Evaluation of miR-107, DAPK1, and KLF4 Expression in Colorectal Tumors and Effect of Oxaliplatin and 5-FU on their Levels in Colorectal Cancer Cell Lines

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Abstract

Background: In recent years, the role of micro-RNAs in the cancer pathophysiology has attracted a great deal of scientific attention. MiRNAs regulate a variety of cellular functions, such as apoptosis, differentiation and migration by targeting oncogenic or tumor suppressor genes. We conducted the current study to assess the expression of miR-107, Krüppel-like factor 4 (KLF4) and death-associated protein kinase (DAPK1) genes in malignant and normal colon tissues and also colorectal cancer (CRC) model cells exposed to oxaliplatin and 5-FU chemotherapy agents.

Method: In this case-control study, the tissue samples from CRC patients were collected during colonoscopy process in 2013 -2016 at Imam Reza hospital. Subsequently, the expression levels of miR-107, KLF4, and DAPK1 were detected with quantitative Real-Time PCR. Furthermore, in the in vitro phase of this study, we investigated the changes in the expression level of miR-107, KLF4 and DAPK1 transcripts after oxaliplatin and 5-FU treatment.

Results: Unlike miR-107, the expression levels of KLF4 and DAPK1 genes decreased in the tumor samples compared to those in the marginal specimens. In addition, both oxaliplatin and 5-FU significantly increased the expression level of miR-107. There were significant correlations between the expression levels of miR-107, KLF4, and DAPK1genes and clinicopathological features, for instance lymph node metastasis and cell differentiation.

Conclusion: The current study suggested a tumor suppressor role for KLF4 and DAPK1 in CRC. The altered expression of miR-107, KLF-4, and DAPK1 genes in CRC tumors and healthy tissues could be utilized for CRC diagnosis and prognosis. Furthermore, the studied genes could be considered as potential therapeutic targets in CRC.

Keywords: Colorectal neoplasms, miR-107, KLF4, DAPK1, Oxaliplatin, 5-FU

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Introduction

Colorectal cancer (CRC) is the third most prevalent malignancy with a significant contribution to cancer-related death rate worldwide.[1,2] Numerous environmental, genetical, and epigenetical factors take part in the pathogenesis of CRC.[3] Consequently, identification of appropriate risk factors and biomarkers could help to provide novel diagnostic, prognostic and therapeutic tools.[4] On the other hand, utilization of proper diagnostic and prognostic biomarkers enables more successful follow-up implementations in patients with CRC. Recently, microRNAs (miRNA) have been identified as new therapeutic biomarkers in patients with CRC.[5,6]

miRNAs, by post transcriptional alteration of gene expression, are considered as key regulatory molecules.[7,8] A variety of miRNAs have been known to be dysregulated in tumor cells.[9,10] miR-107 is an example of miRNAs with a pivotal role in the induction of epithelial-to-mesenchymal transition (EMT) leading to an invasive and metastatic phenotpe.[11] In vitro studies and mice models have indicated that miR-107 targets death-associated protein kinase (DAPK1) and Krüppel-like factor 4 (KLF4), which leads to a weak cell-matrix adhesion and cell-cell connection resulting in invasiveness of CRC cells. In other words, overexpression of miR-107 and down regulation of DAPK1 and KLF4 genes, are associated with poor prognosis in CRC models.[12] DAPK1 is a proapoptotic serine/threonine protein kinase known as a tumor suppressor gene, which is dysregulated in different types of cancers. DAPK1 expression inhibits metastasis by inducing programmed cell death.[13] Furthermore, it augments cell-matrix connection through deactivation of integrin b1protein, and prohibits tumor cell mobility.[13] In line with the antimetastatic function of DAPK1, depletion of its expression in different cancers has been shown to be correlated with more advanced tumor stages and aggressive phenotypes.[14] KLF family members are a group of zinc-finger transcription factors that adjust various biological processes, including proliferation, differentiation,

development, and apoptosis. An increasing body of evidence suggests that KLF4 functions as a tumor suppressor gene in certain cancers, CRC for instance. Loss of KLF4 gene in the intestine results in a rise in proliferation and migration and a reduction in differentiation of epithelial cells.[15] The overexpression of KLF4 is also believed to decrease the transformation, migration, invasion, and tumorigenicity of a CRC cell line.[12]

Chemotherapy is a common therapeutic approach to fighting cancer. Oxaliplatin is a third-generation platinum complex considered as a first-line medication of CRC. It has also revealed efficient functions in patients with metastatic CRC.[16] Oxaliplatin binds specifically to the guanine and cytosine bases of DNA and prevents DNA replication and transcription, which triggers apoptotic cell death. RNA and protein synthesis in cells could also be inhibited by consuming elevated doses of this drug.[17] Studies have also demonstrated that oxaliplatin is of synergistic activities in combination with other medications. For example, the combination of oxaliplatin and 5FU has shown synergistic activities against cancer cells. The largest therapeutic response to 5-FU has been described in CRC.[18] The active form of 5-FU, via multiple pathways including the folate metabolic pathway, disrupts both DNA and RNA synthesis. 5-FU functions in several ways, but mostly as a thymidylate synthase (TS) inhibitor. TS methylates deoxyuridine monophosphate (dUMP) to form thymidine monophosphate (dTMP). Disturbing the action of this enzyme inhibits synthesis of the pyrimidine thymidine, which is required for DNA replication.[19,20] Despite the efficiency of chemotherapy as a treatment strategy, adverse effects on unintended cells, and emergence of drug resistance is frequently observed in its practice.[21] Manipulation of miRNA expression levels via miRNA restoration or using antagomirs has recently attracted a great deal of interest.[22] We expect that engineering the miRNA expression pattern help to lower the drug intake and re-sensitizing the chemo-resistant cells.[23]

In this study, we assessed the expression of miR-107 and its potential targets (DAPK1 and

KLF4) in CRC patients. Moreover, we employed CRC model cell lines in order to investigate the correlation between miR-107, DAPK1, and KLF4 expressions with resistance to oxaliplatin and 5FU.

Materials and Method
Expression analysis in patient derived samples
Patients and samples

This case-control study comprised 47 patients diagnosed with CRC according to their clinical symptoms, colonoscopy, and other Paraclinical assessments conducted in Imam Reza hospital, (Tabriz University of Medical Sciences, Iran) from 2013 to 2016. The tumor samples and specimens from marginal areas (representing healthy tissue) were acquired during the colonoscopy process. Clinicopathological characteristics of the patients are summarized in table 1. Subsequently, the specimens were immediately placed in sterile vials containing RNAlater™ solution (Thermo Fisher Scientific, USA). The vials were stored at -80°C for subsequent RNA extraction. All the patients provided written informed consents, so that we could utilize the left-over tissue samples for scientific researches. The protocol for the use of human tissues was approved by the Research Ethics Committee of Tabriz University of Medical Sciences (License No.: IR.TBZMED.REC.1397.931).

RNA extraction

Total RNA was extracted from tumoral and

Table1. Clinicopathological characteristics of the patients

Age	
<55	21
>55	26
Gender	
Female	21
Male	26
Lymph node metastasis	
Positive	26
Negative	21
Tumor location	
Good	26
Middle	16
Bad	5
Tumor depth	
T2	5
T3	11
T4	31
Venous invasion	
positive	32
negative	15
Stage	
II, III	17
IV, V	30
Liver metastasis	
positive	7
negative	40
Tumor size	
>3cm^3	6
<3	41

healthy marginal colon specimens using TRIzol reagent (Roche diagnostics, Germany) according to the manufacturer's instructions. We investigated the quality and concentration of the RNA samples with agarose gel electrophoresis and NanoDrop
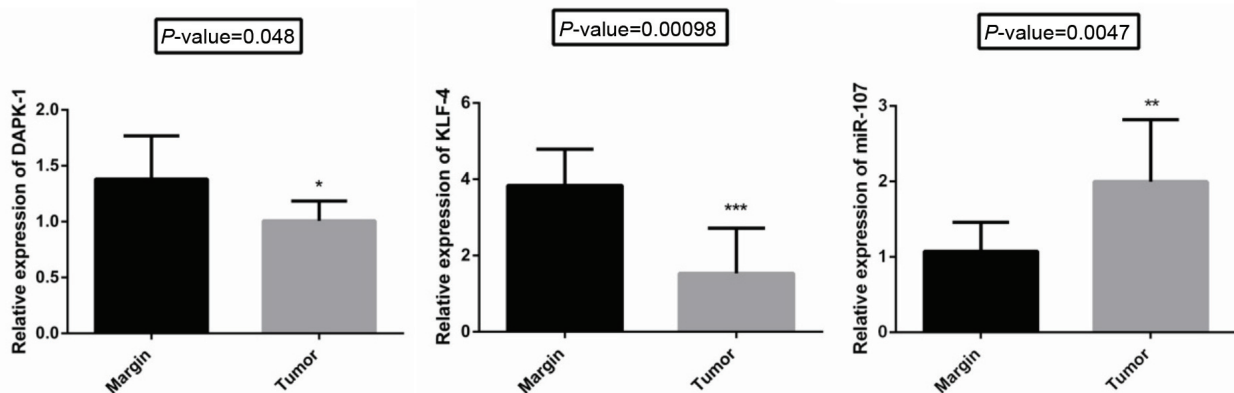
Figure 1. The expression of miR-107, KLF-4, and DAPK-1 transcripts were compared between colon cancer and marginal tissue samples.

KLF 4: Krüppel-like factor 4; DAPK 1: death-associated protein kinase

Table 2. Primer sequences

Gene Symbol	Product length (base pair)	Primer sequences
GAPDH	226	Forward: 5' GAAGGTGAAGGTCGGAGT-3' Reverce:5'-GAAGATGGTGATGGGATTC-3'
KLF4	184	Forward: 5'-GATTATAAATTAAGGGGAGAGTGGG-3' Reverce:5'-TCCCTAAAAAATAACCATATACCAAAA-3'
DAPK1	194	Forward: 5'-TTCGAGATTAGGATCGAGTTC-3' Reverce: 5'-CAATCATAATAAACTATCACGCC-3'

KLF4: Krüppel-like factor 4; DAPK1: death-associated protein kinase

2000c UV-Vis spectrophotometer (THERMO, USA).

cDNA synthesis and quantitative Real-Time PCR (qRT-PCR)

For the assessment of KLF4 and DAPK1 expression, complementary DNA was synthesized from total RNA utilizing a cDNA synthesis kit (TAKARA- Japan) according to the manufacturer's instructions. For this purpose, we utilized random hexamer primer mix and two micrograms of the extracted RNA. qRT-PCR was carried out using Light Cycler 96 system (Roche diagnostics, Germany). PCR was carried out in the final volume of 10 µl with 2X SYBR Green premix (Takara. RR820L), 0.5 µl of cDNA and 0.25 µl of 4 µM primers. We used the GAPDH gene as a normalizer (primer sequences are shown in table 2). In parallel, to perform miRNA quantification, cDNA synthesis kit and miR-107

targeted primers (both from Exiqon company, Denmark) were utilized. U6 was employed for normalization purpose in this process.

In vitro phase of the study
Cell culture

RPMI-1640 culture medium and fetal bovine serum (FBS) were purchased from Gibco Company. The SW480 and HCT-116 cell lines were purchased from Pasteur Institute of Iran (Tehran, Iran), and maintained at 37ºC in humidified 5% CO_2 atmosphere in RPMI-1640 supplemented with 10% (V/V) FBS, penicillin (100 U/ml), streptomycin (100 mg/ml) (Simga-Alderich, St. Louis, MO, USA).

Cell cytotoxicity assay

We measured the cytotoxicity of oxaliplatin and 5-FU on the target cells using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5diphenyltetrazoliumb romide (MTT) assay kit (Sigma-Aldrich,
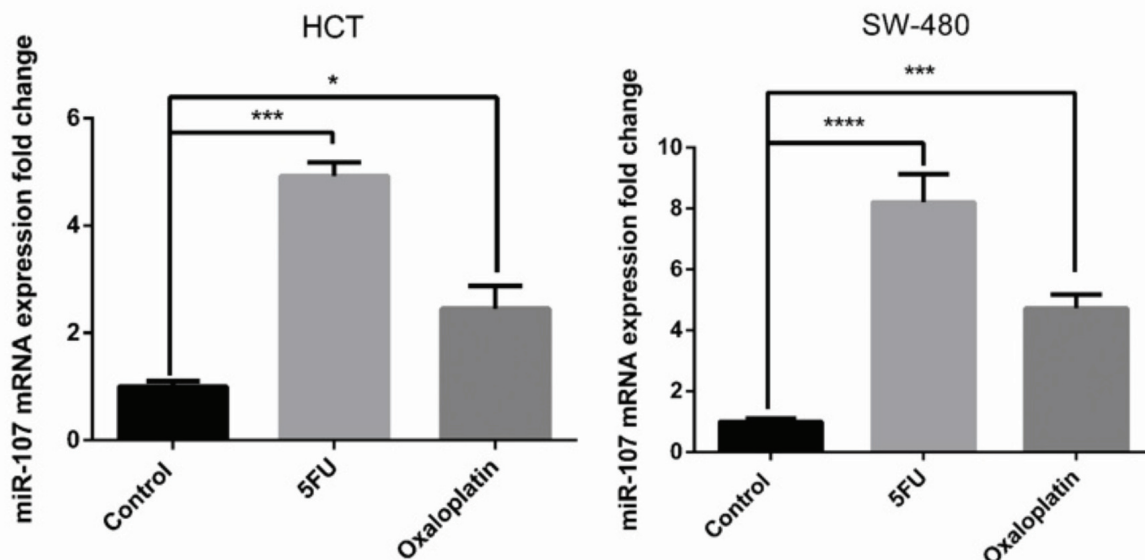
Figure 2. The changes in miR-107 expression were compared between oxaliplatin and 5-FU treated HCT-116 and SW-480 cells.

DMSOUSA). In brief, 15×10^3 cells were initially seeded in 96-well culture plates. Subsequently, the cells were treated with different concentrations of oxaliplatin and 5-FU and were incubated for 48 h in a humidified CO_2 incubator. Following this, 50 µl of MTT reagent (2mg/mL in PBS) was added to each well and the plates were returned to the incubator for 4 h. The water-insoluble formazan crystals were formed during the incubation period and then solubilized by adding 200µl of DMSO and 10 µl of Sorensen's buffer to each well. After 30 min of incubation in the above-mentioned conditions, we measured the optical density (OD) of each well at a wavelength of 570 nm with an ELISA reader (Awareness Technology, USA). The IC50 values were obtained using Graphpad Prism version 6:00 software.

Treatment

Oxaliplatin and 5-FU (98 % purity, Sigma-Aldrich, Germany) were separately dissolved in DMSO and then added to RPMI-1640 cultures at IC50 concentration (mentioned in the table 3). Finally, the cells were harvested after 48 h. RNA extraction, cDNA synthesis, and qRT-PCR were performed as patient samples. Total RNA was extracted with using TRIzol reagent from healthy and tumoral marginal colon specimens. Then, c-DNA was synthesized from total RNA and

Table 3. Drug IC50 concentrations for each cell line (micromol)

Cell line	5-FU	Oxaliplatin
Sw480	652.67724	16.01448
HCT116	518.913	45.84019

qRT-PCR was done using Light Cycler 96 system.

Statistical analysis

We performed the statistical analysis employing Graphpad Prism version 6:00 software. Our data were expressed as mean±SD. The differential expression of miR-107, KLF4, and DAPK1 among malignant colon tissues, healthy marginal tissues, and CRC model cell lines was evaluated with unpaired t-test. $P<0.05$ was considered statistically significant.

Results

IC50 concentrations of drugs for CRC model cell lines

We performed MTT assay in order to determine the IC50 values of oxaliplatin and 5-FU drugs for CRC model cell lines. Graphpad Prism software was utilized for the calculation of IC50 values based on the initial results of spectrophotometry optical densities in 570 nm (The quantities are presented in table 3).

Expression analysis of miR-107, KLF4 and DAPK1 transcripts in CRC tumors and marginal
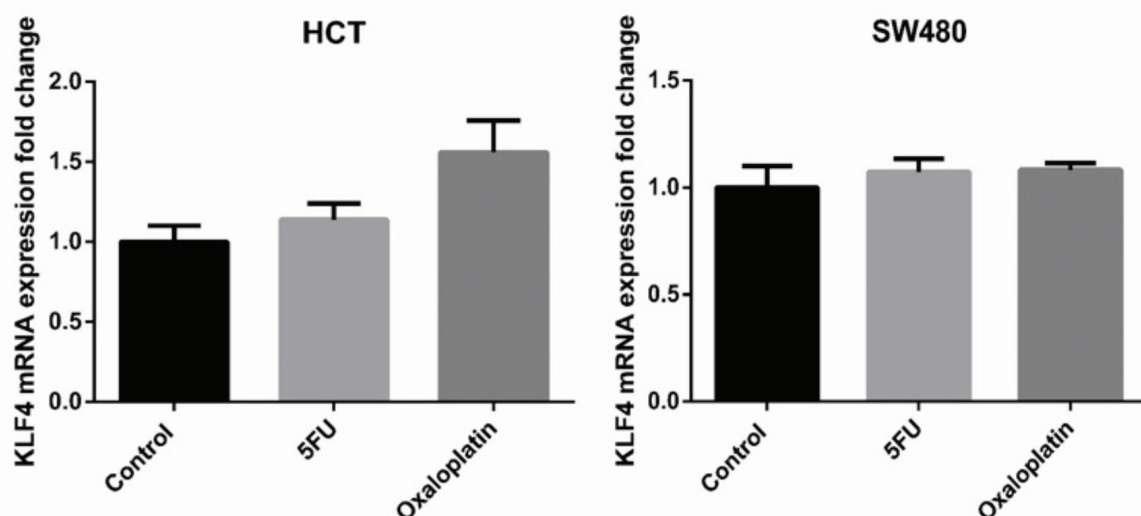
Figure 3. The change in KLF4 expression in response to 5FU and oxaliplatin was assessed in HCT-116 and SW-480 cell line.
KLF4: Krüppel-like factor 4

Table 4. Relation of target genes expression with clinicopathological features

Gene symbol	Related feature	*P* value
miR-107	Cell differentiation	0.034
KLF4	Lymph node metastasis	0.0018
DAPK-1	Lymph node metastasis	0.023

KLF4: Krüppel-like factor 4; DAPK1: death-associated protein kinase

tissues

The expression of miR-107 was examined in CRC tissues and healthy marginal tissues with qRT-PCR. The expression level of miR-107 was significantly up-regulated in tumor tissues compared with that in healthy marginal tissues (*P*=0.004). This could demonstrate an oncogenic function for miR-107 in CRC tissues (Figure 1).

The analysis of KLF4 and DAPK1 mRNA levels indicated that the expression of tumor tissues decreased in comparison to that of healthy marginal tissues (Figure 1). Moreover, the relations between the expression of miR-107, KLF4, and DAPK1 and clinicopathological features of the patients were acquired employing statistical analysis (Table 4).

Effects of oxaliplatin and 5-FU on the expression of miR-107, KLF-4 and DAPK1 in CRC cell lines

We employed real time PCR analysis to assess the effects of oxaliplatin and 5-FU treatment on the expression of miR-107, KLF4, and DAPK1 in SW480 and HCT-116 cells.

According to the obtained results, the expression level of miR-107 significantly increased after 48 h of treatment (Figure 2).

Our results indicated an increase in the expression of KLF4 and DAPK1 genes following oxaliplatin and 5-FU treatment. However, the differences concerning the rates were not statistically significant (Figures 3 and 4).

Discussion

CRC is highly associated with high rates of cancer related mortality and morbidity. Hence, identification of efficient diagnostic, prognostic and therapeutic tools seems to be of central importance.[22]

Dysregulated gene expression is an important cause of the initiation and progression of CRC. The interplay between oncogenes and tumor suppressor genes determines the fate of individual cells. MicroRNAs, by their regulatory roles, depending on their target genes, play oncogenic or tumor suppressor roles in cancer.[6]

In the present work, we compared the expression levels of miR-107, KLF4, and DAPK1
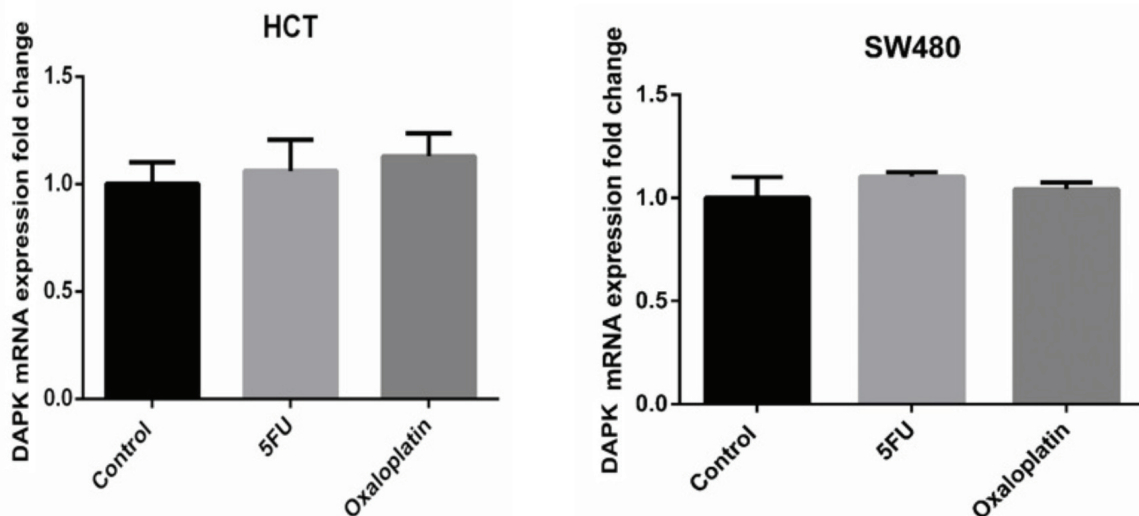
Figure 4. The change in DAPK-1 mRNA expression in response to 5FU and oxaiplatin drugs was assessed in SW480 cell line.

DAPK1: death-associated protein kinase

genes between CRC tumor samples and healthy marginal tissues. According to the qRT-PCR results, we observed a significant increase in the expression of miR-107 in the tumoral samples. Moreover, a significant correlation was found between the expression of miR-107 and cell differentiation. Chen and colleagues reported the involvement of miR-107 in CRC oncogenesis in murine models and in vitro. They indicated that miR-107 negatively affects the expression of KLF4 and DAPK1 genes, which are known as metastasis supressors.[12] Likewise, Boros and co-authors found a correlation between the expression of miR-107 and EMT in a rat model of colitis.[24] In the present study, the down-regulated expression of KLF4 and DAPK1 genes in neoplastic specimens was also evident. In parallel, clinicopathological features of the tumor samples revealed a significant correlation between KLF4/DAPK1 expression and lymph node metastasis in CRC patients. This suggests the suppressor function of these genes in CRC invasiveness. Moreover, the expression levels of KLF4 had an inverse relationship with the rate of recurrence and prognosis of the patients with CRC. On top of antimetastatic effects,[12] Benderska et al. showed that DAPK1 functions as a tumor suppressor gene and triggers TNF-mediated apoptotic cell death in CRC.[25]

Oxaliplatin and 5-FU are the two frequent chemotherapy agents used for the treatment of CRC. Investigation of the genes and pathways targeted or affected by these drugs could provide biology and etiology of cancer with new insights and initiate discovery of more effective therapeutics. In this regard, the effects of the above-mentioned anticancer drugs on the expression of miR-107, KLF4, and DAPK1 were investigated in vitro.

According to our findings, oxaliplatin and 5-FU significantly increased the miR-107 expression. miR-107 is capable of sensitizing cells to chemotherapy in various cancers via regulating genes, such as BCL2 family members.[26,27]

Due to their intrinsic potential in targeting multiple genes, miRNAs could exhibit diverse effects on different cancers and disease stages. On the other hand, these drugs did not significantly affect the expression of KLF4 and DAPK1 genes. It seems that the mentioned drugs exert inhibitory effects without affecting the expression levels of KLF4 and DAPK1 transcripts and cannot be appropriate candidates for the assessment of response to chemotherapy.

Conclusion

Based on our results, an increased expression of miR-107 was observed in the tumor samples while KLF4 and DAPK1 indicated a reduction in their expression levels in these tissues. The down-regulated expression of KLF4 and DAPK1 was coupled with lymph node metastasis. Moreover, dysregulated miR-107 expression was correlated with cell differentiation. In sum, the differential expression of miR-107, KLF4, and DAPK1 genes in CRC tumors and healthy marginal tissues might provide the potential for CRC diagnosis/prognosis and serve as therapeutic targets in CRC.

Conflict of Interest

None declared.

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