EPB49 PROMOTES COLORECTAL CANCER CELL PROLIFERATION AND MIGRATION BY REGULATING WNT AND MAPK SIGNALING PATHWAYS THROUGH TIAMLRACL SIGNALING PATHWAY

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ABSTRACT

Objective: *To investigate the effect and mechanism of erythrocyte membrane protein band 4.9 (EPB49) regulating mitogen activated protein kinase (MAPK) and wingless MMTV family members (Wnt) signaling pathway on colorectal cancer cell proliferation and migration through T-lymphoma invasion and metastasis inducing factor (Tiam1)-Rac1 signaling pathway.*

Methods: *Human colon cancer cell lines SW480 and HT29 were collected for routine culture. An EPB49 overexpression group was constructed using the pSin-EF2-puro vector along with a negative control (NC) group. HT29 was constructed by using PLTHM to create the interference lentivirus group (shRNA group) and its corresponding negative control group. The number of migration cells, Rac1-GTP, EPB49 white expression level, Wnt signaling pathway (p27 and cyclin D1), MAPK signaling pathway related proteins (c-Jun N-terminal kinase (JNK) and p-JNK) expression levels and cell proliferation rate (OD value) of each cell group at 1d, 3d, 5d, 7d were compared, and the interaction between EPB49 and Tiam1 was analyzed.*

Results: *At 5d and 7d, the proliferation rate of SW480 cells in the EPB49 group was significantly lower than the NC group ($P < 0.05$). In HT29 cells, the proliferation rate of the shRNA group was significantly higher than that of the NC group ($P<0.05$). In SW480 cells, the number of migration cells in the EPB49 group was significantly lower compared to the NC group ($P<0.05$). In HT29 cells, cell migration in the shRNA group was significantly higher than that found in the NC group ($P<0.05$). In SW480 cells, the expression level of Rac1-GTP protein in the EPB49 group was significantly lower than that in the NC group ($P<0.05$). In HT29 cells, the expression level of Rac1-GTP protein in the shRNA group was significantly higher than the NC group ($P<0.05$). Interaction between EPB49 and Tiam1 was found using immunoprecipitation. In SW480 cells, the expression levels of EPB49 and p27 in the EPB49 group were significantly higher than those in the NC group, and the cyclin D1 protein expression level was significantly lower compared to the control. In HT29 cells, the expression levels of EPB49 and p27 in the shRNA group were significantly lower than those in the NC group, and cyclin D1 protein expression level was significantly higher than the NC group ($P<0.05$). In SW480 cells, the expression level of p-JNK protein in the EPB49 group was significantly lower compared to the NC group ($P<0.05$), and there was no significant difference in JNK protein expression among all groups ($P>0.05$). In HT29 cells, the expression level of p-JNK protein in the shRNA group was significantly higher than that in the NC group ($P<0.05$), but there was no significant difference in JNK protein expression level among different groups ($P>0.05$).*

Conclusion: *EPB49 can regulate Wnt and MAPK signaling pathways through Tiam1-Rac1 signaling pathway to promote the proliferation and migration of colorectal cancer cells.*

Keywords: *EPB49, Tiam1-Rac1, Wnt, MAPK.*

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Introduction

Colorectal cancer is a malignant tumor of the digestive system with a high incidence, especially in developed countries. The incidence of colorectal cancer and its tumor-related mortality both occupy the third place amongst all cancers[1]. At present, human dietary structure and lifestyle have changed, resulting in increasing year-by-year incidence of colorectal cancer as well as a younger age of onset[2]. Previous studies have confirmed that metastasis is a major cause of death in cancer patients. Nearly 50% of patients who are diagnosed for the first time already have tumor metastases with the liver being the most common site for metastasis. If there are metastases in a colorectal cancer patient, the

treatment may not be timely enough to be effective with estimated survival time being 5-10 months[3-4]. Therefore, revealing the pathogenesis and metastasis of colorectal cancer is beneficial to explore new intervention methods and provide reference value for clinical treatment.

Erythrocyte membrane protein band 4.9 (EPB49) was first discovered in the human erythrocyte membrane skeleton. Clinical studies have shown that it has function maintaining the shape and integrity of red blood cell membranes[5]. Under normal physiological conditions, EPB49 is expressed in hematopoietic system cells. However, it has been reported that it is expressed in cardiomyocytes, epithelial cells, and other cells[6]. Clinical studies have shown that the loss or change of EPB49 expression may be closely associated with the onset of a tumor. T-lymphoma invasion and metastasis factor (Tiam1) belongs to Rac1 and RhoA activity regulator and plays an important role in cytoskeleton recombination as well as tumor onset and progression through Tiam1-Rac1 signaling pathway[7]. Clinical studies have shown that the mechanism of colorectal cancer metastasis is very complex involving mitogen activated protein kinase (MAPK), wingless-type MMTV integration site family members (Wnt), and other signaling pathways. However, the role of EPB49, Tiam1-Rac1, Wnt and MAPK signaling pathways in colorectal cancer remains unclear[8].

Therefore, this study aimed to explore the effects and mechanisms of EPB49 on the proliferation and migration of colorectal cancer cells by regulating the Wnt and MAPK signaling pathways through the Tiam1-Rac1 signaling pathway.

Materials and methods

Experimental materials

Human colorectal cancer cell line SW480 was purchased from Shenzhen Haodi Huatuo Biotechnology Co., Ltd. Human colon cancer cell line HT29 was purchased from Kilton Biotechnology (Shanghai) Co., Ltd.

Main reagents and instruments

Reagents

Rabbit anti-EPB49, JNK and p-JNK monoclonal antibodies were purchased from Shanghai Bohu Biotechnology Co., Ltd. Serum working fluid for sealing was purchased from Wuhan Hualianke Biotechnology Co., Ltd. Transwell chamber was purchased from Corning Life Sciences (Wujiang) Co., Ltd. BCA protein quantitative kit was purchased from Shanghai Kanglang Biotechnology Co., Ltd. ECL luminous liquid was purchased from Beijing Applygen Gene Technology Co., Ltd. The overexpressed lentiviral vector pSin-EF2-puro was purchased from Wuhan Miaoling Biotechnology Co., Ltd. Interfering lentiviral vector PLTHM was purchased from Shanghai HJHAG Biotechnology Co., Ltd.

Instruments

Super clean workbench purchased from Kirton Biotechnology (Shanghai) Co., Ltd. The cell incubator was purchased from Shanghai Xinyu Biotechnology Co., Ltd. Low-temperature overspeed centrifuge was purchased from Shanghai Shiwei Experimental Instrument Technology Co., Ltd. The 6-well plate was purchased from Beijing Kairiji Biotechnology Co., Ltd. The electrophoresis tank was purchased from Shanghai Chenlian Biotechnology Development Co., Ltd. The optical microscope was purchased from Wuhan Purity Biotechnology Co., Ltd.

Methods

• Human colorectal cancer cell lines SW480 and HT29 were routinely cultured. Twenty-four hours before transfection, cells in the logarithmic growth phase were digested with trypsin and transplanted into a 6-well plate at a cell density of 5×105. Transfection was performed when the cell fusion degree reached 80%. EPB49 overexpression (EPB49 group) was constructed using the pSin-EF2-puro vector in SW480 cells along with the corresponding negative control (NC). HT29 used PLTHM to construct the interference lentivirus group (shRNA group), and its NC group was set.

• The time points for measurements were 1d, 3d, 5d and 7d, and the cell proliferation rate (OD value) of each cell line was detected using the MTT assay at the four time points. Transwell method was used to detect the number of cell migrations in each cell line.

• Western blot was used to detect the protein expression levels of Rac1-GTP, EPB49, Wnt signaling pathway (p27 and cyclin D1), MAPK signaling pathway related proteins (c-Jun N-terminal kinase (JNK) and p-JNK) expression levels in each cell line.

• The interaction between EPB49 and Tiam1 was detected using immunoprecipitation. IgG was used as the negative control group while INPUT was used as the positive control group.

Statistical methods

SPSS Statistics version 23.0 was used to analyze the data. The cell proliferation rate and the number of migratory cells in each group were expressed as (mean ± standard deviation). The t-test was used for comparison between two groups and one-way ANOVA was used for comparison between multiple groups.

Results

Comparison of cell proliferation rates in different time periods

At 5d and 7d, the proliferation rate of SW480 cells in the EPB49 group was significantly lower than in the NC group. The proliferation rate of HT29 cells in the shRNA group was significantly higher than that in the NC group (P<0.05). Refer to Table 1 for a summary of the data.

Group		1d	3d	5d	7d
SW480	NC group	0.25 ± 0.01	0.45 ± 0.02	0.87 ± 0.05	1.35 ± 0.06
	EPB49 group	0.26 ± 0.02	0.41 ± 0.03	0.58 ± 0.02[a]	0.92 ± 0.04[a]
HT29	NC group	0.15 ± 0.01	0.31 ± 0.02	0.69 ± 0.02	0.89 ± 0.02
	shRNA group	0.14 ± 0.02	0.38 ± 0.01	0.96 ± 0.05[a]	1.32 ± 0.04[a]

Table 1: Comparison of cell proliferation rates in each group at different time periods (mean ± standard deviation).
Note: Compared with NC group, [a]P<0.05.

Comparison of cell migration ability in each group

In SW480 cells, the number of migrated cells in the EPB49 group was significantly lower compared to the NC group (P<0.05). In HT29 cells, cell migration measured in the shRNA group was significantly higher than the NC group (P<0.05).

Cell migration results are summarized in Table 2 and illustrated in Figure 1.

Group		Number of migrated cells
SW480	NC	142.78 ± 19.67
	EPB49	51.44 ± 14.16[a]
HT29	NC	74.44 ± 12.12
	shRNA	194.44 ± 13.16[a]

Table 2: Comparison of cell migration ability in each group (mean ± standard deviation).
Note: Compared with NC group, [a]P<0.05.

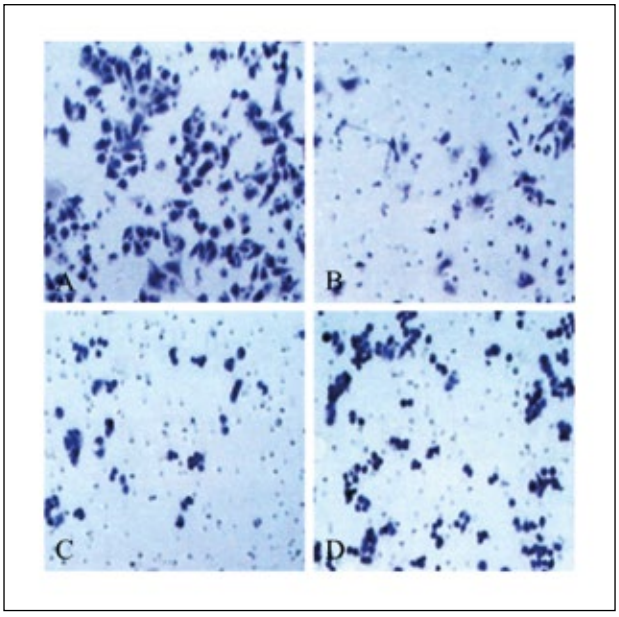

Figure 1: Comparison of cell migration in each group. *A: SW480 NC group; B: SW480 EPB49; C: HT29 NC group; D: HT29 shRNA group.*

Comparison of Rac1-GTP protein expression levels in each group

In SW480 cells, the protein expression level of Rac1-GTP in the EPB49 group was significantly lower than the NC group (P<0.05). In HT29 cells, the expression level of Rac1-GTP protein in the shRNA group was found to be significantly higher compared to the NC group (P<0.05), as is shown in Figure 2.

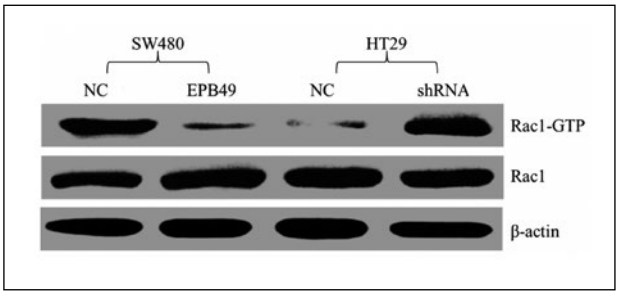

Figure 2: Comparison of Rac1-GTP protein expression levels in each group.

Interaction analysis between EPB49 and Tiam1

Tiam1 can be found by coprecipitation of EPB49 antibody. Similarly, EPB49 was detected by coprecipitation of Tiam1 antibody showing that EPB49 can interact with Tiam1. The results from this experiment is shown in Figure 3.

Comparison of Wnt signaling pathway-related protein expression levels in each group

In SW480 cells, the protein expression levels of EPB49 and p27 in the EPB49 group were

significantly higher than those in the NC group, and the protein expression level of cyclin D1 in the EPB49 group was significantly lower than that in the NC group. In HT29 cells, the protein expression levels of EPB49 and p27 in the shRNA group were significantly lower than those in the NC group, and the protein expression level of cyclin D1 in the shRNA group was significantly higher than that in the NC group (P<0.05).

The Western blots from Wnt pathway-related proteins are shown in Figure 4.

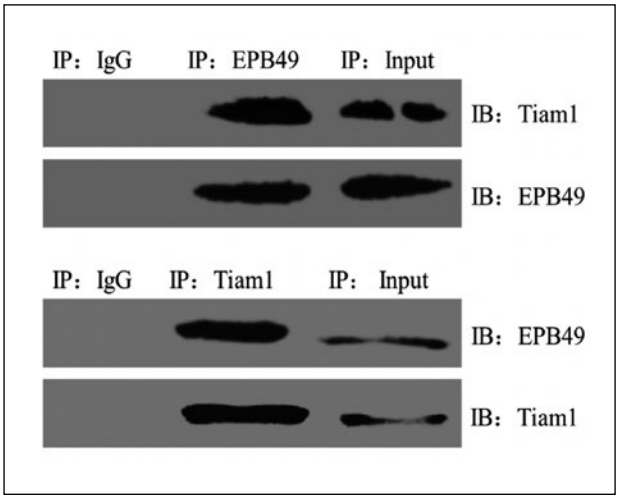

Figure 3: Interaction analysis of EPB49 and Tiam1.

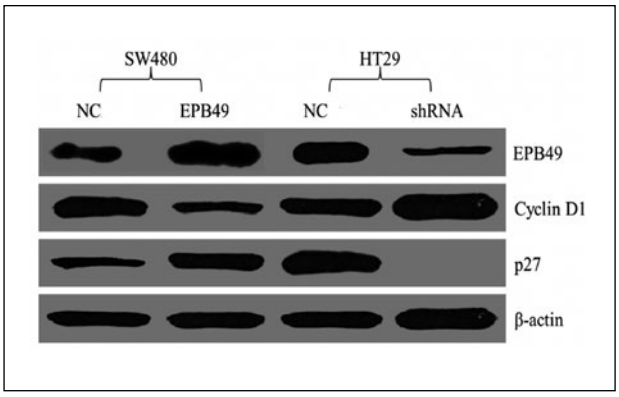

Figure 4: Comparison of expression levels of Wnt signaling pathway-related proteins in each group.

Comparison of MAPK signaling pathway-related protein expression levels in each group

In SW480 cells, the p-JNK protein expression level of the EPB49 group was significantly lower than the NC group (P<0.05). There was no significant difference in JNK protein expression levels among all groups (P>0.05).

In HT29 cells, the p-JNK protein expression level of the shRNA group was significantly higher compared to control (P<0.05). The results are presented in Figure 5.

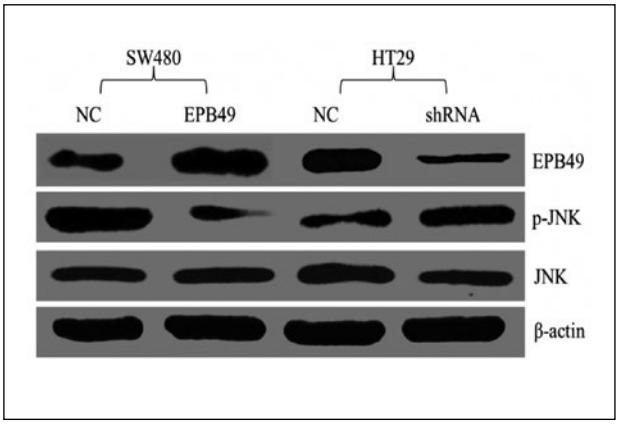

Figure 5: Comparison of expression levels of MAPK signaling pathway related proteins in each group.

Discussion

The onset of colorectal cancer is mainly caused by the stimulation of many tumorigenic factors, which can cause the intestinal mucosal epithelial cells to lose the normal regulation of their own growth at the gene level. In turn, this causes an imbalance of the cell cycle, leading to unrestricted cell proliferation and finally inducing the onset of colorectal cancer[9].

At present, radical surgical measures are an important method for the treatment of liver metastasis in patients with colorectal cancer, but only about 15% of liver metastases can be completely removed with most of these patients already having great vessel involvement leading to a very poor prognosis[10]. Therefore, it is necessary to further explore the mechanism of metastasis in colorectal cancer to improve the prognosis of such patients.

Tumor-related studies have shown that a deletion of EPB49 chromosome region exists in prostate cancer, colon cancer and other tumors[11]. It has been reported that the up-regulation of EPB49 expression in PC-3 cell lines can promote the morphological and phenotypic recovery of epithelioid cells[12]. Some scholars have found that EPB49 can control RhoA activity, and then regulate cell shape, movement, and other links. Clinical reports have confirmed that the changes in Tiam1 expression are closely associated with the onset and metastasis of nasopharyngeal cancer, lung cancer and other tumors. Changes in Tiam1 expression plays an important role in the pathogenesis and metastasis of colorectal cancer[13].

Currently, there are many reports on Tiam1, and its molecular mechanism of action is relatively clear. However, the regulation of its activity is not

studied enough. It has been reported that factors such as extracellular lysophosphatidic acid and calcium ion influx can lead to the activation of Tiam1, and specific proteins can bind to Tiam1-related domains resulting in Tiam1 activation[14]. Conversely, there exists proteins that bind to Tiam1-related domains and block Tiam1 activation. It has been reported that NM23H1 can bind to Tiam1 and negatively regulate its activity, ultimately blocking Rac1 activation[15].

Studies related to colorectal cancer have shown that the decreased EPB49 expression level in this disease leads to activation of the Tiam1-Rac1 signaling pathway and therefore its downstream effector molecules, which ultimately accelerates the onset, progression, and metastasis of colorectal cancer. The Wnt signaling pathway has been found to be activated in colon cancer, resulting in less β-catenin degradation and accumulation in the cytoplasm and the nucleus. This results in accelerated cell proliferation with epithelial mesenchymal transformation, but the mechanism of β-catenin accumulation in the nucleus is not fully understood. Rac1 has been shown to regulate the entry of β-catenin into the nucleus, thus enhancing the role of the Wnt signaling pathway. Clinical reports have also confirmed abnormal activation of the MAPK signaling pathway in colorectal cells.

In this study, we found that abnormal expression of EPB49 plays an important role in the proliferation and migration processes of colorectal cancer cells. Up-regulation of EPB49 expression can block tumor cell metastasis and proliferation, while down-regulation of EPB49 expression can accelerate tumor cell metastasis and proliferation. EPB49 is widely expressed in many types of organs and tissues and is involved in actin skeleton regulation in non-red blood cells and other biological processes.

EPB49 can interact with Tiam1 and regulate Rac1 activity. In SW480 cells, the protein expression levels of p-JNK and cyclin D1 in the EPB49 group were significantly lower than those in the NC group, and the protein expression level of p27 in the EPB49 group was significantly higher compared to the NC group ($P<0.05$). In HT29 cells, the protein expression levels of p-JNK and cyclin D1 in the shRNA group were significantly higher than those in the NC group, and the protein expression level of p27 was significantly lower than the NC group ($P<0.05$). These results indicate that EPB49 can regulate Wnt and MAPK signaling pathways.

In conclusion, EPB49 can promote the proliferation and migration of colorectal cancer cells, and its mechanism may be realized through the regulation of Wnt and MAPK signaling pathways by the Tiam1-Rac1 signaling pathway.

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