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Correlation between PTEN and P62 gene expression in rat colorectal cancer cell

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ABSTRACT

Objective: Autophagy is a cellular pathway that regulates the transportation and degradation of cytoplasmic macromolecules and organelles towards lysosome, which is often related to the tumorigenesis and tumor suppression. Here, we investigate the regulating effect of PTEN gene on autophagy-related protein P62 in rat colorectal cancer (CRC) cells and explore the application value of PTEN gene in clinic.
Methods: Rat colorectal cancer was induced by intraperitoneal injection of 1,2-dimethyl hydrazine in male ACI rats. A total of 20 rats were randomly selected from those successfully induced with CRC as the experimental group, while 10 healthy rats as control. The rat CRC cells were isolated and cultured. After transfecting the rat CRC cells with pEGFP-N1-PTEN plasmid, RT-PCR was adopted to examine that gene expression of p62 and PTEN, while Western blotting was used to detect the protein expression of p62 and PTEN. Also, the proliferation of CRC cells was measured by MTT assay.
Results: The expression of PTEN gene in the experimental group was significantly inhibited as compared with the control group, while the expression of P62 gene was significantly increased ($p < 0.05$). Western blotting demonstrated that the PTEN protein in the experimental group was lower, while the expression of P62 protein was higher. When the CRC cells were transfected with pEGFP-N1-PTEN plasmid, the PTEN expressions were elevated, while p62 was down-regulated. Also, the proliferation of CRC cells was inhibited.
Conclusion: The expression of PTEN gene is negatively correlated with the expression of P62 gene in rat CRC cells. And the expression of PTEN gene can inhibit the occurrence and development of colorectal cancer, thus providing theoretical basis for future clinical treatment.

1. Introduction

Colorectal cancer (CRC), as the most common cancer with a high mortality rate, often results from genetic and epigenetic changes, which cause transformation from normal colorectal mucosa to invasive carcinoma (Chatenoud et al., 2016). There are about 1,235,108 people being diagnosed with CRC each year, and 609,051 of them die from it (Chatenoud et al., 2016; Forastiere et al., 2017). Based on the data obtained from the World Health Organization, it is estimated that the number of CRC cases and the deaths caused by CRC will increase by 77% and 80%, respectively, by 2030 (Steuer et al., 2017). It will bring huge obstacles

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to society development and cause serious economic burden on the patients and their families. Therefore, it is of great importance to develop efficient and simple therapeutic technology.

Mammalian sequestosome-1 (p62/SQSTM1) is a multifunctional ubiquitin-binding protein (Haapaniemi et al., 2016). Also, p62 can activate numerous intracellular signaling pathways that are closely related to cancer, such as nuclear factor-kappa-B (NF-κB), nuclear transcription factor erythrocyte-2 related factor 2 (NRF2), mammalian target of rapamycin (mTOR) pathway and the like (Lipkowitz, 2003). Under normal physiological conditions, p62 participates in the continuous degradation of ubiquitinated proteins by autophagy (Lipkowitz, 2003; Xiao et al., 2015). However, recent studies have shown that abnormal aggregation of p62 protein are often found in the malignant tumor tissues such as gastric cancer (Korn et al., 2007). And it is reported that the accumulation of p62 is strongly associated with the postoperative recurrence of lung cancer (Falchetti et al., 2005).

PTEN (phosphatase and tensin homolog deleted on chromosome ten), a hot research about tumor

suppressor gene, have a negative effect on modulate the phosphoinositide 3-kinase signaling pathway for involved in many cancers' process by dephosphorylating the phosphatidylinositol 3,4,5-triphosphate. As a crucial regulator gene for the process of dephosphorylating, *PTEN* can influence the expression of many genes include *P53*, *PDK1* et al. In CRC, *PTEN* plays a key role in the development process of cancer cell proliferation. The loss of expression of PTEN protein is observed in 30% of sporadic CRC. However, the base of PTEN modulate CRC is influence the expression of downstream genes which is directly effect on CRC.

There has been reports about the p62 protein in colorectal cancer. However, its biological function and clinical significance, including its correlation with other cancer suppressor genes still await clarification (Cheng et al., 2013; Slattery et al., 2010). Therefore, we focus on the immunological reaction activity of p62 in rat CRC as well as its correlation with tumor suppressor gene PTEN.

2. Materials and methods

2.1. Cell culture

The CRC cells were isolated from 1,2-dimethylhydrazine (DMH)-induced CRC rats, and cultured in DMEM medium (American Life Technology) containing 10% FBS at 37 °C, under 5% CO_2.

2.2. Establishment of rat CRC model

The DMH was dissolved in the mixture of 0.9% NaCl and 1% EDTA, producing 0.35% DMH solution. The pH was adjusted within 6.5–7.0, and then filtered through a 0.25 μm filter. The prepared DMH solution was administered to the ACI male rats (Shanghai Experimental Animal Center of Chinese Academy of Sciences) via intraperitoneal injection at a dosage of 25 mg/mL based on body weight, three times a week, for five consecutive weeks. The rats were anesthetized and subjected to colonoscopy to observe whether the colorectal cancer was successfully induced. And 20 rats were randomly selected from those successfully induced with CRC as the experimental group.

2.3. Assessment of physiological status

Eight weeks after the successful establishment of a rat CRC model, five rats were randomly selected from each group for physiological status evaluation, including exercise performance, tumor development, and spleen size.

2.4. Construction and transfection of pEGFP-N1-PTEN plasmid

The sequence of tumor suppressor gene PTEN was downloaded from NCBI, and constructed by Huada Genetics biotech company. A total of 50 μL competent cells were added into 1.5 mL EP tube for ice bath; and 5 μL of the linked plasmid mixture (plasmid containing ampicillin expression gene) was added for 15 min of incubation on ice; then 10 mL LB medium (containing ampicillin) was added and placed at 37 °C for 1 h of vibration. 200 μL of the above culture solution was taken and placed onto an LB plate culture dish containing ampicillin, and colonies were selected for monoclonal culture. Then plasmid extraction was performed using QIAGEN plasmid extraction kit. Finally, 5 μg extracted plasmid, 200 μL optimized medium opti-mem, and 10 μL transfection solution were mixed and added into the cell culture medium.

2.5. Real-time quantitative PCR

Total RNA was extracted from rat CRC tissues and cultured CRC cells using the Tiangen RNA isolation kit. The quality and concentration of RNA were measured using an Evolution 201 ultra-differential spectrophotometer. Extract 1 μL of RNA, and 4x gDNA wiper Mix was used to remove genomic DNA. Then 4 μL of 5 × qRT SuperMix II was added to generate cDNA at 50 °C for 15 min. The SYBR Green Master Mix real-time quantitative PCR kit was used. At the end of the primer reaction, the melting curve analysis was performed to evaluate the quality of the final PCR product (PTEN upstream primer: 5′-CAGCCAAGTCTGTGACTFGCCGTAC-3′, downstream primer: 5′-12CGCTCGAGCAGTCGCTGCAACCATCCA-3′; p62 upstream primer: 5′-TCCTGCAGACCAAGAACTATGACATCG-3′, the downstream primer is: 5′-TCTACGCAAGCTTAACACAACTATGA GACA-3′). The threshold cycle C(t) value is calculated by fixing the base fluorescence at 0.05 units. Each sample was repeated three times and the average C(t) value was calculated. The calculation of the value of ΔC(t) is exemplified by C(t) - C(t) GAPDH. The C (t) GAPDH value was taken as a reference point for n times of increase or decrease ΔΔCt method calculated by the calculation formula.

2.6. Western blotting

The protein sample obtained from CRC tissue and cultured CRC cells were subjected to SDS-PAGE (25 μg/lane), and then transferred to polydiene difluoride membrane that was then incubated with 5% milk in TBST for 1 h at room temperature. Then it was incubated with CD47 antibody Ab (Abcam) overnight at 4 °C, which was terminated by 15 min of wash with TBST. Then appropriate second-antibody was used (1:5000, Jackson Immuno Research) and incubated with horseradish peroxidase. The image was visualized with the use of ECL reagent (Amersham Biosciences). After stripping with Western Blot Antibody Stripping Buffer (Pierce) for 20 min at room temperature, the membrane was similarly treated with anti-actin antibody as a control.

2.7. The proliferation of CRC cells transfected with PTEN gene was detected with MTT assay

After PTEN gene transfection, the CRC cells were plated onto 96-well plates at 1×10^5 cells/mL to observe the growth at 24 h, 48 h, 72 h, and 96 h after transfection. 30 μL of MTT (6 mg/mL) was added into each well 4 h before the end of culture. After incubation for 2 h at 37 °C, under 5% CO2, the medium was discarded and 200 μL DMSO was added into each well. Then after rocking at low speed for 10 min, the cells were measured by a microplate

reader with an OD value of 490 (A), and the cell growth curves were drawn.

2.8. Statistical processing

Data analysis was executed using SPSS software. Quantitative data were expressed as $x \pm s$, and compared through paired *t*-test. $P < 0.05$ was considered as statistically significant. The figures were plotted with Graphpad Prism 7.0.

3. Results

3.1. The expression of p62 gene and protein are negatively correlated with PTEN gene

To understand the interaction and correlation between p62 gene and PTEN gene in rat CRC model, and to detect the anti-tumor effect of highly expressed PTEN gene in CRC cells, we tested

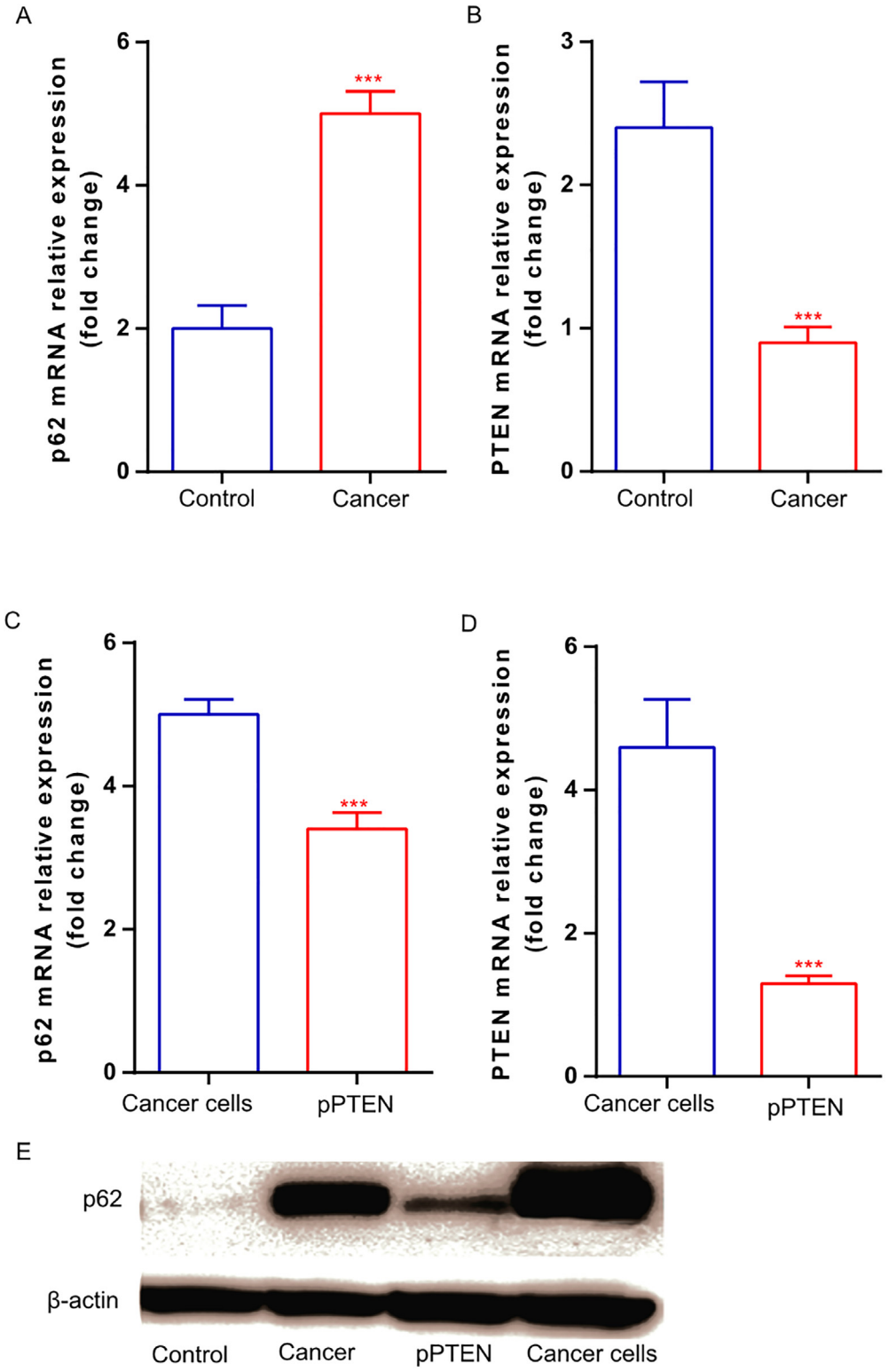

Fig. 1. The mRNA expression level of p62 gene and PTEN gene and the level of p62 protein.

the expression of p62 gene and protein in the experimental group and control group, respectively. We found that both p62 mRNA levels (Fig. 1A) and protein levels (Fig. 1E) were rather high in CRC rats, while the PTEN mRNA levels (Fig. 1B) decreased significantly as compared with the control group ($p < 0.05$). Then direct sequencing and Sanger-PCR sequencing of p62 and PTEN genes were performed. No other DNA mutations and abnormal methylation were found in the promoters of these two genes. Therefore, we believe that the differential expression of these two genes may occur at the transcriptional level, and the expression of these two genes is correlated. To further verify that the p62 and PTEN genes are closely related to the occurrence and development of CRC, we examined the protein expression of p62 and PTEN in non-transfected CRC cells and pEGFP-N1-PTEN plasmid transfected CRC cells. And we found that both gene and protein expression of p62 were markedly decreased after transfection (Fig.-1C, Fig.-1E), while the expression of PTEN was increased within certain range (Fig.-1D) ($p < 0.05$). And the gene and protein expression of p62 in non-transfected CRC cells were still high ($p < 0.05$). In vitro cell transfection experiments further proved that the expression of PTEN gene was negatively correlated with the p62 gene. Also, it demonstrated the inhibitory effect of PTEN gene on p62 oncogene at the molecular level.

3.2. Detection of the physiological indicators

In order to find out the significant difference in other physiological indicators between the experimental group and control group, we examined the exercise performance and the degree of involvement of various organs in rats after 5 weeks of CRC induction. The exercise performance was measured by using video to record the motion trails of the rats in a circular container for 30 min. And we found that the exercise performance of the experimental group was significantly decreased (Fig. 2A and B), indicating that CRC would also affect the patient's behavior. Subsequently, the rats were dissected and the morphology of its organs were observed. Except for the abnormal morphology of intestine, we found that the spleen was also involved in CRC rats, which could be related to the fact that the spleen is the main immune organ. Abnormal edema of the spleen was found in CRC rats, and the size was significantly different from the control group (Fig. 2C).

3.3. PTEN gene transfection inhibits the growth of cancer cells

To further verify the inhibitive effect of tumor suppressor gene PTEN on CRC, we employed pEGFP-N1-PTEN plasmid for *in vitro* transfection of the rat CRC cells. We found that the gene expression and protein level of PTEN gene and p62 gene were affected. Also, with MTT assay, we demonstrated that the growth and proliferation of CRC cells were inhibited after pEGFP-N1-PTEN plasmid transfection. As compared with the non-transfected group, it took more time for the cells transfected with pEGFP-N1-PTEN plasmid to reach the same OD value (Fig. 3, $p < 0.05$).

4. Discussion

In this study, we employ ACI rats which are susceptible to cancer as animal models, and explore the correlation between PTEN gene and p62 gene in CRC. We found that in the CRC tissue, the higher the expression of p62, the lower the PTEN gene. And this correlation still exists when potential confounding factors

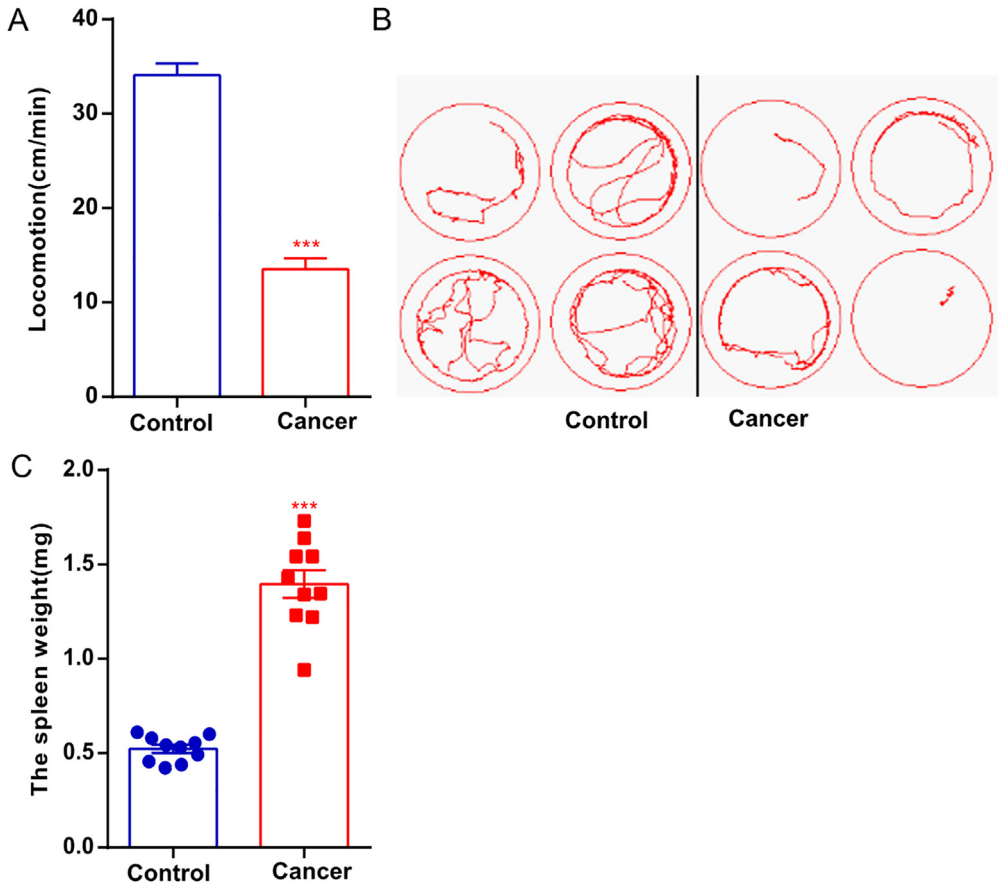

Fig. 2. Comparison of the physiological indicators between control group and experimental group.

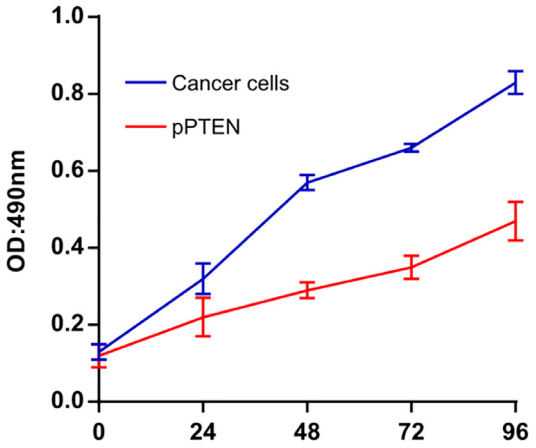

Fig. 3. The growth curves of non-transfected CRC cells and pEGFP-N1-PTEN plasmid transfected CRC cells.

including SNP gene and gene methylation level were excluded, indicating that the expression of tumor suppressor PTEN is closely related to p62 in CRC. Based on our data, though this results are needed to be verified in large samples, the tumor suppressor PTEN potentially regulates gene p62 in tumor cells, thereby affecting the host immune status in the microenvironment of CRC.

The occurrence and development of CRC was not only driven by the alterations of tumor cell genome and epigenomics, but also affected by the tumor-host interaction (Ortegamolina and Serrano, 2013). The importance in analyzing the tumor characteristics in human body and the immunological function of the host is on the rise. Actually, the higher the p62 expression in CRC tissue, the higher the recurrence rate, which suggests that down-regulation of p62 has anti-tumor effects. Therefore, it is necessary to identify potential molecular targets that may affect the expression of p62 (Zhu et al., 2012).

The PTEN gene is considered to be a classic tumor suppressor gene that has various functions and multiple anti-tumor characteristics (Laguë et al., 2008). Increasing evidence suggests that the PTEN gene regulates the growth and proliferation of cancer cells and adapts to different immune environments. And the functional role of PTEN in the occurrence and development of cancer (pro- and anti-tumor effect) depends on whether PTEN is activated, which may also reflect the changes in the phosphorylation state of PTEN gene caused by tumor microenvironment (Nuala et al., 2016). Although the function of PTEN gene and the effects of various cancer gene expression are not yet defined, in patients suffered from CRC, those with high expression of PTEN gene usually have good prognosis (Zhang et al., 2018). Some scholars have suggested that PTEN gene plays an important role in inhibiting tumor-regulated protein kinase B (PKB), which may explain that high expression of PTEN gene in CRC patient can produce good prognosis (Zhao et al., 2017).

5. Conclusion

To sum up, it seems reasonable to conclude that the PTEN gene regulates the p62 gene in CRC by regulating PKB, thereby inhibiting the CRC progression. One of the limitations of this study is that the evidence for the association between the PTEN gene and the p62 gene is not sufficient. Therefore, we cannot rule out other possible causal relationships. Though the tumor autophagy could be changed by the p62 gene, our experimental hypothesis is based on the evidence from previous researches, which indicates that the autophagy activity of tumor cells can be regulated by PTEN gene. However, its specific correlation still awaits further verification. Another limitation is that our study only explored the association between the p62 gene and the PTEN gene, but other tumor suppressor genes. Future researches are needed for correlation evaluation of other tumor suppressor genes. The advantage of this study lies on the rat CRC model that we adopted to fully understand the clinical pathological characteristics, tumor molecular characteristics and immune response status in CRC tissues. This rat-based CRC model allows us to rigorously examine the relationship between p62 gene and the PTEN gene in tumor, with potential confounders under controlled. Moreover, the accuracy of sample construction and multiple physiological verifications have added the reproducibility of our findings. In the end, the possibility correlation between the PTEN gene and the p62 gene lays profound theoretical foundation for the future treatment of colorectal cancer.

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