Original Research Article

BCL9 enhances the development of cervical carcinoma by deactivating CPEB3/EGFR axis

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Abstract

Purpose: To investigate the differential expression of BCL9 in cervical carcinoma samples, analyze its biological functions in regulating malignant phenotypes of cervical carcinoma cells, and to explore its potential molecular mechanism.

Methods: Expression levels of BCL9 in 58 pairs of cervical carcinoma tissues and paracancerous tissues were determined using quantitative real time-polymerase chain reaction (qRT-PCR). Kaplan-Meier curves were used to analyze the prognostic potential of BCL9 in cervical carcinoma. After knockdown using BCL9 by lentivirus transfection, proliferative and migratory changes in Siha and HeLa cells were determined by CCK-8, colony formation and Transwell assays. Cytoplasmic polyadenylation element binding protein 3 (CPEB3), the potential downstream target of BCL9, was confirmed via dual-luciferase reporter assay. Western blot analyses were conducted to determine the protein levels of CPEB3, EGFR, AKT and p21 in Siha and HeLa cells with BCL9 knockdown. The co-regulation of BCL9 and CPEB3 on phenotypes of cervical carcinoma cell was investigated.

Results: BCL9 was upregulated in cervical carcinoma tissues. The high level of BCL9 was predicted by the tumor size, advanced stage and poor prognosis. The knockdown of BCL9 significantly weakened proliferative and migratory abilities of Siha and HeLa cells (p < 0.05). CPEB3 was the downstream target of BCL9, and was lowly expressed in cervical carcinoma tissues. The knockdown of BCL9 upregulated CPEB3, and downregulated EGFR, AKT and p21 (p < 0.05). The knockdown of CPEB3 also reversed the influence of silenced BCL9 in regulating its proliferative and migratory abilities in cervical carcinoma cells (p < 0.05).

Conclusion: BCL9 drives the deterioration of cervical carcinoma by inhibiting the CPEB3/EGFR axis. Thus, BCL9 may be a novel molecular target for cervical carcinoma treatment.

Keywords: B-cell lymphoma 9, Cytoplasmic polyadenylation element binding protein 3 (CPEB3), Cervical carcinoma

INTRODUCTION

Cervical carcinoma has become the third most-common malignant tumor, and the second leading cause of death in females globally. Annually, approximately 530,000 people die of cervical carcinoma worldwide [1,2], and up to 460,000 new diagnoses of cervical carcinoma are recorded every year globally, with about 50 % of them from Asia [2-4]. Potential pathogenic

factors for cervical carcinoma include premature sexual activity, sexual life disorder, premature birth, multiparous births, racial and geographical causes, poor economy, human papillomavirus (HPV) infection, etc. In particular, HPV infection is a major reason for the carcinogenesis of cervical carcinoma [5-7]. It induces the integration of viral DNAs of cervical carcinoma into chromosome DNAs by activating oncogenes and/or inactivating tumor suppressors [7]. However, not all HPV infections develop into cervical carcinoma. The therapeutic effects of HPV-related cervical cancer are not satisfactory [8,9]. It is therefore of great importance to develop a specific diagnosis and therapeutic target for cervical carcinoma [10,11]. The B-cell lymphoma 9 (BCL9) was initially detected in a patient with pre-B cell acute lymphoblastic leukemia. It is located on chromosome 1q21, and is prone to translocation of the chromosome (1:14) (q21;q23) [12,13]. B-cell lymphoma has been identified as a novel oncogene in the Wnt pathway. Abnormally expressed BCL9 triggers the abnormal activation of the Wnt pathway, and thus induces tumorigenesis [12]. In recent years, the involvement of the Wnt pathway in malignant phenotypes of tumor cells has been well concerned [14,15]. Previous studies have reported that BCL9 is differentially expressed in many types of tumors [13,16,17]. The clinical significance of BCL9 in cervical carcinoma remains unclear, which will be explored in this paper.

METHODS

Cervical carcinoma samples

Cervical carcinoma and paracancerous tissues were collected from 58 patients, and preserved in liquid nitrogen for isolating RNAs. The TNM staging was defined according to the criteria proposed by UICC/AJCC (8th edition). This study was approved by the research ethics committee of The Second Affiliated Hospital of Zhejiang University School of Medicine, and complied with the guidelines of Helsinki Declaration. Informed consent was obtained from the patients prior to collecting samples and clinical data.

Cell lines and reagents

Cervical carcinoma cell lines (HeLa, Siha, Caski, HCC94 and C33-A) and the immortalized keratinocyte line (HaCaT) were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). They were cultivated in DMEM at 37 ℃ with 5 % CO_2. Fetal bovine serum (FBS) (10 %) (Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 μg/mL streptomycin were added to culture medium. Cell passage was conducted every 2 - 3 days.

Transfection

Cells were seeded in 6-well plate with 2 mL of medium per well. Transfection of shRNAs, which were constructed by GenePharma (Shanghai, China), was conducted using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). Fresh medium was replaced at 4 - 6 h.

Cell proliferation assay

Cells were inoculated in a 96-well plate with 2.5 × 10^3 cells/well. At days 1, 2, 3 and 4, absorbance value at 450 nm of each sample was measured using the cell counting kit-8 (CCK-8) kit (Dojindo Laboratories, Kumamoto, Japan) for plotting the viability curves.

Colony formation assay

Cells were inoculated in 6-well plate at 200 cells/well. Visible colonies were washed by phosphate buffered saline (PBS), fixed in methanol for 20 min and stained with 0.1 % crystal violet for 20 min. Colonies were captured in a well-lit environment.

Transwell migration assay

Transfected cells were prepared in serum-free suspension at 2.0 × 10^5/mL. In the Transwell chamber, 200 μl of suspension and 500 μl of complete medium were added into the upper and bottom chamber. After 24 h of incubation, cells in the bottom were fixed in methanol for 15 min, stained with crystal violet for 30 min and counted under a light microscope.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Cellular or tissue RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and reversely transcribed into cDNA using Primescript RT Reagent (TaKaRa, Otsu, Japan). SYBR®Premix Ex Taq™ (TaKaRa, Otsu, Japan) was used for qRT-PCR. The relative level was calculated by $2^{-\Delta\Delta Ct}$. The primers used were shown below: BCL9: Forward: 5'-ATACCA GGAGGCCAGGGATT-3', Reverse: 5'-GGGCCC ACATTCAGTCCTTT-3'; CPEB3: Forward: 5'-TTGCCAGAGCGGTCACATAA-3', Reverse: 5'-TTTGCGCAGCTTTGAGTGTC-3'; GAPDH: Forward: 5'-GCACCGTCAAGGCTGA GAAC-3', Reverse: 5'-ATGGTGGTGAAGACG CCAGT-3'.

Western blot

The cells were lysed in radioimmunoprecipitation assay (RIPA) on ice for 30 min, and centrifuged at 14000 rpm × g for 15 min, and at a temperature of 4 ℃. The concentration of cellular protein was determined by bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). Protein samples were resolved with sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto a polyvinylidene fluoride (PVDF) membrane (Roche, Basel, Switzerland). The membrane was cut into small pieces according to the molecular size and blocked in 5 % skim milk for 2 h. Thereafter, they were incubated with primary antibodies (anti-CPEB3, anti-EGFR, anti-AKT, anti-p21 and anti-GAPDH), followed by incubation with the secondary goat anti-rabbit (HRP) IgG antibody. All the antibodies were purchased from Abcam (Cambridge, MA, USA).

Dual-luciferase reporter assay

The BCL9-WT and BCL9-MUT were constructed based on the predicted binding sites in the 3'UTR of BCL9 and CPEB3. They were co-transfected into cells with either pcDNA-CPEB3 or pcDNA-NC. After co-transfection for 48 h, luciferase activity was determined according to the manufacturer's protocols.

Statistical analysis

SPSS statistical analysis software (version 26.0) was used for statistical analysis. Data are expressed as mean ± SD, and compared using the *t*-test. Kaplan-Meier survival curves were plotted based on BCL9 and CPEB3 levels in cervical carcinoma patients. Chi-square test was applied for analyzing the influence of BCL9 on pathological parameters in cervical carcinoma patients. $p < 0.05$ was considered as statistically significant.

RESULTS

Clinical significance of BCL9 in cervical carcinoma

A total of 58 cases of cervical carcinoma tissues and paracancerous ones were collected. The BCL9 was upregulated in cervical carcinoma tissues (Figure 1). Based on the detected level of BCL9 in cervical carcinoma tissues, the patients were classified into low BCL9 expression group and high BCL9 expression group. The influence of BCL9 level on pathological parameters in cervical carcinoma was analyzed using the Chi-square test. Significant differences in tumor size and tumor stage were identified between groups (Table 1). In addition, Kaplan-Meier curves demonstrated that poor prognosis was predicted in cervical carcinoma patients overexpressing BCL9. The above results indicated that BCL9 may be a promising biomarker for cervical carcinoma.

Knockdown of BCL9 weakened the proliferative and migratory abilities of cervical cancer ball

To explore the role of BCL9 in cell behaviors of cervical carcinoma, the *in vitro* expression of BCL9 was first examined.

Table 1: Association of BCL9 expression with clinicopathologic characteristics of cervical carcinoma

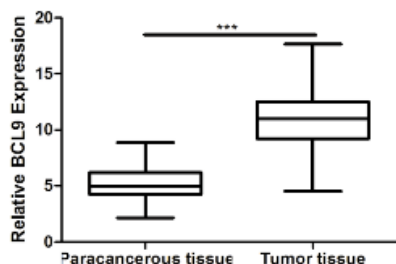| Parameter	Number of cases	BCL9 expression		*P*-value
		Low (n=35)	High (n=23)	
Age (years)				0.539
<60	23	15	8	
≥60	35	20	15	
Tumor size (cm)				0.031
≤5	44	30	14	
>5	14	5	9	
T stage				0.006
T1-T2	33	25	8	
T3-T4	25	10	15	
Lymph node metastasis				0.956
No	28	17	11	
Yes	30	18	12	
Distance metastasis				0.778
No	34	20	14	
Yes	24	15	9	

Figure 1: Clinical significance of BCL9 in cervical carcinoma. Differential expressions of BCL9 in cervical carcinoma tissues and paracancerous tissues; ***$p <$ 0.001, vs paracancerous tissues

Results showed that BCL9 was significantly upregulated in cervical carcinoma cell lines compared with the immortalized keratinocyte line (Figure 2 A). In particular, BCL9 level remained highest in Siha and HeLa cell lines, and they were used for constructing BCL9 knockdown models by the transfection of sh-BCL9 (Figure 2 B). The CCK-8 assay uncovered the finding that the knockdown of BCL9 reduced the viability of Siha and HeLa cells (Figure 2 C). Similarly, transfection of BCL9 significantly decreased colony number, indicating that the inhibited proliferative ability (Figure 2 D). In addition, migratory cell number declined due to the knockdown of BCL9, suggesting an attenuated migratory ability (Figure 2 E).

CPEB3 was the downstream target of BCL9

Using the bioinformatic software, CPEB3 was predicted as the potential target of BCL9. Dual-luciferase reporter assay showed that overexpression of CPEB3 reduced the luciferase activity in BCL9-wt, but did not affect luciferase activity in BCL9-mut, confirming that BCL9 targeted CPEB3 (Figure 3 A). Subsequently, qRT-PCR data revealed lowly expressed CPEB3 in cervical carcinoma tissues (Figure 3 B). Kaplan-Meier survival curves suggested poor survival in cervical carcinoma patients expressing low level of CPEB3. Western blot analyses showed that the knockdown of BCL9 upregulated CPEB3, and downregulated EGFR, AKT and p21 in Siha and HeLa cells (Figure 3 C). It has been shown that the CPEB3/EGFR axis could be involved in the BCL9-regulated progression of cervical carcinoma.

CPEB3 was involved in BCL9-regulated progression of cervical carcinoma

The involvement of CPEB3 in the progression of cervical carcinoma was further investigated. The transfection of si-CPEB3 in Siha and HeLa cells with BCL9 knockdown effectively downregulated

protein level of CPEB3, and upregulated BCL9 (Figure 4 A). Compared with cells with BCL9 knockdown, cell viability and clonality were much higher in Siha and HeLa cells, with the co-silence of BCL9 and CPEB3 (Figure 4 B and C). As expected, a higher migratory cell number was detected in the cells co-transfected with sh-BCL9 and si-CPEB3, relative to cells co-transfected with sh-BCL9 and si-NC (Figure 4 D). Therefore, CPEB3 was able to reverse the regulatory effects of BCL9 on proliferative and migratory abilities in cervical carcinoma.

DISCUSSION

As a highly prevalent tumor in the female reproductive system, cervical carcinoma seriously endangers women's health and lives [1-3]. Cervical carcinoma progressively deteriorates to cervical intraepithelial neoplasia (I-III), carcinoma *in situ* and infiltrating carcinoma [2,4], and it is prone to develop local infiltration and lymphatic metastasis.
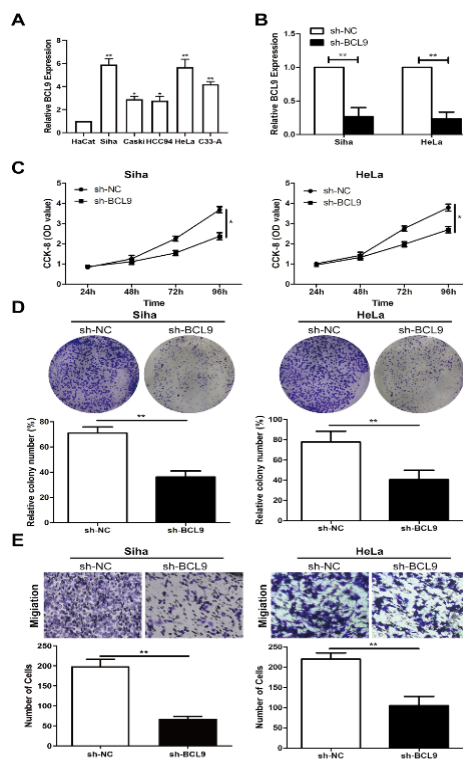

Figure 2: Knockdown of BCL9 weakened the proliferative and migratory abilities in cervical carcinoma. (A) BCL9 levels in cervical carcinoma cell lines, (B) Transfection efficacy of sh-BCL9 in Siha and HeLa cells, (C) Viability in Siha and HeLa cells with BCL9 knockdown, (D) Colony number in Siha and HeLa cells with BCL9 knockdown; (E) Migration in Siha and HeLa cells with BCL9 knockdown. *$P < 0.05$, **$p < 0.01$
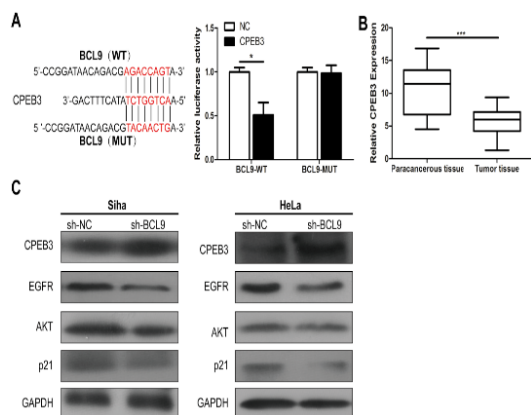
Figure 3: CPEB3 was the downstream target of BCL9. (A) Luciferase activity in co-transfected Siha and HeLa cells, (B) Differential expressions of CPEB3 in cervical carcinoma tissues and paracancerous tissues, (C) Protein levels of CPEB3, EGFR, AKT and p21 in Siha and HeLa cells with BCL9 knockdown. *p* < 0.05, ***p* < 0.001
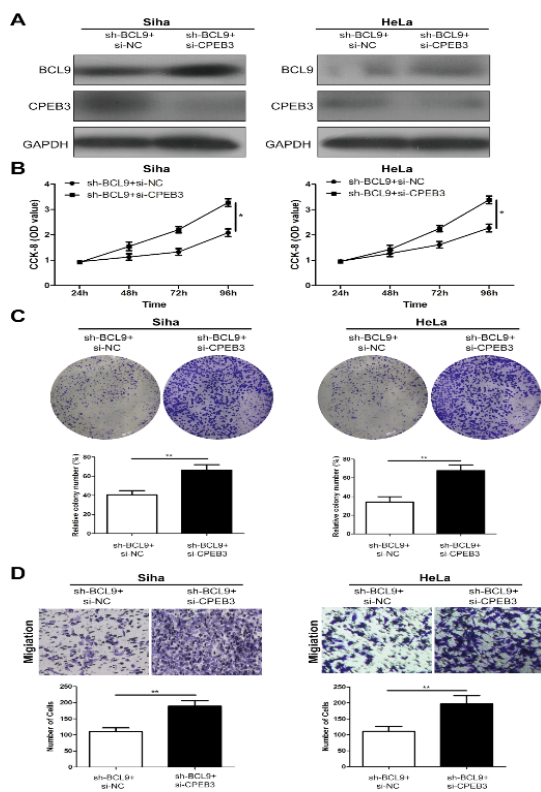

Figure 4: CPEB3 was involved in BCL9-regulated progression of cervical carcinoma. (A) Protein levels of BCL9 and CPEB3 in Siha and HeLa cells with BCL9 knockdown or co-knockdown of BCL9 and CPEB3, (B) Viability in Siha and HeLa cells with BCL9 knockdown or co-knockdown of BCL9 and CPEB3, (C) Colony number in Siha and HeLa cells with BCL9 knockdown or co-knockdown of BCL9 and CPEB3; (D) Migration in Siha and HeLa cells with BCL9 knockdown or co-knockdown of BCL9 and CPEB3. *P* < 0.05, **p* < 0.01

It is estimated that the 5-year survival rate in cervical carcinoma patients with lymphatic metastasis is only 50.8 % [5]. Although the screening method of cervical carcinoma has been widely popularized, the 5-year survival rate is not good [2,5,6]. Surgery, chemotherapy and radiotherapy are the major therapeutic strategies for cervical carcinoma. Nevertheless, adverse events caused by these traditional treatments pose great physical and psychological burdens on cervical carcinoma patients [7,8]. The recently emerged immune therapy and gene therapy are promising in clinical application [9-11].

The BCL9 has been reported as an oncogene in many types of tumors [13,16,17]. In this study, 58 cases of cervical carcinoma samples were collected, and B-cell lymphoma 9 was upregulated in the carcinoma tissues, and its level positively correlated to tumor size, tumor stage and overall survival. It was believed that BCL9 may exert a carcinogenic role during the progression of cervical carcinoma. By generating BCL9 knockdown models *via* sh-BCL9 transfection, proliferative and migratory abilities in Siha and HeLa cells were markedly weakened. This indicated that BCL9 was responsible for the malignant phenotypes of cervical carcinoma cells, but the molecular mechanism was unclear.

The B-cell lymphoma 9 is an adapter protein in the classic Wnt pathway, which is responsible for the formation of the tetramer: TCF/LEF-β-catenin-BCL9-PYGO, by connecting Aramdillo/β-catenin and PYGO in the nucleus. Besides, BCL9 activates β-catenin-binding cofactor Pygo, and induces the nuclear aggregation of β-catenin, thereby further stimulating the formation of the tetramer and activating the Wnt pathway [13]. BCL8 is a vital regulator during embryo development, stem cell growth and tumorigenesis [12-14]. B-cell lymphoma 9-2 is the homologous product of BCL9. In addition to being a cofactor for transcription of β-catenin, BCL9-2 can also affect the cell adhesion of β-catenin, thus maintaining the normal development of the body. Otherwise, it leads to tumor growth, invasiveness and metastasis [13-15]. This study confirmed that CPEB3 was the downstream target of BCL9. CPEB3 was downregulated in cervical carcinoma tissues, and it was a favorable factor for the prognosis in cervical carcinoma. Notably, Western blot analysis demonstrated that the knockdown of BCL9 in Siha and HeLa cells activated the CPEB3/EGFR axis, suggesting that the CPEB3/EGFR axis regulated by BCL9 may be involved in the deterioration of cervical carcinoma. Through rescue experiments, it was found that co-silencing of BCL9 and CPEB3

resulted in higher proliferative and migratory abilities in cervical carcinoma cells than those with BCL9 knockdown. As a result, it may be concluded that BCL9 aggravated the malignant progression of cervical carcinoma by inhibiting the CPEB3/EGFR axis.

CONCLUSION

BCL9 is upregulated in cervical carcinoma samples, and can be used for predicting prognosis in cervical carcinoma. BCL9 drives the deterioration of cervical carcinoma by inhibiting CPEB3/EGFR axis. Therefore, BCL9 may be a novel molecular target for cervical carcinoma treatment.

DECLARATIONS

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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REFERENCES

1. Munoz N, Bravo LE. Epidemiology of cervical cancer in Colombia. Salud Publica Mex 2014; 56(5): 431-439.
2. Gottlieb SD. U.S. Cervical Cancer Mortality. J Adolesc Health 2016; 58(3): 372.
3. Tao Y, Mao F, Gu W, Wu L, Guo J, Xu P. Mir-23b down-regulates the expression of target gene of acetaldehyde dehydrogenase 1a1 and increases the sensitivity of cervical cancer stem cells to cisplatin. Trop J Pharm Res 2021; 20(1):29-34
4. Di J, Rutherford S, Chu C. Review of the cervical cancer burden and population-based cervical cancer screening in China. Asian Pac J Cancer Prev 2015; 16(17): 7401-7407.
5. Johnson CA, James D, Marzan A, Armaos M. Cervical Cancer: An overview of pathophysiology and management. Semin Oncol Nurs 2019; 35(2): 166-174.
6. Ramadan WS, Sait KH, Anfinan NM. Anticancer activity of aqueous myrrh extract alone and in combination with cisplatin in HeLa cells. Trop J Pharm Res 2017; 16(4):889-896
7. Goodman A. HPV testing as a screen for cervical cancer. BMJ 2015; 350: h2372.
8. Fang J, Zhang H, Jin S. Epigenetics and cervical cancer: from pathogenesis to therapy. Tumour Biol 2014; 35(6): 5083-5093.
9. Dukic A, Lulic L, Thomas M, Skelin J, Bennett SN, Grce M, Banks L, Tomaic V. HPV Oncoproteins and the Ubiquitin Proteasome System: a signature of malignancy? Pathogens 2020; 9(2): 133.
10. Wang J, Wang Y, Shen F, Xu Y, Zhang Y, Zou X, Zhou J, Chen Y. Maternal embryonic leucine zipper kinase: A novel biomarker and a potential therapeutic target of cervical cancer. Cancer Med 2018; 7(11): 5665-5678.
11. Curty G, de Carvalho PS, Soares MA. The Role of the cervicovaginal microbiome on the genesis and as a biomarker of premalignant cervical intraepithelial neoplasia and invasive cervical cancer. Int J Mol Sci 2019; 21(1): 222.
12. van Andel H, Kocemba KA, Spaargaren M, Pals ST. Aberrant Wnt signaling in multiple myeloma: molecular mechanisms and targeting options. Leukemia 2019; 33(5): 1063-1075.
13. Jiang M, Kang Y, Sewastianik T, Wang J, Tanton H, Alder K, Dennis P, Xin Y, Wang Z, Liu R, et al. BCL9 provides multi-cellular communication properties in colorectal cancer by interacting with paraspeckle proteins. Nat Commun 2020; 11(1): 19.
14. Zhan T, Rindtorff N, Boutros M. Wnt signaling in cancer. Oncogene 2017; 36(11): 1461-1473.
15. Duchartre Y, Kim YM, Kahn M. The Wnt signaling pathway in cancer. Crit Rev Oncol Hematol 2016; 99: 141-149.
16. Gay DM, Ridgway RA, Muller M, Hodder MC, Hedley A, Clark W, Leach JD, Jackstadt R, Nixon C, Huels DJ, et al. Loss of BCL9/9l suppresses Wnt driven tumourigenesis in models that recapitulate human cancer. Nat Commun 2019; 10(1): 723.
17. Yao B, Li Y, Wang L, Chen T, Niu Y, Liu Q, Liu Z. MicroRNA-3194-3p inhibits metastasis and epithelial-mesenchymal transition of hepatocellular carcinoma by decreasing Wnt/beta-catenin signaling through targeting BCL9. Artif Cells Nanomed Biotechnol 2019; 47(1): 3885-3895.