

# *The Expression of LINC00673 in Colon Cancer and the Melatonin's Effect on Its Expression*

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**Abstract:** Objective: In this study, the cell lines SW620 and DLD-1 which belongs to colon cancer were employed with the aim of observing melatonin's effect on the growth of colon cancer cells and exploring the influence of melatonin about the autophagy of colon cancer. Besides. It uses qRT-PCR to identify LINC00673 (LINC00673) expression in colon cancer and the influence of melatonin on LINC00673 expression. For the purpose of providing a fundamental theory for melatonin in anti-cancer therapy and also presenting as a biomarker for the early detection of colon cancer. Method: 1) Cultivating the colon cancer cell lines SW620 and DLD-1, and subdividing them in accordance with distinct concentrations of melatonin: 0 mM, 1mM, and 2mM, to detect the impact of melatonin for migration and growth of colon cancer cells by Cell Scratch Test and Colony Formation Assay. 2) Detecting the expression level of autophagy-related molecules in colon cancer cells in response to melatonin employing Western Blot Assay. 3) Utilizing QT-PCR, extract RNA from two groups of cells and detect the expression of LINC00673 and LINC00673 following melatonin treatment. Result: 1) The Cell Scratch test and Colony Formation Assay indicated that compared with the normal group, the scratch healing of SW620 cells and DLD-1 cells decreased. Furthermore, following melatonin administration, the number of cell colonies decreased. And the results are statistically significant. 2) Western blotting results illustrated that after the interferon of melatonin, the expression level of the autophagy-related molecule Beclin-1 increased significantly, whereas the expression of P62 decreased. 3) QT-PCR results demonstrated that the expression of LINC00673 in colon cancer cells SW620 and DLD-1 was significantly increased. Moreover, melatonin had an inhibiting effect on it. Conclusion: These in vitro experiments managed to prove that melatonin possesses a powerful anti-colon cancer effect and can inhibit the growth and spread of colon cancer. Western blotting indicated that melatonin can inhibit colon cancer through autophagy. Besides which, it is discovered that melatonin may exert its anticancer effect by influencing LINC00673.

## 1. Introduction

According to the study's published cancer statistics, the incidence of colon cancer among individuals was 10.2% in 2018 and the mortality rate hit 9.2%, moving colon cancer up to the second position from the fourth.<sup>[1,2]</sup> Initially, combination chemotherapy was effective for the majority of

patients. Nonetheless, due to drug resistance, about 50% of colon cancer patients experience recurring disease, and individuals with advanced disease are experiencing a 10% lower 5-year survival rate.<sup>[3,4]</sup> At the time of their initial diagnosis, Approximately 25% of people with colon cancer had liver metastases, and 50% develop them within three years of their original surgery<sup>[5]</sup>.

Both the incidence and mortality rates of colorectal cancer (CRC) are increasing. According to the Statistical Report, colorectal cancer has the third-highest incidence rate of all malignant tumors, with 376,000 new cases, and the fifth-highest mortality rate, with 191,000 fatalities. Moreover, urban areas have much greater incidence and fatality rates than do rural areas, with the incidence rate showing a definite upward trend. In accordance with the screening results of the city's population aged over 50 years old and at high risk, the incidence of colorectal cancer appears to be continuously increasing. Given that most patients are only detected in the middle or late phases of the disease, early screening enables the early detection and prevention of colorectal cancer. The predominant methods include screening high-risk groups on the basis of age, family history, fecal occult blood test, etc., and subsequently proceeding to endoscopic screening. The treatment process of colorectal cancer can involve various treatment methods including surgery, radiation, chemotherapy, endoscopy, imaging analysis, and pathological analysis. Studies indicate that the model of multidisciplinary treatment collaboration (MDT) can significantly enhance the treatment of colorectal cancer. Medical professionals are required to boost the level of colorectal cancer diagnosis and treatment in medical institutions, improve the prognosis of colorectal cancer patients, and maintain medical quality and safety through the development of precise specifications in order to further standardize colorectal cancer diagnosis and treatment behavior in China.<sup>[6]</sup>

Long-stranded non-coding RNA (lncRNA) is a class of RNAs that are longer than large 200 nucleotides and do not encode proteins<sup>[7]</sup>. A substantial amount of data suggests that lncRNAs are involved in tumor biology through a variety of molecular processes, including interactions with DNA, RNA, and proteins<sup>[8,9]</sup>. To date, a small subset of lncRNAs has been indicated to play key roles in cancer's pathological and physiological processes. The lncRNA LINC-PINT, for example, inhibits tumor cell invasion by controlling the availability of free PRC2 close to the core genomic motif.<sup>[10]</sup> By controlling the AKT/mTOR pathway, the long non-coding RNA MetaLnc9 encourages lung cancer metastasis<sup>[11]</sup>. The lncRNA FAL1, which is overexpressed in epithelial malignancies and regulates the transcription of numerous genes to promote tumor growth, is related to the epigenetic blocker BMI1<sup>[12]</sup>. Through interacting with EZH2, lncRNA FAL1 contributes to the advancement of renal cell carcinoma<sup>[13]</sup>. The lncRNA EGFR-AS1 controls drug resistance in squamous cell carcinoma and mediates epidermal growth factor receptor dependence<sup>[13]</sup>.

Numerous studies have determined that LINC00673 affects the tumorigenesis of many cancers. It has been determined that LINC00673 plays a part in non-small cell lung cancer.<sup>[14]</sup> Moreover, Huang et al.<sup>[15]</sup> demonstrated that activated SP-1 LINC00673 (SP-1 activated LINC00673) exerts tumorigenic effects and inhibits KLF2 through LSD1 and EZH2, and LAST2 expression. Except for that, Xia et al<sup>[16]</sup> reported that through altering the expression of B7-H6, LINC00673 may promote the migration, invasion, and epithelial-mesenchymal transition (EMT) of breast cancer cells.

The pineal gland, retina, brain, heart, and digestive system all create an indoleamine known as melatonin (n-acetyl-5-methoxytryptamine)<sup>[17]</sup>. The primary source of melatonin has been reported to be the gastrointestinal tract, which generates higher than 400 times the pineal gland, according to estimates. As a free radical scavenger and antioxidant enzyme that shields the body from oxidative damage, indoleamine has remarkable pharmacological benefits<sup>[18,19]</sup>. Previous research have shown that melatonin can slow the growth of a number of human malignancies, including leukemia<sup>[20,21]</sup>, liver cancer<sup>[22 23]</sup>, breast cancer<sup>[24,25]</sup>, lung cancer<sup>[26,27]</sup>, as well as gastric cancer<sup>[28,29]</sup>.

## 2. Materials and Methods

### 2.1 Reagents and Antibodies

The melatonin was purchased from (Sigma Aldrich, St. Louis, MO, USA), dissolved as a stock solution at a concentration of 1M in DMSO, and diluted with culture media to acceptable quantities before use. Cell Signaling Technologies sold the anti—actin, anti-Beclin-1, and anti-P62 antibodies that were purchased (Danvers, MA, USA)

### 2.2 Cell Culture and Treatment

The Chinese Academy of Sciences Cell Bank provided the human colon cancer cell lines DLD-1SW620 and the human colonic epithelial cell line FHC (Shanghai, China). 10% fetal bovine serum was supplied to the RPMI-1640 media used to maintain the cells on a regular basis (Gibco, USA). Moreover, the stated culture system was supplemented with streptomycin (100 U/mL) and penicillin (100 U/mL) and placed to 37 °C in humid air with 5% CO<sub>2</sub>.

### 2.3 Cell Scratch Test

Replated cells at a density of  $1 \times 10^5$  per well were grown for 24 hours at 37 °C in an incubator with 5% CO<sub>2</sub> to produce monolayers of cells. We created a scratch to represent a wound using a sterile clear tip (200ul), and cells were maintained in culture with the full medium. The scratch images were recorded using an inverted fluorescence microscope (Nikon, Tokyo, Japan) at the time point of 0 hours. The cells were then given a variety of medium melatonin doses individually. An optical microscope was used to observe cell migration after 24 or 48 hours of treatment.

### 2.4 Colony Formation Assay

The 6-well plates with the full medium were replated with 1000 cells per well. According to the experimental plan, the necessary medications were applied to 6-well plates, and cells were cultured at 37 °C in a 5% CO<sub>2</sub> incubator for around 2 weeks, or until the colonies in the control group could be seen with the naked eye. Individual colonies (those with more than 50 cells) were counted after the cells had been stained with crystal violet and the colonies had been preserved with 4% paraformaldehyde. The number of colonies in each group was calculated by using the Image J software.

### 2.5 Western Blot Assay

After taking melatonin, all cell proteins were taken out and lysed on ice in a solution called RIPA lysis buffer, which contains phosphatase inhibitor and phenylmethylsulfonyl fluoride. The BCA Protein Assay Kit was used to determine the quantity of protein. Using 8–12% SDS polyacrylamide gel electrophoresis, the equivalent protein from each specimen was extracted and transferred to a PVDF membrane. The membrane was then blocked overnight at 4 °C using the appropriate primary antibodies. Secondary antibodies conjugated with horseradish peroxidase (1:5000) were also used for an extra hour at room temperature. Using an improved chemiluminescence detection kit, proteins were located, and Imaging Lab software was used to determine antibody band densities.

### 2.6 RT-qPCR

Total RNA was extracted from cells and purified with TRIzol Reagent as directed by the

manufacturer (Invitrogen, USA). The following primers were used in quantitative PCR using an ABI 7500 Sequence Detection System, Applied Biosystems analytic software, and a complementary DNA synthesis kit (Invitrogen; Thermo Fisher Scientific, Inc.) to produce complementary DNA (designed and synthesized by Sangon Biotech Co, Ltd, Shanghai, China): LINC00673 , TCAGAAGACCCACGACCTCT (F) and AATACCTCCAGCTTGGCGA (R) ; GAPDH , CCTTCCGTGTTCTACCC (F) and AAGTCGCAGGAGACAACC (R) and ChamQ SYBR qPCR Master Mix(Vazyme Biotech Co.,Ltd, Nanjing China).The cycling parameters used in the RT-qPCR were 50° C for 2 minutes, 40 cycles at 95° C for 30 seconds, 95° C for 5 seconds, and 60° C for 30 seconds. The samples were analyzed in triplicate, with GAPDH serving as the internal standard.the relative differences in messenger RNA (mRNA) expression levels were calculated using the 2- $\Delta\Delta$ CCT method.

## 2.7 Statistical Analysis

Employing GraphPad Prism 7.0, all experimental data were statistically analyzed in this experiment. All detail of data was measured and presented as mean standard deviation. One-way ANOVA was used for comparisons between several groups, and the t-test or paired t-test was used for comparisons between two groups. The test level  $\alpha=0.05$  and the distinctions were statistically essential at  $P<0.05$ .

## 3. Results

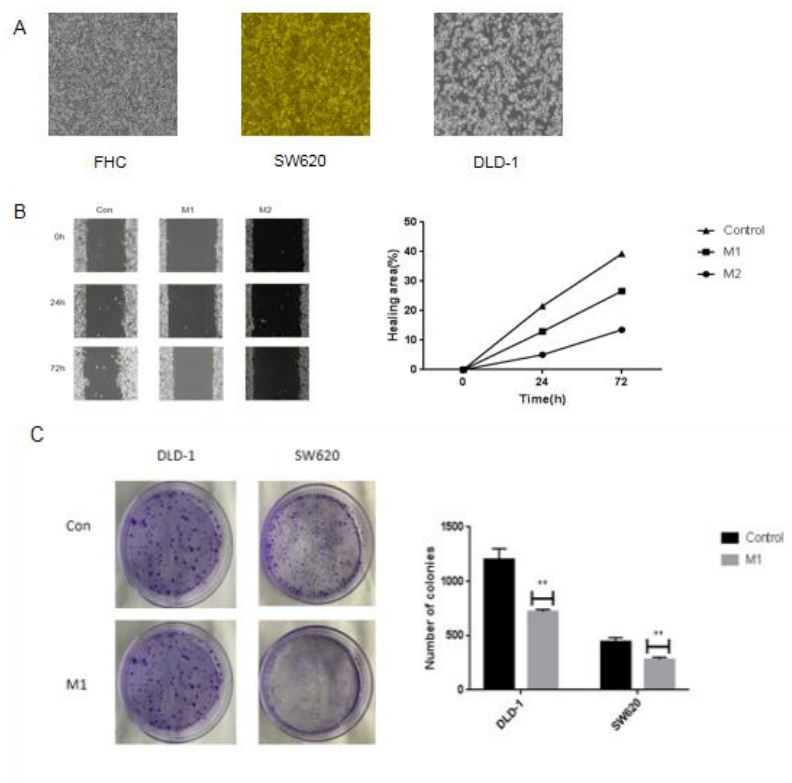


Figure 1:(A)Cell line in this experiments(B) Results of the scratch examination showed that the melatonin group's cell migration was inhibited.(C)Colony formation and colony number statistics of DLD-1 and SW620 cells upon treatment with 0, and 1 mM melatonin. The chart shows data as means  $\pm$ SD, with n=3. The control and student's t-tests were compared.\* $P<0.05$ , \*\* $P<0.01$ .

Abbreviations: M1, 1 mM melatonin; M2, 2 mM melatonin; Con, control trip

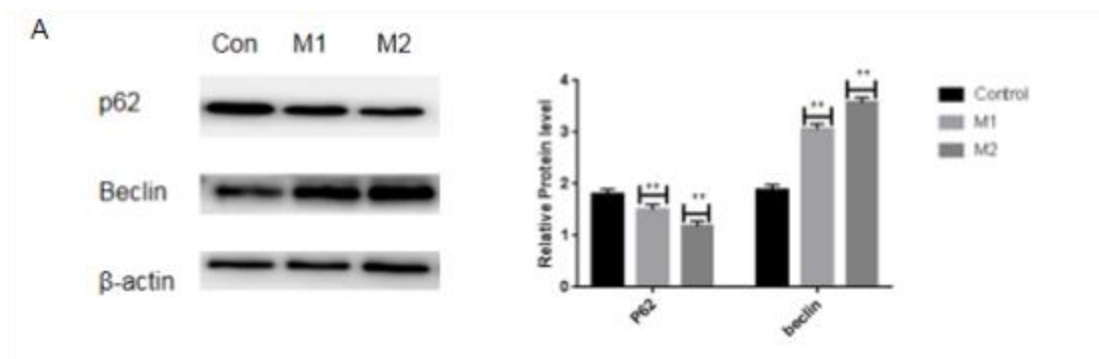


Figure 2: (A) Analysis of autophagy-related proteins by Western blot after melatonin treatment. (B) Results of the Western blot were quantified and expressed as a percentage of the control. The test was performed three times. Here, data are presented as the means  $\pm$  SD,  $n=3$ . Student's t-test was compared to control. \* $P<0.05$ , \*\* $P<0.01$  compared to the control.

### 3.1 Changes in migration and invasion ability of colon cancer cells after melatonin intervention

Since migration and invasion ability is crucial for tumor progression, colon cancer cells are capable of rapid migration and invasion. We employed cell scratch assay to detect this phenotype as an assessment of melatonin's anti-cancer effect. Human colon cancer SW620 cells were treated with distinct concentrations of melatonin (M1 and M2) and the migration of SW620 cells were recorded at three times of 0h, 24h, and 72h after melatonin intervention. Figure 1B demonstrates that the M1 and M2 groups significantly slowed down cell repair and that the inhibitory effect got stronger as concentration rose.

### 3.2 Effect of melatonin on the colony-forming ability of colon cancer cell lines SW620 and DLD-1

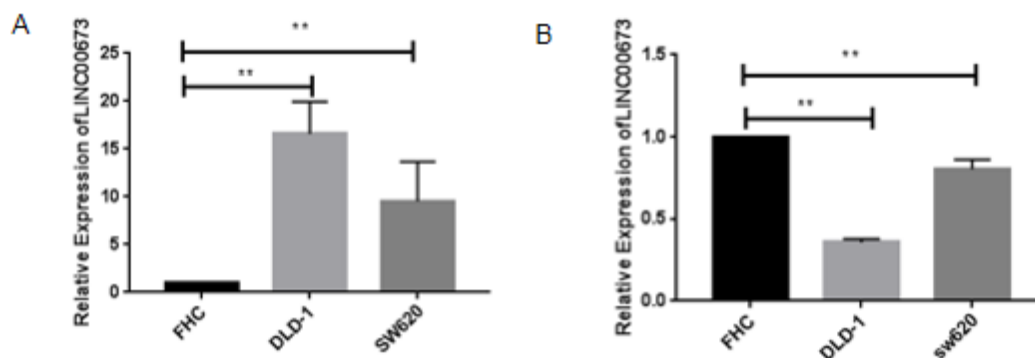
One of the traits of tumor cells' increased stemness, which can reflect their population dependence and proliferation ability, is their enhanced capacity for colony formation. And the results of the plate colony formation assay showed that in Figure 1C, the number of tumor cell colony formations in each group of M1 was over the Control group and the colony was relatively larger. The number of colony formations in SW620 and DLD-1 likewise decreased from  $445.0 \pm 26.6$  and  $1215 \pm 56.9$  in the Control group to  $285.6 \pm 16.7$  and  $720.3 \pm 36.5$ . The findings imply that melatonin can prevent the formation of cell colonies by influencing cell growth and consequently cell formation.

### 3.3 Altered protein expression profile after melatonin intervention in colon cancer cells

Lysosomes degrade cytoplasmic elements such as proteins, aggregates, and entire organelles through the process of autophagy. Many different forms of cancer develop and progress due in large part to autophagy. The expression of molecules (P62, Beclin-1) associated to autophagy was examined using the Western blot approach. Moreover, Figure 2A shows that as compared to the control group, the melatonin-treated group had significantly higher expression levels of the autophagy-related protein Beclin-1 and lower expression levels of P62. This means that the activity of melatonin induces autophagy in colon cancer cells.

### 3.4 LINC00673 expression was significantly increased in colon cancer cell lines SW620 and DLD-1, and melatonin reduced its expression

For the purpose of detecting the expression level of LINC00673 in SW620 and DLD-1, we examined the expression of LINC00673 in these two cell lines by qRT-PCR, and the results demonstrated that LINC00673 was highly expressed in SW620 and DLD-1 cells in comparison with normal colonic epithelial cells FHC in Figure 3A. Additionally, we co-cultured two tumor cells with healthy colonic epithelial cells in melatonin at a M1 concentration before running qRT-PCR on the LINC00673 of the tumor cells.



The figure indicates data as the means  $\pm$  SD,  $n=3$ . Student's t-test was compared to the control, \* $P<0.05$ , \*\* $P<0.01$ .

Figure 3: (A) expression of LINC00673 in cells (B) expression of LINC00673 in cells after treatment of M1 melatonin.

## 4. Discussion

In most cases, early-stage colon cancer is treated with tumor resection. Combinations of chemotherapeutic agents are frequently administered to colon cancer patients at all stages, but especially when it is most evolved. Cytotoxic (5-fluorouracil, oxaliplatin, irinotecan, and capecitabine) and biologic drugs are included in chemotherapy (bevacizumab, panitumumab, and cetuximab) [30]. Moreover, the majority of patients initially respond well to combined treatment. Nonetheless, approximately 50% of patients with colon cancer develop recurrent disease and 5-year survival is reduced in more than 10% of patients with advanced disease owing to drug resistance [31,32]. After three years of first surgery, 25% of patients with colon cancer will acquire liver metastases, and 50% will do so at the time of initial diagnosis. [33]. Hence, early diagnosis of colon cancer is crucial for the treatment of colon cancer.

LncRNAs are a class of RNA transcription products that are greater than 200 bp in length and have more spatiotemporal specificity and lower interspecies conservation than mRNAs [34]. Prior to recently, lncRNAs were thought to be transcriptional by-products. Nonetheless, it is now commonly acknowledged that lncRNAs play a role in cancer etiology, cancer cell differentiation and development, and many other disorders [35]. The classification of lncRNAs based on their position in relation to protein-coding genes in the genome includes sense, antisense, bidirectional, intronic, intergenic, and enhancer lncRNAs [36]. The importance of lncRNAs in regulating gene expression at the epigenetic, transcriptional, and post-transcriptional stages has just recently been recognized [37].

Although lncRNAs are widely distributed in mammalian cells, our understanding of their carcinogenic consequences has been constrained by conceptual and technical barriers. First, there needs to be a conceptual shift away from the molecular biology dogma, which holds that RNA's only function is to encode proteins. Additionally, the technical inability to query non-coding regions of the

genome needed to be addressed, resulting in a lack of reliable lncRNA annotation. The widespread use of deep sequencing technologies and the ensuing functional examination of lncRNAs have overcome these constraints. The translation and maintenance of cancer phenotypes may be clinically affected by the simultaneous presence of a high number of lncRNAs. It provides an entirely new avenue for the detection and treatment of cancer [38]. For instance, by comparing prostate cancers to healthy tissues, the first lncRNAs prostate cancer associated 3 (PCA3 also known as DD3)15 [39] and prostate-specific transcript 1 (PCGEM1)16 [40] linked to prostate cancer were discovered, and their aberrant expression allowing PCA3 to be currently adopted as a biomarker for prostate cancer [41]. The early identification of lncRNA metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) expression as a predictive marker for lung cancer survival [42]. Currently, MALAT1 expression is linked to cancer in a number of tumor types, including liver, breast, and colon cancer [43], indicating a role in cell proliferation. LINC00673 was noted to be associated with medium tumorigenesis in numerous articles on cancer. Huang et al. observed that SP1 can activate LINC 00673, a lncRNA that exerts oncogenic effects in gastric cancer by binding LSD 1 and EZH 2 [44]. Moreover, Wei Lu et al. noted that LINC 00673 was upregulated. In non-small cell lung cancer and it can sponge miR-150-5p and indirectly regulate ZEB1 [45]. Ubaidat Abdul-Rahman et al. identified LINC 00673 in breast cancer as an indicator of overall survival [46]. Hence, for the early diagnosis of colon cancer, LINC00673 may be used as a biomarker.

When first discovered, melatonin was believed to be a circadian rhythm regulator which produced by the pineal gland. Nonetheless, subsequent in-depth studies have revealed that it can be synthesized in numerous (perhaps all) animal and plant tissues, acting as a local redox regulator [47]. In an essence, the efficiency of melatonin as an antioxidant is on the basis of the fact that its amphiphilic nature enables it to quickly navigate all biological obstacles and enter the mitochondria [48]. Melatonin and its metabolites such as cyclic-3-hydroxymelatonin, 6-hydroxymelatonin, and N(1)-acetyl-5-methoxyamine (AMK) exhibit substantial antioxidant effects by scavenging reactive oxygen species (ROS) and free radical reactants [49]. Additionally, the effects of melatonin on the cell cycle, apoptosis, oxidative stress, immune stimulation, and growth signaling exhibit intrinsic antitumor properties through receptor-mediated or direct interactions with free radicals, proteins, or lipids [50,51]. Linoleic acid plays an essential role in the proliferation of tumor cells and its oxidation by 15-lipoxygenase generates 13-hydroxy-octadecadienoic acid (13-HODE), a source of energy for tumor signaling molecules. Since adenylate cyclase and cyclized AMP (cAMP) are both inhibited by melatonin receptors MLT1 and MLT2, lowering cAMP production lowers linoleic acid uptake. Melatonin may produce antiproliferative effects by inhibiting linoleic acid [52]. Besides, melatonin can reduce estrogen receptor expression (ER $\alpha$ ) and estradiol (E2-ER) binding. Melatonin can also deactivate calmodulin, which is involved in the start of anticancer activity in the estrogen signaling pathway [53]. And in various clinical trials on melatonin anticancer, several data have shown that the cytotoxicity of melatonin is low in a range of doses. Patients with advanced lung cancer receiving two concurrent chemotherapeutic agents and 40 mg/day of melatonin did not experience any myelotoxic side effects [54]. Melatonin use in a phase II research in patients with metastatic breast cancer not only reduced serum IGF-1 levels, which lessened anxiety symptoms, but also did not increase the toxicity of tamoxifen [55]. Moreover, oral administration of 0.5 mg or more of melatonin is immediately detectable in plasma, and yet it does not mimic endogenous conditions [56]. Melatonin can also increase the effectiveness and side effects of some anticancer medications. In non-small cell lung cancer (NSCL) patients, combining interleukin-2 and melatonin significantly increased 1-year survival when compared to an interleukin-2-only regimen [57]. Melatonin can play a crucial part in the eradication of cancer cells when combined with chemotherapeutic agents and similarly as an adjuvant. Likewise, on the condition that given with chemotherapeutic agents, melatonin enhances the therapeutic effect, lessens chemotherapy-induced side effects, enhances antioxidant effects, and



strengthens intracellular immunostimulatory mechanisms [58].

In summary, this study revealed the anti-colon cancer effect of melatonin through in vitro experiments, which could partially inhibit its migratory effect, and also demonstrated that a portion of the anti-colon cancer effect of melatonin was achieved by promoting autophagy in colon cancer cells. We also noticed that LINC00673 was highly expressed in colon cancer cells by qRT-PCR and melatonin reduced the expression of LINC00673 in colon cancer cells. This may provide additional evidence that melatonin is an effective therapeutic candidate in anti-cancer therapy and a biomarker for colon cancer early diagnosis.

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