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Polyphyllin I inhibit cell viability, migration and invasion of human colon cancer cells by regulating MiR-26a/TGF-β signaling pathway.

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Abstract

miR-26a is reported to be involved in both tumor suppressor or oncogenic action in various types of cancer progression. Colon cancer is the fourth leading lethal form of cancer and cause large number of deaths worldwide. Despite its various health beneficial effects against abnormalities, Polyphyllin I (PPI), has lack of scientific evidence on its role in colon cancer therapeutic efficacy were entice our attention. This study is designed to identify the role of miR-26a in the PPI mediated treatment for colon cancer. Further, Gene Ontology (GO) data on miR-26a biological process of its association with Transforming Growth Factor (TGF-β) signaling also hypothesized. In the present study, the protective role of PPI in cell migration and invasion of human colon cancer SW-948 and HT-29 cells was determined assays method. Further, reverse transcription-quantitative Polymerase Chain Reaction (qRT-PCR) analysis was carried out to determine the expression pattern of miR-26a and TGF-β, subsequently western blot analysis was performed to determine the TGF-β expression pattern. This experimental analysis validates the therapeutic efficiency of PPI that whether the anticancer potential was mediated by Transforming Growth Factor-β (TGF-β) cascade signaling and microRNA (miR) 26a. Earlier, Cell viability assay was performed to identify the Minimum inhibitory concentration M (IC50) of PPI on colon cancer cells. Our results suggest that cell proliferation, migration and invasion of cancer cells (SW-948 and HT-29) was significantly suppressed by PPI treatment, further the effect was observed in a dose-dependent manner. Moreover, following PPI treatment, miR-26a was up-regulated were further induce the tumor suppressor property also. This process consequently alters the TGF-β signaling cascade process that reportedly complicated, were associate with cell proliferation and differentiation. Findings of this study elucidated that, the over expression of miR-26a subsequently downstream the TGF-β signaling pathway, consequence of this effect was found to be PPI treated colon cancer cells. This might be the possible mechanism of action of PPI by which appears to be produce effective therapeutic potential in colon cancer therapy.

Keywords: Polyphyllin I, miR-26a, Transforming growth factor (TGF-β), Colon cancer.

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Introduction

Colon cancer is a malignant tumor originates from epithelial cells lining on inner mucous layer of large intestine or rectum. It is the fourth major cause of cancer related death worldwide. Every year 1.4 million people newly diagnosed with colon cancer as the third most prevalence rate than other cancer types around the world. The rate of colon cancer incidence is rapidly increase over 60% until 2030. Despite recent advances in the diagnosis and treatment include surgery and chemotherapy for colon cancer, the relative level of complete cure or life increase is very low when they diagnosed in the stage of advanced carcinoma or typical metastasis. Lack of findings about efficient targets, late diagnosis and unavailability of potential chemotherapeutic agents are the major risk factors in the colon cancer treatment. Accumulating evidences suggests the pivotal role of microRNAs(miRNAs) in the involvement and various types of human cancer. Further, number of studies frequently deduced that dysregulation of miRNAs closely related to initiation, development and progression of malignancy related to metastasis of various cancer including colon cancer in human. Generally, microRNAs are non-coding in nature and occur endogenously and length of around 18-24 nucleotide sequence, which regulate the post transcriptional expression of many genes by degradation and repression of mRNA.

miRNAs regulate the progression of cancer metastasis through control the expression of various oncogenic and tumor suppressor property of genes. More often, recent studies,

proposed the possibility of using miRNAs as biomarker in various cancer diagnosis and therapeutic target too10. The microRNA 26a belongs to the family of miR-26 and located in the 3 and 12 respectively with subtypes 1 and 2. Pharmacological studies on human hepatocellular carcinoma cells results has find out the association of miR-26a in the process of cell cycle arrest, partially through downregulation of cyclin D2 and cyclin E2. In addition, down regulation of cyclins D3 and E2, c-myc, and cyclin-dependent kinases 4 and 6 were observed an in vivo study of nasopharyngeal cells with tumorigenesis.

Moreover, in vivo study demonstrated that miR-26a directly up-regulate the expression of Metadherin (MTDH) and EZH2 which further inhibit tumorigenesis of breast cancer cells by induce apoptosis and colony formation. Increased level of miR-26a found in human cholangiocarcinoma cell line and tissues were accompanied by down regulation of GSK-3β and further activation of β-catenin. This increase is restored when compared with noncancerous epithelial cells of biliary. Recent study explores the replace of miR-26a as potential therapeutic strategy to treat metastatic melanoma. Further stated the over expression of miR-26a in melanoma cells found when compare with primary melanocytes and the miR-26a mimic treatment to the melanoma cell line which efficiently promote apoptosis.

In Traditional Chinese Medicine (TCM) Paris polyphylla widely used as an agent for the treatment of various illness including inflammatory associated diseases. Rhizome of Paris polyphylla is the source of Polyphyllin I (PPI), classified as a small molecular steroidal saponin, commonly known as “Chonglou”, In many Asian countries PPI is broadly used to treat various illness such as bacteriostasis inflammation, abscess, and hemorrhage-related diseases. In the year of 2015, Chinese Pharmacopoeia, has officially recorded PPI is the potent anti-inflammatory and hemostatic agent, isolated from Paris polyphylla. Previously, in vitro studies using HepG2 cell line to establish hepatocellular carcinoma and osteosarcoma has exhibited the potential role of PPI in downregulation of p65 phosphorylation, an NF-kB subunit.

Furthermore, recent studies substantiate that PPI could inhibit NF-kB signaling-mediated over production of pro-inflammatory cytokines. Despite its positive effect in various illness, there is no study directly explore the effect of PPI on colon cancer cells. In addition, there is no scientific evidence on role of miR-26a in colon cancer regulation by TGF-β. Previous study clearly demonstrates the role of miR-26a on regulation of TGF-β signaling cascade in smooth muscle cell proliferation. Moreover, Gene Ontology (GO) analysis of miR-26a reveals which is highly relevant to transforming growth factor beta receptor signaling pathway (TGF-β) in biological process. Therefore, this study is aimed to identify the therapeutic potential of PPI on colon cancer cells, and for the first time this study explores the role of miR-26a and TGF- β in colon cancer.

Materials and Methods

Cell culture and reagents

PPI (purity ≥ 98%) was obtained from Goren Biotechnology Company (Nanjing, China). In order to obtain the desired concentration of PPI, initially the compound was dissolved in DMSO at 50 mM concentration. This consider as stock and then used to extracellular solutions preparation. Less than 0.1% concentration of DMSO was maintained in all the experimental need to avoid the toxic effect of DMSO. Primary Antibodies against TGF-β were purchased from Cell Signaling located at Beverly, MA. HRP coupled secondary antibodies were purchased from ZSGB-BIO (Beijing, China).

Colon cancer cell lines SW-948, SW480 and HT-29 along with normal colon CCD-18Co were obtained from Chinese Academy of Sciences typical culture collection cell bank (Shanghai, China). Culture medium supplied by Invitrogen, CA US, which contains 10% fetal bovine serum, streptomycin of (100 μg/ml) and penicillin at a concentration of 100 (IU/ml) were using for culturing of cell lines in RPMI-1640) medium were purchased from Sigma Aldrich at St. Louis, MO, USA. Further cells were incubated at room temperature (37°C) with 5% CO2 and 95% air in humidified atmosphere.

Cell proliferation assay

Exponential phase culture of SW-948 and HT-29 cells were used to determine the cells viability by MTT assay method. Briefly, after trypsin treatment the cancer cells seeded at a concentration 4 × 104 cells per well in 96-well plates and pre-incubated for 24 h before the PPI treatment. The cells were then exposed to gradient of PPI (5, 2.5, 1.25, and 0.625), and wells used for control group incubated without PPI. After 24 h of incubation, 20 μL of 5 g /L MTT solution was added. Following 4h incubation medium was removed and replaced with blue formazan crystal solution dissolved in 100 ml of DMSO. Thus, this procedure was followed for after 48 h treatment also. The absorption was measured at 490 nm using a microplate reader supplied by Thermo Varioskan Flash Multiskan Mk3 from USA. All experiments related to cell viability assay was performed independently and triplicate also.

RNA extraction and RT-qPCR analysis

Following successive treatment with different concentrations of Polyphyllin I (0,1, 2 and 4 μM) for 24 h, RNA iso small RNA kit was used to extract total small RNA from treated cells. After isolation, total small RNA reverse transcription process was performed with a miRNA First-Strand Synthesis kit followed by manufacturer's protocol. The primers for miR-26a (cat. no. HP300272), TGF-β (cat. no. HP200609)
were obtained from OriGene Technologies (Rockville, MD, USA). cDNA obtained from reverse transcription process was further used to determine the levels of miR-26a and TGF-β in Fast PCR systems of ABI 7500. Premix Ex Taq II dye used for quantification according to the manufacturer's directions. U6

and 36B4 was used as an internal control. Relative quantification measurement was performed using the $2^{-\Delta\Delta}$Cq method. Each PCR amplification and quantification was carried out in biological sample triplicate.

Cell migration and invasion assay

To evaluate potential of cell migration and amount of invasion, Transwell assays were conducted using 8 µm pore filters made transwell cell culture chambers obtained from Corning Life Sciences. Following treatment with 0, 1, 2, and 4 µM of PPI for 24 h, cells were (HT-29 colon cancer cells) harvested from wells and grown in serum-free RPMI-1640 medium without PPI. Upon 24 h PPI treatment, survival cells were adjusted to ~5 × 104 cells were further seeded into the upper chambers. 10% FBS contains RPMI-1640 medium is filled in the upper chamber is served as a chemoattractant in cell migration assay. Next 12 hours of migration assay, cells were permitted to move towards the complete medium, the non-migrated cells in the upper chamber were wiped while remaining migrated cells were quantified. During cell count measurement, the average was calculated from three randomly selected fields and number of migrated cells was assessed under a phase contrast microscope at a magnification of 200 from Leica Microsystems (GmbH, Geramny). The cell invasion assay also carried out to follow the similar protocol for cell migration assay, in addition during invasion assay upper chambers were covered with Matrigel matrix (BD Biosciences). Methanol used for fixation and the cells were stained with crystal violet.

Western blot analysis

Total protein was harvested from the cultured human colon cancer (HT-29 and SW-948) cells using Protein extraction reagent obtained from ThermoFisher, USA. The concentration of extracted protein was estimated by using BCA protein assay kit purchased from Beyotime, China. 12% SDS-PAGE gel used for separation of protein (20 g), and the separated protein electroblotted onto poly-vinylidene difluoride (PVDF) membranes obtained from Millipore, Bedford, MA). TBST (Tris-buffered saline with 1% Tween 20) solution contains 5% of skimmed milk was used as a blocking solution to block the membrane from false signals. This membrane has subsequently incubated (4°C overnight) with primary antibody of TGF-β with a dilution of 1:500, (Abcam Company, Cambridge, UK) against human or β-actin from Beyotime, China with a dilution of 1:800 against human were followed by washing with TBST. β-actin protein sample were used to be a sample loading control. The transfer membranes were further incubated with respective HRP-conjugated secondary antibody at 37°C for 1 h. The protein molecule band signals were detected using ECL detection reagent from Amersham Biosciences of NJ at USA, and band intensity was measured by using ImageJ software developed by NIH, Bethesda, MA.

Statistical analysis

Graph-Pad Prism 7 (La Jolla, CA, USA) used for statistical analysis. Statistical data were presented as the mean ± SD. All the analysis was performed using one-way ANOVA or Student's t-test. Hence in the case of more than two samples, Tukey's post-hoc test after one-way ANOVA was performed for statistical analysis. P-value of <0.05 to be considered statistically significant.

Results

MTT assay was performed to evaluate the inhibitory effect of Polyphyllin I on the growth of SW-948 and HT-29 colon cancer cells. After successive treatment with Polyphyllin I for 24 and 48h the half maximal inhibitory concentration (IC50) values of PPI were calculated to be and 3.8. µM against SW-948 and respectively. Polyphyllin I treatment shows considerable change in cell viability in both cells culture in time and dose dependent pattern. As well as, PPI exhibit the inhibit cell proliferation in SW-948 and HT-29 cells with the IC50 of 2.412 ± 0.32 and 1.164 ± 0.14, 2.116 ± 0.22 and 1.97 ± 0.17 and µM after 24 and 48 h of treatment respectively. SW-948 and HT-29 cells showing fragmented and condensed nuclei are designated as abnormal and supressed its growth pattern. DMSO treated wells consider as control and the cells were visualized as healthy and attached to the culture plate with confluent monolayer. In contrast PPI incubation notably reduced the confluence of SW-948 and HT-29 cell culture and resulted in detached as well as fragmented cells, in a dose-dependent pattern. Therefore, these results demonstrated that PPI suppress the proliferation of HCT116 and HCT-8 cells in statistically significant (Figure 1).

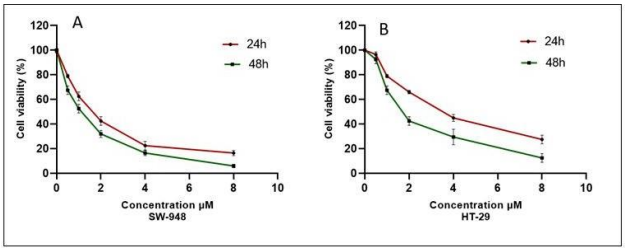

Figure 1. *Proliferative effect of PPI on SW-948 (A) and HT-29 (B) cells was measured by MTT assay. Cells incubated with sub-micromolar concentration of PPI that inhibit cell proliferation in a dose-dependent pattern.*

miR-26a is up regulated in PPI treated colon cancer cells

Gene ontology analysis of miR-26a revealed as it's primarily involved in the biological process of transforming growth factor beta receptor signalling. However, to identify the association between mir-26a and TGF- β receptor signalling the level of miR-26a expression was measured in PPI (10–40 µM) treated SW-948 and HT-29 cells to determine the role on colon cancer mechanism by qRT-PCR. The results showed that miR-26a is significantly up regulated upon PPI treatment ($p<0.05$) in both the colon cancer cell lines. Furthermore, the level of miR-26a was found to be considerably less in colon cancer cell lines relative to normalization with U9. The lowest expression was observed in cells not treated by PPI during the

24-hour treatment. In contrast, TGF-β transcription level was significantly supressed by PPI treatment, thus expression level was gradually up-regulated with respect to increase in the concentration of PPI. This data could demonstrate the miR26a/ TGF-β relatively contribute the development of colon cancer (Figure 2).

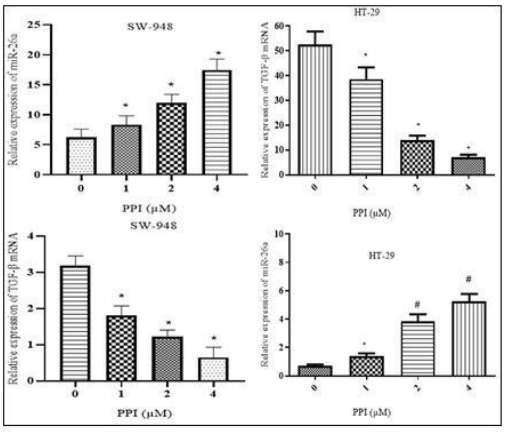

Figure 2. *Following 0, 1, 2, and 4 μM PPI treatment for 24 h, the RNA expression of miR-26a and TGF-β in SW-948 and HT-29 cells was determined by using qRT-PCR experiment. U6 and 36B4 was served as the internal control. *P<0.05 vs. untreated control cells.*

PPI prevents invasion and migration of HT-29 cells

To identify the migration potential and invasiveness of HT-29 cells, transwell assay was performed. In the case of cell migration, the PPI treatment notably reduce the migration of (23.3 ± 4.2-91.3 ± 1.2%) HT-29 cells compared with DMSO treated control cells. The result of transwell assay was clearly demonstrated that treatment with 0-4 μM PPI significantly reduce the rate of invasion (25.5 ± 2.4-98.8 ± 0.2%) of HT-29 cells, when compared with DMSO treated control cells. All together, these results strongly support the potential of PPI in moderate the abnormal property of cell migration and invasive of HT-29 colon cancer cells in a dose dependent manner (Figures 3 and 4).

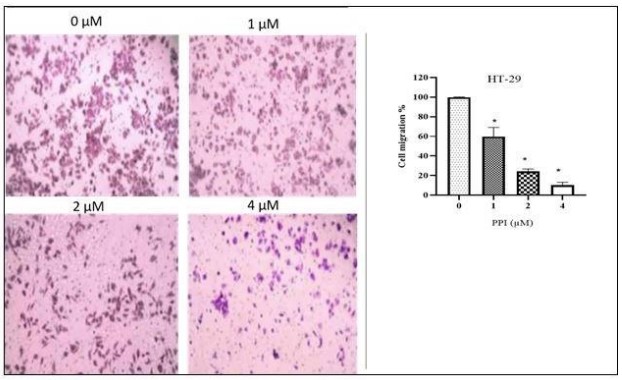

Figure 3. *Protective effect of PPI treatment with 0, 1, 2, 4 μM for 24 h on the migration of HT-29 cells was determined using Transwell assays. 200X magnification was used in the microscope for image collection. HT-29 average number of migrated cells were counted in membrane lower surface and the three randomly selected fields were normalized against untreated cells. Data are expressed as Mean ± SD and *P<0.05 vs. untreated control cells.*

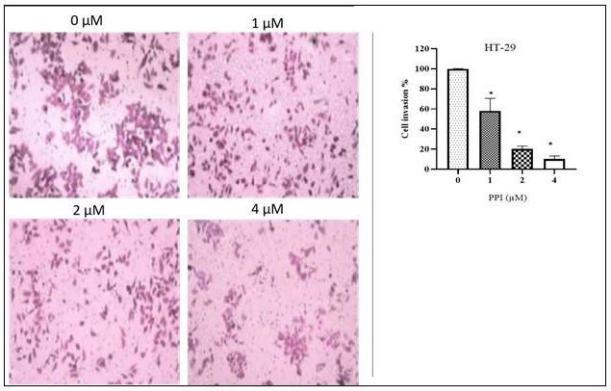

Figure 4. *Successive 24 h incubation of HT-29 cells with PPI (0, 1, 2, and 4 μM) cell invasion was determined using Transwell assays. The cell invasion images were captured at a magnification of 200X. HT-29 invasive cells average was counted in membrane lower surface at three randomly selected fields and normalized against control cells. Data are expressed as the Mean ± SD and *P<0.05 vs control cells.*

PPI treatment selectively mediated TGF-β expression in colon cancer cells

miR-26a expression is essential for TGF-β receptor and signalling in cell differentiation and many essential cellular functions. Our study intends to know the overexpression of miR-26a could reverse effects of colon cancer cell differentiation and on the proliferation of the SW-948 and HT-29 cells. The blotting results showed that TGF-β could almost completely supressed by the effects of miR-26a overexpression on the proliferation of the SW-498 and HT-29 cells indicating that miR-26a is essential for the tumour suppressive effects of the PPI (Figure 5).

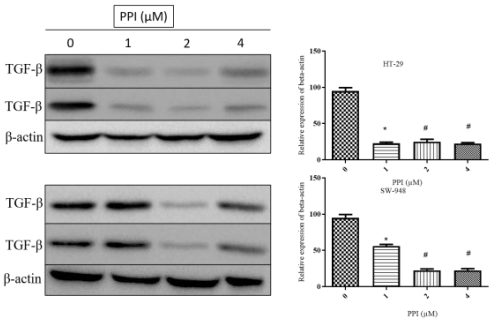

Figure 5. *Inhibitory effect of PPI on the expression of TGF-β signalling in HT-29 and SW-948 cells. Following treatment with 0, 1, 2, and 4 μM PPI for 24 h the TGF- β protein expression pattern was determined by blotting analysis. β-actin was used as served as internal loading control. Images are collected from three independent blotting experiments. Relative*

*densitometric analysis of bands is shown in graph and *P<0.05 vs. control cells.*

Discussion

The rhizome of *Paris polyphylla* extracted Polyphyllin I (PPI), exerts numerous beneficial effects and preclinical level anticancer drug research on various cancer models through by blocking cell cycle phases, inducing apoptosis, and cause impairing of mitochondrial function in various cancer cells. In traditional Chinese medicine PPI has long been used in for the treatment of various forms of cancer were explored by researchers in various human cancer cells. More recently, the identification and validation of potent anti-cancer compounds from traditional herbs has attracted more attention in research community. Till date number of polyphyllins with diverse range of molecular weights have been identified including polyphyllin I. These polyphyllins have been reported to have immune modulation related to anti-cancer, hemostatic, analgesic, bacteriostasis, positive inflammatory regulation properties.

However, recent studies explored the efficacy of polyphyllin I an inhibition of human lung cancer cells, while another study found that polyphyllin I strongly suppress the hepatocellular carcinoma in cells. Colon cancer is a hazardous cancer which has predicted that will cause more casualties in the upcoming days. The outcome of this clinical data is not persuading and treatment pattern have many discrepancies. Numerous studies demonstrated that expression of miRNAs modulate the majority of human genes function which has intricate in wide variety of cellular processes. The vital role of miRNAs in various biological including cellular and physiological processes made them as strategic molecule for therapeutic research. Studies have explored the certain role of miRNAs will facilitate the identification and validation of therapeutic targets for the treatment of various cancer. In this study, the role of miR-26a and therapeutic potential of PPI were investigated in colon cancer cells.

Emerging evidences suggest that dis regulation of miRNAs play a critical role in tumor progress and metastasis. Studies about miR-26a inferred that could acts as either tumor suppressor role or oncogene in several types of human cancers through suppressing its different target genes. It was reported that breast cancer cells proliferation and migration has inhibited by miR-26a through the MCL-1 expression and regulation. In vivo studies about gastric cancer by Deng et al revealed that miR-26a suppressed the metastasis and tumor growth by FGF9 gene.

In contrast, some pharmacological studies regarding cancer, reported that miR-26a was significantly upregulated ovarian cancer and glioblastoma. Further, it inhibits PTEN/AKT1 pathway which promote the growth of lung cancer cells. In particular, the biological effect of miR-26a was identified in prostate cancer carcinogenesis is reported to be divergence.

Above mentioned study reports provide the conclusion that depending on the cancer type and organ specific miR-26a possess dual effects on its target genes. In the present study the cell proliferation assay data explores that various concentration of PPI exhibit the inhibitory effects in SW-948 and HT-29 cells with the IC50 of 2.412 ± 0.32 and 1.164 ± 0.14, 2.116 ± 0.22 and 1.97 ± 0.17 and μM after 24 and 48 h of treatment respectively. In this present study, expression of miR-26a was significantly upregulated in both SW-948 and HT-29 cells with respect to increasing concentration of PPI gradient, while significantly low expression of miR-26a was observed in less concentration that relative to the colon cancer cell growth and regulation of TGF-β signaling. Our results demonstrate that upregulation of miR-26a could plays a tumor suppressor which further could suppress the expression of TGF- β cascade. This action could negatively regulate the TGF-β mediated cell differentiation and proliferation. Our mRNA expression data also could clearly support the hypothesis of miR-26a mediated tumor suppression by PPI. This data agrees with the findings tumor-suppressive miR-26a studies on colon cancer cell. Further, the transwell asaay experiment to determine the cell proliferation and colon cancer cell invasion in the presence of PPI also could support the miR-26a tumor suppressor activity. Upregulated expression of miR-26a significantly control the cell migration and invasive property of HT-29 colon cancer cells relative to metastasis of cancer. HT-29 cells migration is effectively controlled by higher dose of PPI, which also effectively safeguard the cells from invasion. As, observed inhibitory activity of miR-26a in colon cancer cells also agrees with the findings on inhibits the human hepatocellular carcinoma cells proliferation and metastasis by regulating the DNMT3B-MEG3 axis.

Conclusion

In order to find out the expression of transforming growth factor signaling receptor (TGF-β) during the upregulation of miR-26a, as we performed the blotting analysis of TGF-β protein molecule in both SW-948 and HT-29 cells. As expected, blotting data also supports the inhibitory effect of miR-26a which highly concur with mRNA expression data of TGF-β. The higher dose of PPI significantly suppresses the expression of TGF-β, this consequently alter the action of TGF-β in the proliferation and differentiation of colon cancer cells. Altogether, PPI could strongly suppress TGF-β, further it controls cascade signaling for cell differentiation and proliferation via inducing the tumor suppressor activity of mir-26a. Studies on PPI in various cancer prevention and treatment were shows significant lead for further search. In summary our study also strongly supports the miR-26 tumor suppressor activity in the presence PPI also control the TGF-β over expression in colon cancer cells. Further studies on expression TGF- β isofroms and it signaling cascade mechanism would provide more insight on understanding about mir-26 a and TGF- β in colon cancer mechanism and treatment.

Conflict of Interest

The authors declare there is no conflict of interest.

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References

1. Siegel R, DeSantis C, Jemal A. Colorectal cancer statistics 2014 CA. Cancer J Clin. 2014;64:104-17.
2. Arnold M, Sierra MS, Lavers Anne M. Global patterns and trends in colorectal cancer incidence and mortality. Gut. 2017;66:683- 91.
3. Saleh M, Trinchieri G. Innate immune mechanisms of colitis and colitis-associated colorectal cancer. Nat Rev Immunol. 2011;11:9–20.
4. Seshagiri S, Stawiski EW, Durinck S. Recurrent R-spondin fusions in colon cancer. Nature. 2012;488:660-64.
5. Potter JD, Slattery MI, Bostick RM. Colon cancer: A review of the epidemiology. Epidemiol Rev. 1993;15:499-45.
6. Gomes CC, De Sousa SF, Gomez RS. MicroRNAs: Small molecules with a po-tentially role in oral squamous cell carcinoma. Curr Pharm Des. 2013;19:1285-291.
7. Nohata N, Hanazawa T, Kinoshita T. MicroRNAs function as tumor suppressors or oncogenes: aberrant expression of microRNAs in head and neck squamous cell carcinoma. Auris Nasus Larynx. 2013;40:143-49.
8. Wu BH, Xiong XP, Jia J. MicroRNAs: New actors in the oral cancer scene. Oral Oncol. 2011;47:314-319.

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