the cells were sufficiently lysed by 250 μL of radioimmunopre-

cipitation assay (RIPA) containing phenylmethylsulfonyl fluoride (PMSF; Beyotime, Shanghai, China). After centrifugation at 4°C and 12,000 g for 10 min, the supernatant was aspirated to obtain total protein. The concentration of extracted total protein was determined using the bicinchoninic acid (BCA) protein assay kit (Beyotime, Shanghai, China). 30 μg of proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes after electrophoresis. Non-specific antigenic sites were blocked in 5% bovine serum albumin (BSA) at 4°C overnight. After incubation with primary antibodies at room temperature for 2 h and secondary antibodies at 4°C overnight, the membranes were washed with 1×Phosphate-Buffered Saline-Tween (PBST) for 5 min × 3 times. Immuno-reactive bands were finally exposed by the enhanced chemiluminescence (ECL) method.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 software (IBM Corp., Armonk, NY, USA) was used for all statistical analysis. Experimental data were represented as mean ± SD (Standard Deviation). The *t*-test was used for analyzing measurement data. Differences between two groups were analyzed by Student's *t*-test. One-way ANOVA was applied to compare the differences among different groups, followed by post-hoc test (Least Significant Difference). The association between Rap2A expression and clinicopathological characteristics was analyzed by Chi-square test. $p<0.05$ was considered statistically significant.

Results

Expression of Rap2A in Tumor Tissues

Paired BC tissues and adjacent normal tissues were first collected in this study, and the expression of Rap2A was detected. As a result, we found that the expression level of Rap2A in BC tissues was significantly higher than that of normal tissues ($p<0.05$, Figure 1A). To detect this result, Rap2A expression in four BC cell lines and one normal cell line was determined by qRT-PCR as well (Figure 1B). The results indicated that the expression level of Rap2A in BC cell lines was significantly higher than that of normal cells ($p<0.05$). MCF-10A and ZR-75-1 cells expressed the highest level of Rap2A, which were chosen for subsequent experiments.

Association Between Rap2A Expression and Clinicopathological Characteristics of BC Patients

Based on the median expression of Rap2A, we divided BC patients into two groups, including: low-expressing Rap2A group and high-expressing Rap2A group. The relationship between Rap2A expression and clinicopathological characteristics of BC patients was analyzed. The results showed that Rap2A expression was correlated with tumor size, clinical stage, and distant metastasis, whereas not with age, differentiation, lymph node metastasis, and HER-2 (Table I).

Rap2A Was Inhibited After Transfection of siRNA

Subsequently, we constructed two BC cell lines with low expression of Rap2A *in vitro*. qRT-

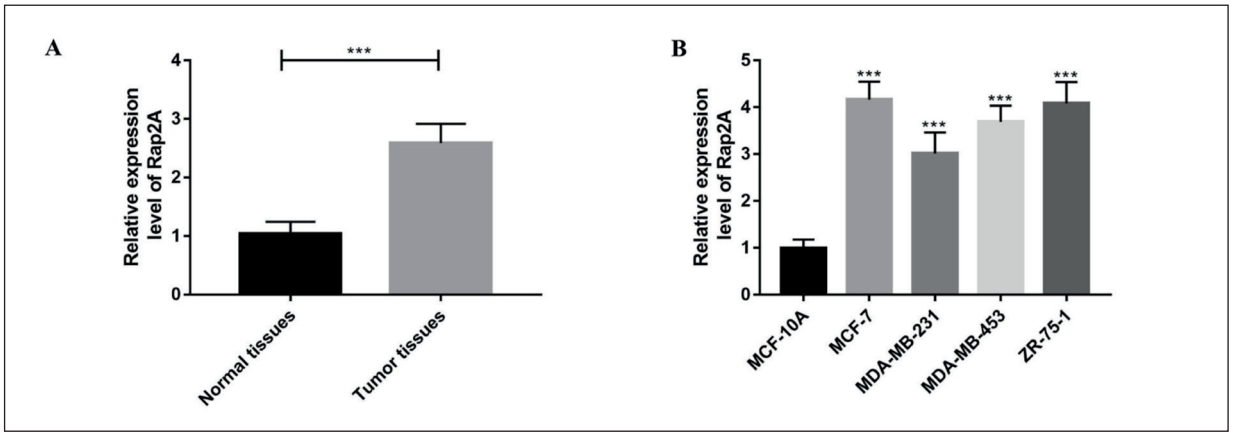

Figure 1. Expression of Rap2A in BC tissues and cells. **A,** Rap2A was upregulated in BC tissues compared with adjacent normal tissues. **B,** Rap2A was over-expressed in BC cell lines compared with normal epithelial cells. (***$p<0.001$).

Table I. Correlations between Rap2A expression and different clinicopathological characteristics of patients with breast cancer.

Characteristics	Cases n = 156	Rap2A mRNA expression		*p*-value
		Low (n = 72)	High (n = 84)	
Age (years)				
< 60	70	23	33	0.403
≥ 60	86	49	51	
Tumor size				
< 2	82	30	51	0.024
≥ 2	74	42	33	
Clinical stage				
I-II	68	22	49	< 0.001
III-IV	88	50	35	
Differentiation				
Well-moderate	89	43	37	0.351
Poor	67	39	47	
Distant metastasis				
No	72	32	52	0.036
Yes	84	40	32	
Lymph node metastasis				
Negative	75	30	45	0.151
Positive	81	42	39	
HER-2				
Negative	65	39	40	0.427
Positive	91	33	44	

PCR and Western blot proved the transfection efficiency. Compared with MCF-10A and ZR-75-1 cells in si-NC group, Rap2A expression was significantly reduced in cells in si-Rap2A group ($p<0.05$, Figure 2).

Si-Rap2A Weakened the Proliferation Ability of BC Cells

Next, we performed colony formation assay based on the above cell model. As shown in Figure 3, compared with normal BC cells, the number of formed colonies in cells with low expression of Rap2A was significantly reduced ($p<0.05$). These findings suggested that inhibition of Rap2A expression could reduce the proliferation ability of BC cells.

Si-Rap2A Decreased the Invasion Ability of BC Cells

Transwell assay was then explored to detect the effect of Rap2A on cell migration. The results demonstrated that compared with si-NC group, low expression of Rap2A significantly inhibited the invasion of BC cells per field in si-Rap2A group ($p<0.05$, Figure 4). The above results suggested that BC cells with inhibition of Rap2A were accompanied with weakened invasion ability.

Discussion

Characterized by high invasiveness, low cure rate, and proneness to recurrence or metastasis, BC seriously threatens human health worldwide[20,21]. Different molecular types of BC manifest diversified biological behaviors. Therefore, therapeutic methods for BC vary prominently from case to case[22]. Currently, the systematic and comprehensive treatments of BC have become more complete[23-25]. However, traditional therapeutic modes, such as surgery, chemotherapy, and radiotherapy cannot satisfy the demands of BC patients for individualized treatments[26]. Therefore, actively seeking for new therapeutic targets is the focus of research on BC.

Rap2A was initially discovered by screening the Burkitt lymphoma library of drosophila-originated Dras3 gene probes. Numerous studies have found that Rap2A can participate in different cellular events *via* cell signaling[27,28], such as cell adhesion, proliferation, and gene activation. Meanwhile, Rap2A requires more accurate spatial-temporal control to exert its effects. When stimulated by upstream signaling molecules, the GDP-bound inactive form of the catalyzed Rap2A kinase transforms into the GTP-bound active form. Such a transformation

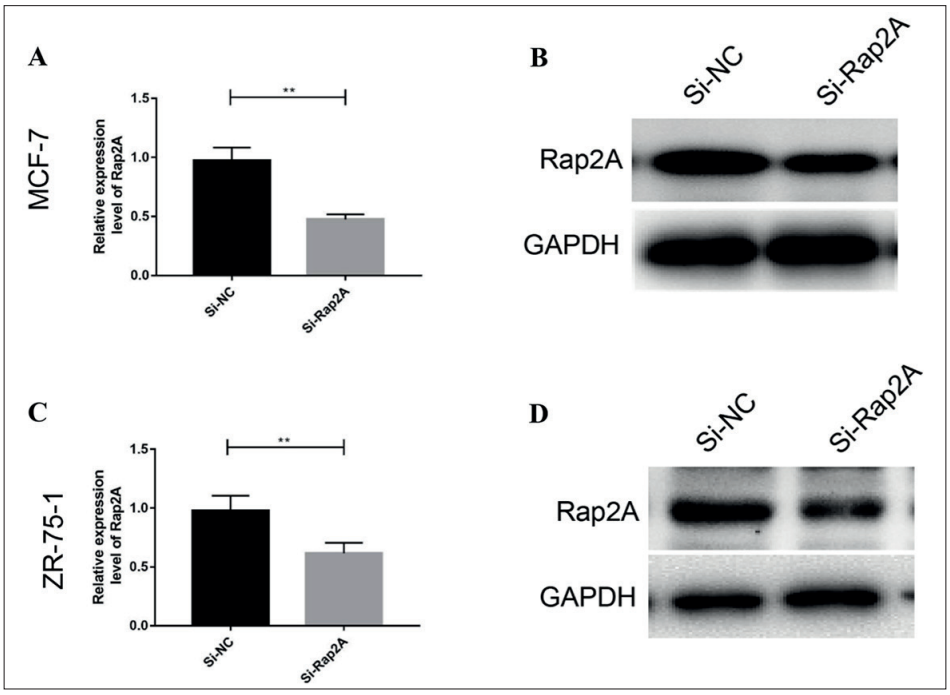

Figure 2. Rap2A expression was inhibited after transfection of siRNA. RT-qPCR and Western blot results indicated that the mRNA and protein expressions of Rap2A were both significantly inhibited in MCF-10A cells (**A, B**) and ZR-75-1 cells (**C, D**) after transfection of siRNA. (**p<0.01).

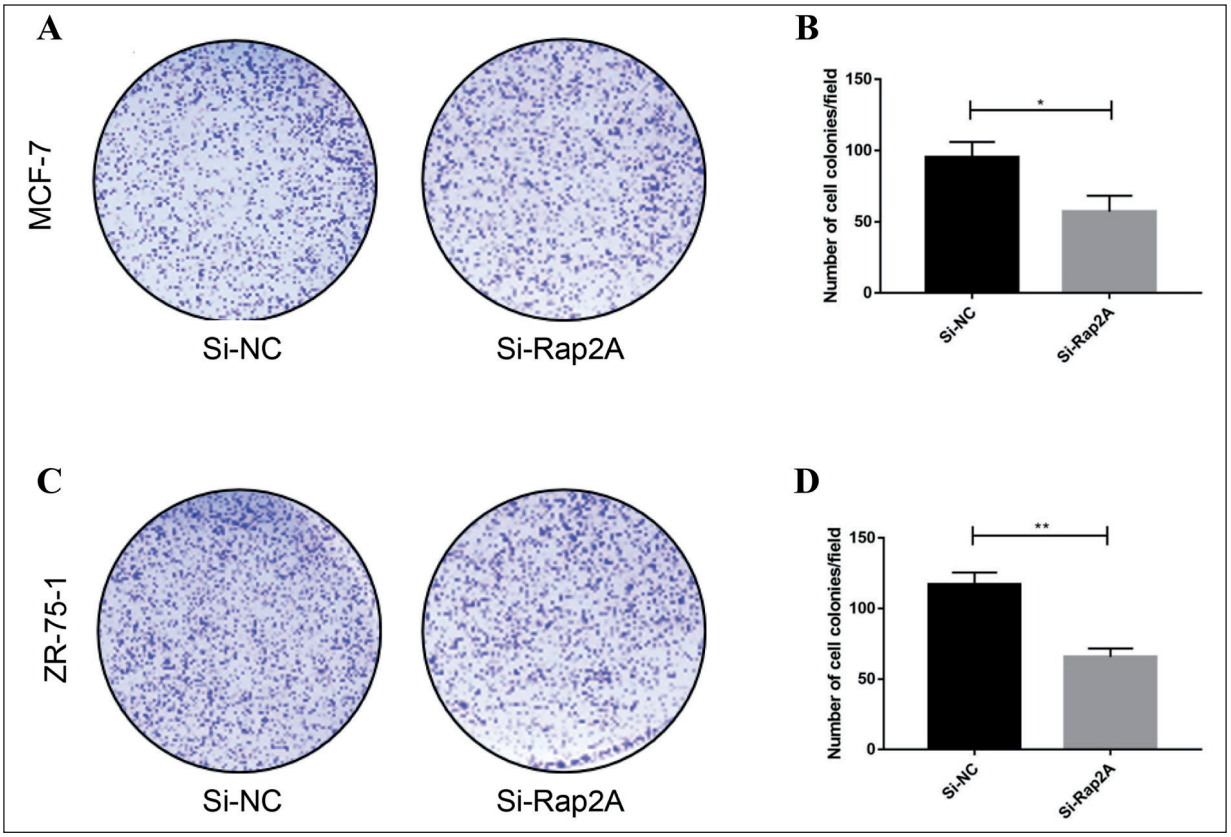

Figure 3. Si-Rap2A weakened the proliferation ability of BC cells. The proliferation ability of MCF-10A cells (magnification: 40×) (**A, B**) and ZR-75-1 cells (**C, D**) in si-Rap2A group decreased significantly compared with cells in NC group detected by colony formation assay (magnification: 40×) (*p<0.05, **p<0.01).

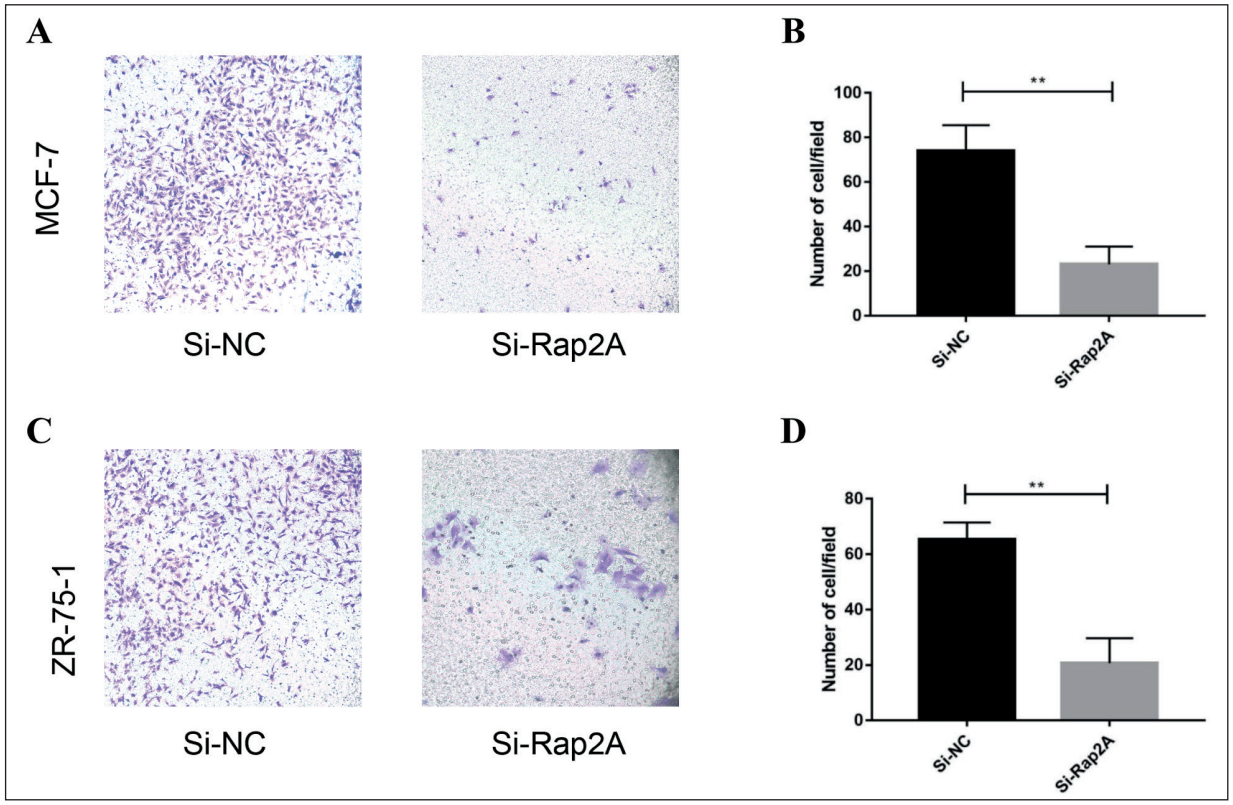

Figure 4. Si-Rap2A decreased the invasion ability of BC cells. The invasion ability of MCF-10A cells (magnification: 400×) (**A, B**) and ZR-75-1 cells (**C, D**) in si-Rap2A group was obviously reduced compared with cells in si-NC group (magnification: 40×). (**p<0.01).

between GDP and GTP can alter the conformation and function of downstream target genes. This enables the Rap2A-GTP active form to stimulate downstream signaling pathways, thus regulating cytoskeleton-actin dynamics and related gene expression[14].

Concerning the role of Rap2A in regulating tumorigenesis, Prabakaran et al[18] have found that Rap2A is significantly highly expressed in micro-dissected carcinoma cells that have invaded through the thyroid capsule compared with thyroid tumor cells growing under the capsule. Wu et al[29] have shown that Rap2A is overexpressed in many types of tumors, which enhances the migration and invasion of cancer cells and increases the activities of MMP2 and MMP9. However, inhibition of Rap2A inhibits cell invasion and activities of MMP2 and MMP9. In addition, Zhang et al[30] have revealed that Rap2A promotes the viability, migration, and invasion abilities of gastric cancer cells, and inhibits cell apoptosis and DNA damage, thereby increasing cisplatin resistance. Moreover, Rap2B, also a member of GTP-binding proteins, has been detected to play a crucial role in the proliferation, migration, and invasion of human BC[31].

Herein, the expression level of Rap2A was found significantly upregulated in BC tissues when compared with adjacent normal tissues. Similarly, the expression level of Rap2A in BC cell lines was remarkably higher than that in normal epithelial cells. Further analysis indicated that high expression of Rap2A was significantly correlated with clinicopathological characteristics of BC patients, including tumor size, clinical stage, and distant metastasis. In order to better clarify the effect of Rap2A on BC, siRNA was transfected into BC cells to construct a Rap2A low expression model *in vitro*. Transfected efficiency was verified by qRT-PCR and Western blot. On this basis, we conducted a series of *in vitro* experiments, including colony formation assay and transwell assay. The results showed that compared with BC cells in si-NC group, the number of clones and the number of invasive cells in si-Rap2A group remarkably decreased.

All these findings indicated that low expression of Rap2A significantly inhibited the proliferation and invasion of BC cells.

Conclusions

Taken together, the aforementioned results demonstrated that Rap2A was highly expressed in BC tissues and was tightly corrected with clinicopathological characteristics of BC patients. All these findings suggested that Rap2A might be a new potential biomarker for the diagnosis and treatment of BC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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