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Prognostic relationship between TIPIN and colorectal cancer and experimental validation

Cao Yusheng[1], Tan Xijuan[1], Liang Haoyuan[1], Huang Tianfu[1], Huang Xusen[2,*]

[1]Youjiang Medical University for Nationalities, Baise, Guangxi, China
[2]Affiliated Hospital of Youjiang Medical University for Nationalities, Baise, Guangxi, China
[*]Corresponding author: hxsfy@163.com

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Abstract: Colorectal cancer (CRC) is the third leading cause of death in the world, and there is no good treatment for it. The purpose of this experiment is to investigate the relationship between TIPIN and prognosis in colorectal cancer, as well as its effects on migration, proliferation, and epithelial-mesenchymal transition (EMT) in colorectal cancer. The expression of TIPIN in colorectal cancer was verified by Western blotting; the expression of E-calmodulin was analyzed by WB assay after transfection of TIPIN plasmid; the effects of TIPIN on the proliferation and migration of colorectal cancer cells were evaluated by cell scratch assay and CCK8 cell growth assay. High expression of TIPIN was closely related to patients' distant organ metastasis (P=0.018), lymph node invasion (P=0.038), significant correlation with tumor stage (P=0.024), no correlation was found with the diameter size of tumors, and patients with low expression of TIPIN had a better survival prognosis; at the same time, TIPIN has a close relationship with DNA damage and repair; TIPIN is highly expressed in colorectal cancer tissues, and high levels of TIPIN inhibit the expression level of E-calmodulin and promote the proliferation and migratory ability of colorectal cancer. TIPIN is associated with patient prognosis and affects proliferation, migration, and the EMT process in colorectal cancer.

1. Introduction

Colorectal cancer (CRC) is a highly malignant tumor of the digestive system, as well as one of the malignant tumors with the worst prognosis. Early diagnosis and treatment of colorectal cancer is extremely, and the age of the patients is decreasing while its morbidity and mortality rates are showing an increasing trend year by year. Colorectal cancer is the third most commonly diagnosed malignancy and the second leading cause of cancer death in the world[1]. Colorectal cancer poses a significant global burden in terms of complications, mortality, treatment side effects, healthcare utilization and healthcare costs. Data project that by 2040, there are likely to be more than 3 million new cases of colorectal cancer globally and approximately 1.6 million deaths per year due to colorectal cancer[2]. Colorectal cancer is associated with a number of factors, including age, lifestyle habits, genetics, environment, etc[3-5]. Lynch syndrome and familial adenomatous polyposis all increase the risk of colorectal cancer. A family history of colon cancer in a first-degree relative increases the risk of colon cancer by about 20%, even in the absence of the hereditary colon cancer syndromes described

above[6].

TIM-interacting protein (TIPIN) is a 301-aa protein that interacts with TIM (TIMELESS) and was originally discovered in yeast[7]. A current study found that TIPIN is aberrantly expressed in melanoma and triple-negative breast cancer, and that low levels of TIPIN promote apoptosis in melanoma and triple-negative breast cancer[8,9]. However, no study has yet found whether there is a relationship between TIPIN and the development of colorectal cancer. This study combined bioinformatics and related experiments to analyze the effect of TIPIN on colorectal cancer.

2. Methods and Materials

2.1. Bioinformatics predictive analytics

Based on TCGA (The Cancer Genome Atlas Program) public database, we analyzed the expression of TIPIN in colorectal cancer and predicted the relationship between TIPIN and patients' distant organ metastasis, the number of lymph node metastasis, tumor stage, tumor size, and patients' prognosis.

2.2. GO and KEGG enrichment analysis

GO (Gene Ontology) functions enrichment and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway enrichment analysis of TIPIN and its related genes were performed using a combination.

2.3. Cell culture and transfection

The LOVO cell line of human colorectal cancer was taken out from the -80 ℃ refrigerator, rapidly thawed in a 37 ℃ water bath, resuspended in complete culture medium after centrifugation, the supernatant was removed, and then transferred to T25 with complete culture medium for further cultivation in an incubator. The cells were seeded into a 6-well plate, and after the cell fusion reached 90%, they were subjected to starvation treatment for 2 hours before transfection with the TIPIN plasmid into the LOVO cell line. After 6 hours, the complete culture medium was replaced for continued cultivation.

2.4. Patient sample collection

Twelve pairs of colorectal carcinoma specimens and correspondent normal tissues were acquired from the Affiliated Hospital of Youjiang Medical University for Nationalities. We obtained all specimens from hospitalized patients who received operative resections, with all pathological data from the Pathology Department. Approval was obtained from the Institutional Review Board of Affiliated Hospital of Youjiang Medical University for Nationalities.

2.5. Western blot experiment

We obtained cancerous and normal tissues from colorectal cancer patients, extracted tissue proteins and heated them for denaturation. The electrophoresis conditions for the experiment were 80V/120V, and the transfer conditions were 200mA/70min. After 48 hours of transfection, we extracted cellular proteins, measured protein concentration, and heated them for denaturation. The electrophoresis conditions for the experiment were 80V/120V, and the transfer conditions were 200mA/140min. Subsequently, we used a protein-free rapid blocking solution to block for 2 hours, followed by washing with 1×TBST. After the end of the blocking step, incubate the primary antibody at room temperature for 1.5 hours; incubate the secondary antibody at room temperature for 1 hour. After the

antibody incubation, we washed the protein bands again. Finally, we performed development and calculated the grayscale values.

2.6. Cell scratching assay

After 6 hours of cell transfection, we counted three times for each group and took the average value. We placed the scratch insert into each well of a 6-well plate and added a cell suspension containing 2×105 cells to each well. Then, we continued to culture the cells. After the cells grew to fill the wells, we removed the inserts, washed them twice with PBS, added 1ml of serum-free medium to each well of the 6-well plate, and then measured the scratch width under the microscope and recorded it. We recorded again after 24 hours.

2.7. Cell proliferation assay

After 6 hours of cell transfection, we took out 4 pieces of 96-well plates. We added 100ul of cell suspension to each well of the 96-well plate, with a cell density of 1×105 per well and 3 replicates per group. Then we added 100ul of PBS to the outermost well of each 96-well plate. After the cells attached to the wall, we added 10ul of CCK8 reagent to each well of the first 96-well plate and placed the cells in an incubator for further incubation for 2 hours. Finally, we measured the cell OD value at a wavelength of 450nm using a spectrophotometer. Thereafter, we measured the cell OD value in one 96-well plate every 24 hours using the same method.

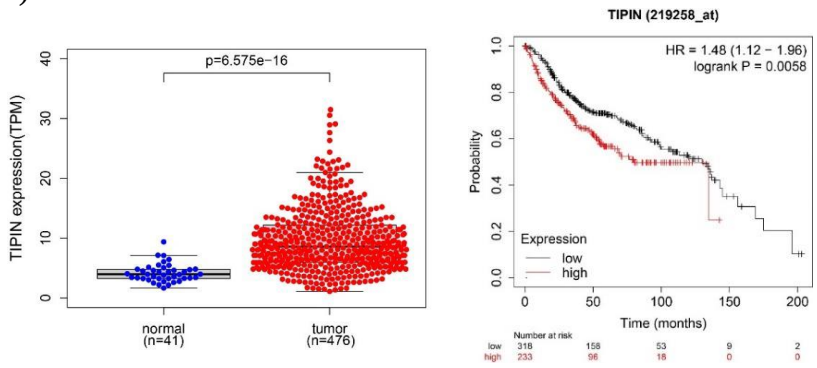
2.8. Statistical analysis

Data were analyzed and plotted using SPSS 25.0. Differences between the two groups were tested using t-tests, and differences between more than two groups were compared using one-way ANOVA. $p<0.05$ (*); $p<0.01$ (**).

3. Results

3.1. Predictive analysis of TIPIN for colorectal cancer

Based on the clinical information data of colorectal cancer patients in the TCGA database, which were collated and analyzed using R, it was found that high expression of TIPIN was closely associated with distant organ metastasis in patients (P=0.018), with lymph node invasion (P=0.038), with significant correlation with the tumor stage (P=0.024), and no correlation was found with the diameter size of the tumor. Survival prognostic analysis of patients using the online site Kaplan Meier showed poorer survival in patients with high TIPIN expression relative to those with low TIPIN expression (P=0.0058)(Figure 1).



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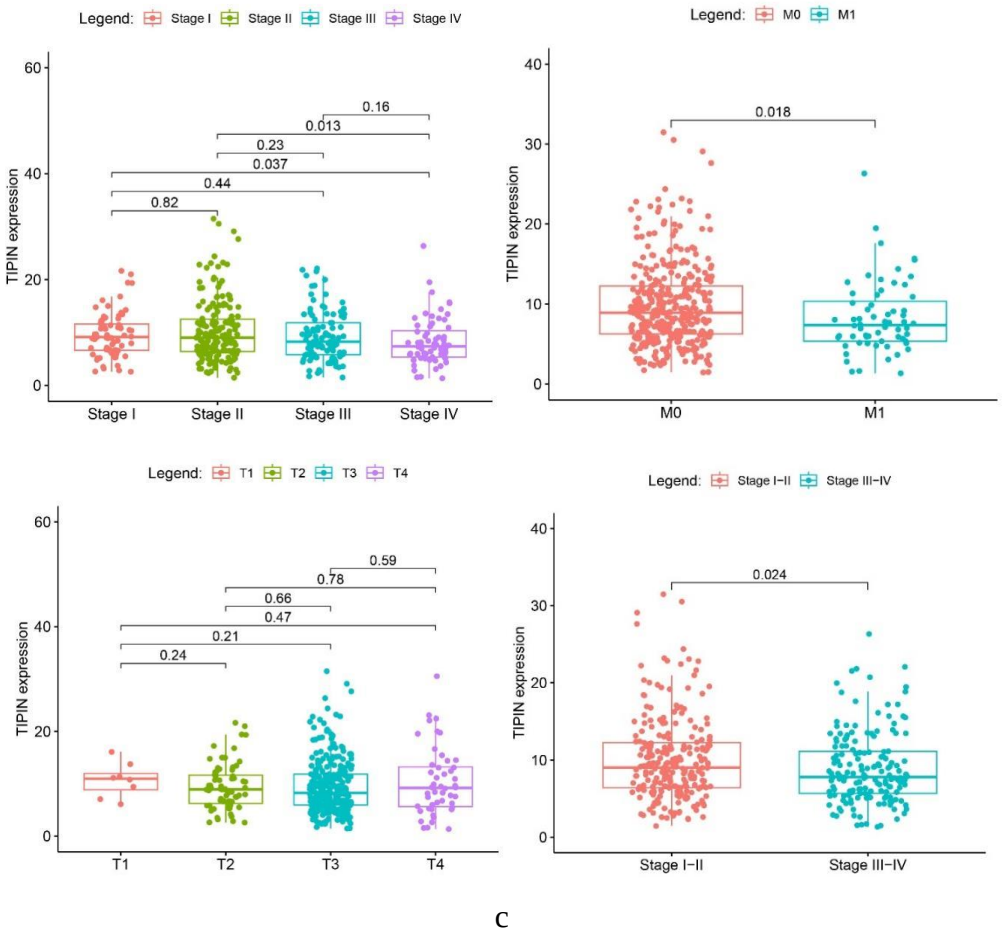

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Figure 1: a) Expression of TIPIN in colorectal cancer; b) Impact of TIPIN on survival prognosis of patients; c) Relationship between TIPIN and tumor stage of colorectal cancer patients

3.2. GO and KEGG enrichment analysis

The related genes of TIPIN were found in the STRING website, and the constitutive gene set was analyzed by GO and KEGG enrichment in the DAVID website, and the results of GO enrichment analysis showed that it was mainly enriched in DNA unwinding involved in DNA replication, DNA replication, and cellular response to DNA damage stimulus in the Biological Process (Biological Process). In Molecular Function, it was mainly enriched in single-stranded DNA binding, single-stranded DNA-dependent ATP-dependent DNA helicase activity, DNA binding, etc. The results of KEGG enrichment analysis showed that it was mainly enriched in CMG complex, nucleoplasm, chromosome and telomeric region, etc. In Cellular Component, it was mainly enriched in CMG complex, nucleoplasm, chromosome, telomeric region, etc. KEGG enrichment analysis showed that it was mainly enriched in DNA replication, Cell cycle, Nucleotide excision repair, etc. It shows that TIPIN has a close relationship with DNA damage and repair, (Figure 2).

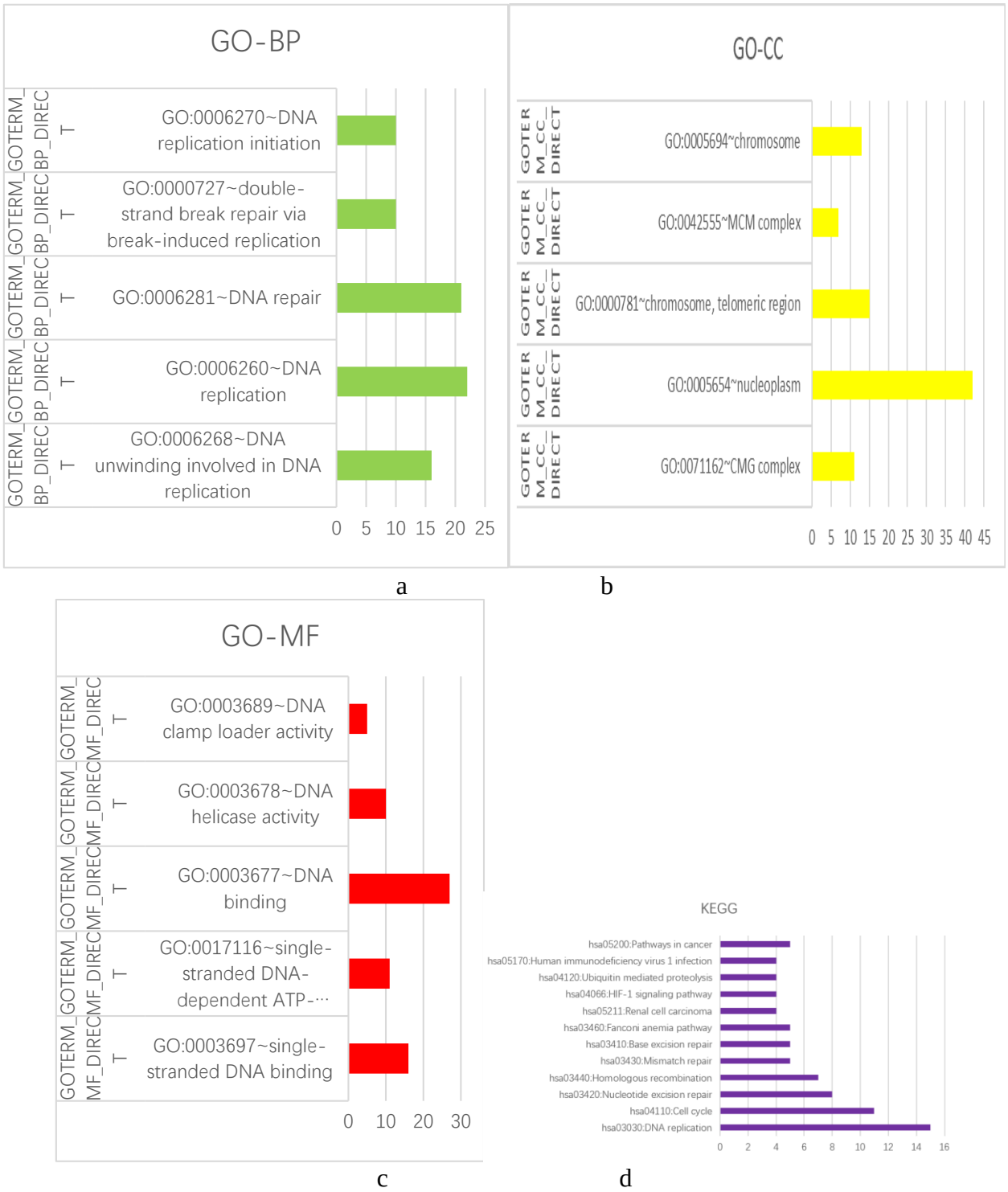

Figure 2: a) TIPIN enrichment in GO bioprocesses; b) TIPIN enrichment in GO molecular functions; c) TIPIN enrichment in GO cellular fractions; d) TIPIN enrichment in KEGG

3.3. Expression of TIPIN in patients with colorectal cancer and its effect on E-calmodulin

The WB experiment was performed to verify whether there was a difference in the expression of TIPIN in colorectal cancer cancerous tissues and normal tissues. The results showed that the expression of TIPIN in colorectal cancer cancer tissues was significantly higher than that in normal

tissues, and the difference was statistically significant ($p < 0.05$), (Figure 3); After transfection of TIPIN plasmid, the expression of E-calmodulin in the experimental group was significantly lower than that in the control group ($p < 0.05$),(Figure 4).

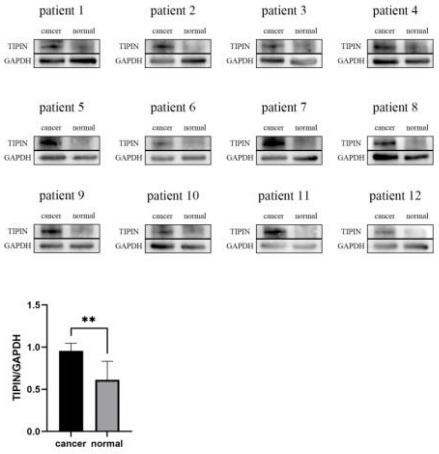

Figure 3: TIPIN expression in colorectal cancer

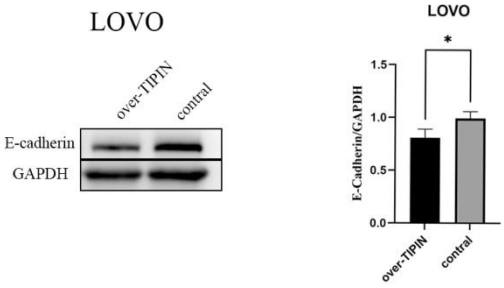

Figure 4: Effect of TIPIN on E Calcineurin

3.4. Effect of TIPIN on the proliferative capacity of colorectal cancer

After transfection of TIPIN plasmid, by CCK8 cell proliferation assay, the author found that the proliferation ability of colorectal cancer cells in the experimental group was significantly stronger than that of the control group, and the difference was statistically significant ($p < 0.05$), which indicated that TIPIN was able to promote the proliferative ability of colorectal cancer cell (Figure 5).

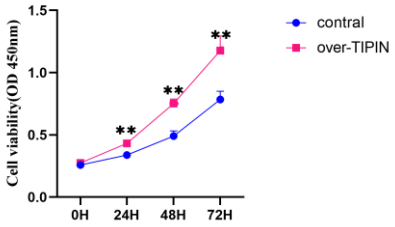

Figure 5: Effect of TIPIN on the proliferative capacity of colorectal cancer

3.5. Effect of TIPIN on the migratory capacity of colorectal cancer

By conducting scratch experiments, the author investigated the effect of TIPIN on the migration ability of colorectal cancer cells. The experimental results showed that under the influence of high levels of TIPIN, the migration ability of colorectal cancer cells in the experimental group was

significantly stronger than that of the control group, and the difference was statistically significant ($p < 0.05$). It indicates that TIPIN can promote the migration ability of colorectal cancer cells, (Figure 6).

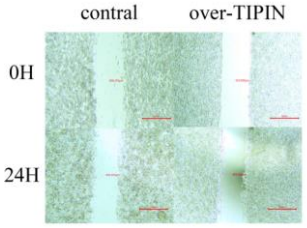

Figure 6: Effect of TIPIN on the migration ability of colorectal cancer cells

4. Discussion

Colorectal cancer is a tumor originating from the colon and is one of the most common malignant tumors in humans[10]. The colon is the part of the body's digestive system that is primarily responsible for absorbing water and salt, making feces solid and pushing it out of the body. Colorectal cancer occurs as a result of a combination of factors. Its causes, classification, symptoms, diagnosis and treatment have been well researched and are gradually becoming more accepted and recognized. The causes of colorectal cancer are complex, including genetic factors, quality of life and environment. In recent years, factors such as smoking, dietary habits and lack of exercise have become important causes of increased human morbidity and mortality rates[6]. TIPIN, which normally forms a complex with TIM, plays an important role in cell cycle arrest in response to the DNA checkpoint response and is involved in normal DNA replication to maintain genomic stability[11]. The TIM/TIPIN complex also regulates cell proliferation when interacting with RPA[12,13]. The yeast direct homolog Tof1 is a core component of the replicasome and is required for the S-phase DNA damage checkpoint, fork stalling during disturbed DNA replication, and fork pausing during replication disorders, whereas Tipin and its yeast direct homolog Csm3 have the same role as Tof1 in DNA synthesis[14,15]. Some studies have found that TIPIN is highly expressed in melanoma, knockdown of TIPIN inhibits melanoma proliferation and promotes apoptosis, and TIPIN depletion reduces tumorigenicity of melanoma xenografts in immunocompromised mice[8]. Similarly in triple-negative breast cancer samples mRNA levels of TIPIN were significantly higher than specimens from other breast cancer subgroups and healthy tissues, protein levels of TIPIN were able to make breast cancer subtypes more invasive and proliferative, and TIPIN depletion reduced the tumorigenicity of triple-negative breast cancer cells[9]. In addition TIPIN is overexpressed in hepatocellular carcinoma at both mRNA and protein levels, and high expression of TIPIN is also associated with poor prognosis in patients with hepatocellular carcinoma[16]. In addition TIPIN expression was downregulated in renal tumors compared to normal tissues and may be associated with dysregulation of cell cycle checkpoints[17].

In this study, we predicted the high expression of TIPIN in colorectal cancer by bioinformatics, analyzed it by GO and KEGG enrichment, and found that it had a close relationship with DNA damage repair, and analyzed it by survival prognosis, and found that patients with high expression of TIPIN had a poorer survival prognosis; and we also found that the high expression of TIPIN had a close relationship with distant organ metastasis of the patients ($P=0.018$), a close relationship with lymph node invasion ($P=0.038$), and an important correlation with tumor stage ($P=0.024$).

By testing samples from colorectal cancer patients, it was found that the expression of TIPIN was higher in colorectal cancer cancer tissues than in normal tissues. The current study suggests that epithelial-mesenchymal transition (EMT) is a cellular process in which epithelial features are transformed to a mesenchymal phenotype by epithelial cells undergoing a variety of factors and exhibiting corresponding behaviors[18]. Epithelial-mesenchymal transition (EMT) is common in the physiological activities of various tumor cells, and EMT is involved in tumorigenesis and influences

the behaviors of tumor cells such as invasion and metastasis, and has a relationship with drug resistance[19]. In this study, by overexpressing TIPIN in colorectal cancer LOVO cell line, we detected that the expression level of EMT-related protein E calcineurin in the experimental group was significantly lower than that in the control group. This suggests that high levels of TIPIN can potentially promote the development of EMT in colorectal cancer. In addition, after overexpression of TIPIN, the proliferation and migration abilities of colorectal cancer cells in the experimental group were significantly stronger than those in the control group.

In summary, this study, based on bioinformatics analysis as well as in vitro cellular experimental validation, found that there was a significant difference in the expression level of TIPIN in cancerous tissues and normal tissues of colorectal cancer patients, and that high levels of TIPIN suppressed the expression level of E-calmodulin, while the proliferative and migratory abilities of colorectal cancer were both promoted. Therefore, TIPIN has the potential to become a prognostic marker and drug target in colorectal cancer. However, this study only explored part of the cellular functions at the cellular level, and the exact role of TIPIN and its specific mechanism of action still need to be further explored and verified.

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