Department of Colorectal Cancer Surgery[1], Yunnan Cancer Hospital, the Third Affiliated Hospital of Kunming Medical University; Department of Oncology[2], the First People's Hospital of Yunnan Province, the Affiliated Hospital of Kunming University of Science and Technology; Department of General Surgery[3], the Second People's Hospital of Yunnan Province; Department of Hepatopancreatobiliary Surgery[4], the First People's Hospital of Yunnan Province; Department of Radiotherapy[5], the First People's Hospital of Yunnan Province, the Affiliated Hospital of Kunming University of Science and Technology, Kunming, P. R. China

MiR-761 inhibits colorectal cancer cell proliferation and invasion through targeting HDAC1

Wei Xiong[1], Shu Yang[2], Wanfu Zhang[3], Yesheng Chen[4,*], Feng Wang[5,*]

Received September 6, 2018, accepted October 13, 2018

**Corresponding authors: Yesheng Chen, Department of Hepatopancreatobiliary Surgery, The First People's Hospital of Yunnan Province, the Affiliated Hospital of Kunming University of Science and Technology, No. 157 Jinbi Road, Xishan District, Kunming 650032, P. R. China*
dr_chenyesheng@163.com
Feng Wang, Department of Radiotherapy, The First Affiliated Hospital of Kunming Medical University, No. 295 Xichang Road, Wuhua District, Kunming 650032, P. R. China
2368854900@qq.com

Pharmazie 74: 111–114 (2019) *doi: 10.1691/ph.2019.8756*

Colorectal cancer (CRC) is one of the most common cancer diagnoses. Histone deacetylase (HDAC) overactivity in CRC could promote cancer progression. HDAC1, a member of the HDAC family, is found aberrantly expressed in CRC, but it remains unclear whether the expression of HDAC1 can be regulated by microRNA. In the present study, we confirmed the overexpression status of HDAC1 in CRC tissues and cell lines, and its overexpression could promote CRC cell proliferation and invasion *in vitro*. We saw that HDAC1 was a direct target gene of miR-761 in CRC by bioinformatic and luciferase reporter analyses. HDAC1 expression could be regulated and was negatively correlated with miR-761 in CRC. We also indicated that the expression of miR-761 was abnormally downregulated in CRC. Transfection with a miR-761 mimic impeded the growth and invasion of CRC cells. In addition, we showed that ectopic expression of miR-761 mitigated HDAC1 stimulation of CRC cell proliferation and invasion. Our results demonstrate that miR-761 represents a potential strategy against CRC.

1. Introduction

Colorectal cancer (CRC) is the third-most commonly diagnosed cancer in males and the second-most commonly diagnosed in females, with an estimated number of 1.4 million new cases and 693,900 deaths occurring in 2012 (Ferlay et al. 2015). Incidence and mortality rates of CRC have been steadily declined in the past decades owing to the alteration in life style and improvement in screening methods (Edwards et al. 2010; Siegel et al. 2017). However, incidence and mortality of CRC in Asian countries are rapidly increasing (Sung et al. 2015; Chen et al. 2016).

The initiation and progression of human cancers are accompanied with abnormal epigenetic modifications (Kanwal et al. 2012). Epigenetic alterations in cancers including aberrant DNA methylation, abnormal histone modifications, and altered expression levels of various non-coding RNAs (Okugawa et al. 2015). Of note, acetylation is the most frequently studied histone modification type (Grunstein et al. 1997). The acetylation status in the chromosome is maintained by the balance of histone acetyltransferases (HATs) and histone deacetylases (HDACs) (Grunstein et al. 1997). Importantly, the use of HDAC-inhibitors, either alone or in combination with conventional chemical reagents, has demonstrated to be a promising method for the treatment of multiple cancers (Lane et al. 2009).

Currently, 18 HDAC enzymes are identified in mammalian cells (Ververis et al. 2013). Among these, HDAC1 has been found aberrantly expressed in several human cancers and associated with the survival of tumor cells (Balliu et al. 2016; Li et al. 2018; Liu et al. 2018). It was found that HDAC1 overexpression is a crucial event for drug resistance of ovarian cancer cells, highlighting the potential to use HDAC1 as an effective strategy to control chemoresistance (Liu et al. 2018). Meanwhile, apoptosis-stimulating protein of p53-2 (ASPP2) can be regulated by HDAC1 in renal cell carcinoma to regulate tumor growth and drug resistance (Li et al. 2018). In CRC, HDAC1 was found capable to modulate cancerous inhibitors of protein phosphatase 2A expression and thus inducing cell growth arrest and apoptosis (Balliu et al. 2016). Despite study results showed that HDAC1 plays an important role in tumor progression, there is limited information regarding HDAC1 regulation in the progression of CRC.

Recently, the regulation of HDAC1 expression by microRNAs (miRNAs) has been reported in the carcinogenesis of human cancers. Wu et al. (2014) showed that HDAC1 was negatively controlled by miR-34a in breast cancer to modulate therapy resistance. Zhang et al. (2017) demonstrated that HDAC1 expression could be regulated by miR-137 in human non-small cell lung cancer to modulate cell proliferation or migration (Zhang et al. 2017). Here we report that miR-761 was downregulated in CRC tissues and cell lines and might function as tumor suppressor in CRC. Notably, ectopic expression of miR-761 suppressed CRC cell proliferation and invasion through targeting the expression of HDAC1, highlighting the potential to use miR-761 as therapeutic target in CRC.

2. Investigations and results

2.1. miR-761 expression was reduced in CRC tissues and cell lines

Levels of miR-761 in CRC tissues and cell lines were analyzed by qRT-PCR. According to the analysis results, miR-761 expression was downregulated in CRC tissues compared with the adjacent noncancerous tissues (Fig. 1A). These 48 enrolled patients were

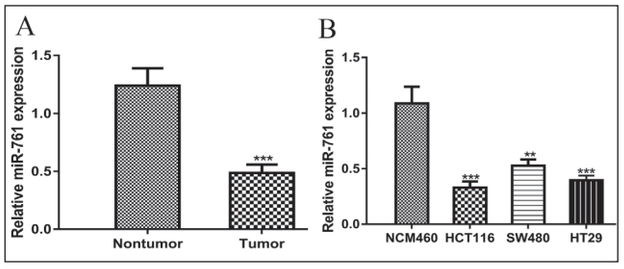

Fig. 1: miR-761 decreased in CRC tissues and cell lines. Expression of miR-761 in A, CRC tumor tissues and nontumor tissues and B, CRC cell lines (HCT116, SW480 and HT29) and normal colon cell line (NCM460) was detected by qRT-PCR. (**P<0.01, ***P<0.001) miR-761: microRNA-761; CRC: colorectal cancer; qRT-PCR: quantitative real-time PCR.

classified into two groups. We found that low miR-761 expression was correlated with tumor size (P=0.020) and tumor stage (P=0.021), but did not have any association with age and gender (P>0.05, Table). Moreover, we performed qRT-PCR to analyze miR-761 expression in CRC cell lines (HCT116, SW480 and HT29) and normal colon cell line (NCM460). We found miR-761 expression was significantly lower in CRC cell lines than in NCM460 cell line (Fig. 1B). SW480 cell line was found to have the highest miR-761 expression in the CRC cell lines investigated (Fig. 1B). Therefore, HCT116 and HT29 cell lines were used for the following functional experiments. These results indicated miR-761 may be involved in CRC progression.

Table: Correlations of miR-761 and clinicopathological features of CRC patients

Clinicopathological 1 features	No.	Low miR-761 (n=26)	High miR-761 (n=22)	P value
Age				
≥ 60	25	13	12	ns
< 60	23	13	10	
Gender				
Male	23	12	11	ns
Female	25	14	11	
Tumor size				
≥ 5	29	16	13	0.020
< 5	19	10	9	
Tumor stage				
I-II	18	11	7	0.021
III	30	15	15	

miR-761: microRNA-761; CRC: colorectal cancer; ns: not significant.

2.2. *Overexpression of miR-761 inhibits cell proliferation, colony formation, and invasion*

To understand the role of miR-761 in CRC, we transfected a set of synthesized miRNAs into the CRC cell lines. qRT-PCR results showed that miR-761 expression in CRC cell lines was reduced by miR-761 inhibitor but enhanced by miR-761 mimic compared with miR-crtl (Fig. 2A). CCK-8 assay, colony formation assay and transwell invasion assay were conducted to evaluate the roles of miR-761 in CRC cells. The observation on cell proliferation showed that cells transfected with miR-761 mimic had a low cell proliferation rate compared with those transfected with miR-crtl (Fig. 2B). Moreover, we found that a miR-761 mimic inhibits colony formation capacity compared with miR-crtl; while miR-761 inhibitor promotes the formation of colonies (Fig. 2C). In addition, the results of transwell invasion assay also demonstrated the suppressive effect of miR-761 on cell invasion (Fig. 2D). These results revealed that miR-761 could inhibit CRC cell growth, colony formation, and cell invasion.

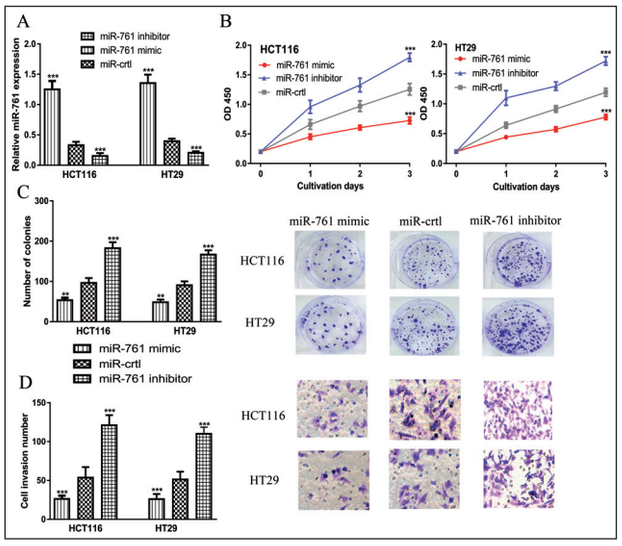

Fig. 2: miR-761 inhibits the proliferation, colony formation and invasion of CRC cells. A, qRT-PCR detection of miR-761 expression; B, CCK-8 detection cell proliferation; C, Colony formation capacity; and D, Transwell invasion assay detection cell invasion in CRC cells with miRNAs transfection. (**P<0.01, ***P<0.001) miR-761: microRNA-761; CRC: colorectal cancer; qRT-PCR: quantitative real-time PCR; miR-crtl: negative control miRNA; CCK-8: cell counting kit-8.

2.3. *miR-761 repressed HDAC1 expression*

Targetscan predicted that miR-761 binds to the 3'-UTR of HDAC1 (Fig. 3A). The luciferase activity reporter assay was conducted to validate HDAC1 as a target of miR-761, the results revealed miR-761 mimic inhibits luciferase activity of HDAC1-wt (Fig. 3B). On the contrary, miR-761 mimic exhibited no effect on the activity of the firefly luciferase with HDAC1-mut (Fig. 3B). Then, western blot showed that miR-761 mimic reduced the expression of HDAC1 in CRC cells (Fig. 3C). Moreover, it was observed HDAC1 expression in CRC tissues was higher than in the adjacent noncancerous tissues (Fig. 3D). Meanwhile, we found miR-761 and HDAC1 expression in CRC tissues was inversely correlated (Fig. 3E).

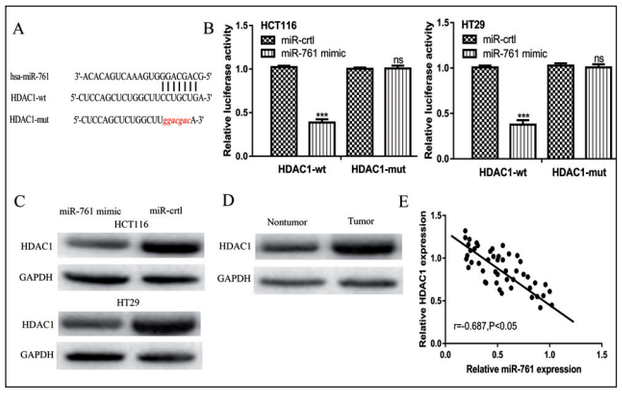

Fig. 3: HDAC1 was a direct target of miR-761 in CRC. A, The miR-761 binding site in the 3'-UTR of HDAC1. B, miR-761 targeted HDAC1-wt but not HDAC1-mut. C, Western blot analysis HDAC1 expression in CRC cells with miRNAs transfection. D, Western blot analysis HDAC1 expression in CRC tumor tissues and nontumor tissues. (E) Correlation between miR-761 and HDAC1 expression in CRC tissues. (ns not significant, ***P<0.001) miR-761: microRNA-761; CRC: colorectal cancer; miR-crtl: negative control miRNA; HDAC1: histone deacetylase 1; UTR: untranslated region; wt: wild type: mut:mutant.

2.4. *HDAC1 overexpression promotes cell proliferation, colony formation, and invasion*

We further explored the role of HDAC1 in CRC cells. To this end, CRC cells were co-transfected with HDAC1 construct and miR-761

mimic to modulate HDAC1 expression. Results showed that HDAC1 expression was increased in CRC cells transfected with HDAC1 construct, but its expression could be partially reversed by miR-761 mimic co-transfection (Fig. 4A). CCK-8 assay revealed that cell proliferation was enhanced by HDAC1 construct but was partially abolished by HDAC1 construct and miR-761 mimic co-transfection (Fig. 4B). The colony formation ability in HDAC1 construct was lower than in HDAC1 construct and miR-761 mimic co-transfection group (Fig. 4C). Moreover, re-introduction of HDAC1 antagonized the inhibitory effect of miR-761 on cell invasion (Fig. 4D). These results suggested that HDAC1 is a functional target of miR-761.

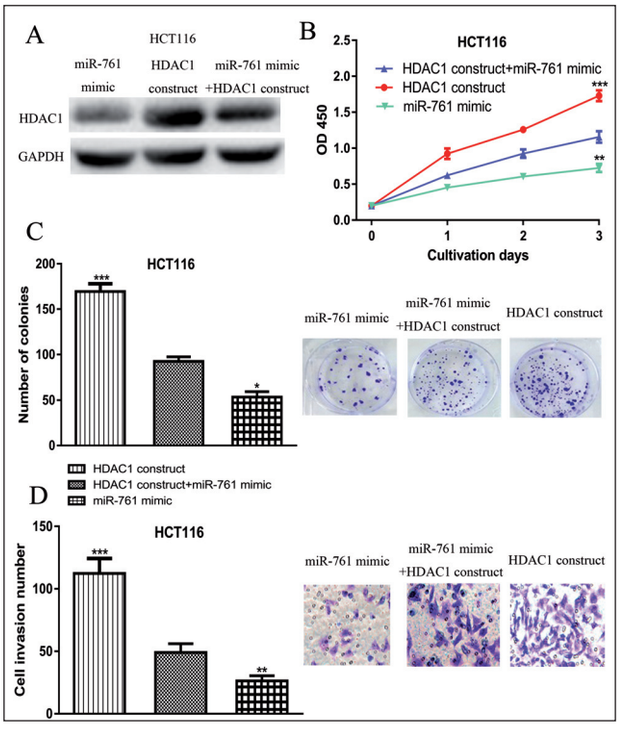

Fig. 4: HDAC1 reintroduction reverses the inhibitory effect of miR-761. A, Western blot analysis HDAC1 expression; B, CCK-8 detection cell proliferation; C, Colony formation capacity; and D, Transwell invasion assay detection cell invasion in HCT116 with miR-761 mimic and HDAC1 construct transfection. (*P<0.05, **P<0.01, ***P<0.001) miR-761: microRNA-761; CRC: colorectal cancer; HDAC1: histone deacetylase 1; CCK-8: cell counting kit-8.

3. Discussion

Tumor initiation and progression is characterized by the accumulation of numerous genetic and epigenetic alternations (Hanahan et al. 2000, 2011; Gupta et al. 2006). During the development of human cancers, it was often accompanied with a series of cancer hallmarks including the activation of cell proliferation, invasion, migration, and metastasis, induction of vascular formation, induction of genome instability and the evasion of immune surveillance, and the accumulation of these hallmarks in turn promote tumor progression (Hanahan et al. 2000; Gupta et al. 2006; Hanahan et al. 2011).

miRNAs have been reported to play crucial roles in human cancers to function as tumor suppressor or oncogene (Farazi et al. 2013; Drusco et al. 2017). Even though the role of miRNAs in cancer progression has been widely investigated, their biological roles and significance in human cancers remain unclear in detail (Farazi et al. 2013; Drusco et al. 2017). miR-761 has a controversial role in the progression of human cancers but our understanding regarding its role in tumor pathogenesis is still limited (Shi et al. 2016; Zhou et al. 2016). It has been reported that miR-761 could either promote or inhibit tumorigenesis through regulating multiple tumor-associated genes (Shi et al. 2016; Zhou et al. 2016). Here, we present a study to demonstrate the expression status and clinical significance of miR-761 expression in CRC. We showed that miR-761 expression was downregulated in CRC tissues compared with the adjacent noncancerous tissues. Moreover, miR-761 expression was found closely correlated with advanced tumor stage and large tumor size. We also showed that miR-761 could regulate CRC cell growth, colony formation, and cell invasion, which can further trigger the malignancy of CRC.

Several studies have investigated target genes of miR-761 in human cancers and several target genes regulated by this miRNA are uncovered (Shi et al. 2016; Zhou et al. 2016). To explore the mechanism by which miR-761 regulates cell behavior, miR-761 targets were predicted through TargetScan. HDAC1 was demonstrated to be a potential target of miR-761. This prediction was further validated by luciferase reporter assay and western blot assay. Functionally, we demonstrated that HDAC1 overexpression could reverse the inhibitory effects of miR-761 mimic on CRC cell proliferation, colony formation, and cell invasion.

Collectively, we found miR-761 expression was downregulated but HDAC1 expression was upregulated in CRC tissues. We also found their expression was negatively correlated in CRC tissues. Additionally, we demonstrated miR-761 overexpression could inhibit CRC cell proliferation, colony formation, and cell invasion through targeting HDAC1. The identification of miR-761/HDAC1 will help us understand the mechanism underlying CRC progression.

4. Experimental

4.1. Tissues samples from patients

Forty-eight pairs tumor and adjacent noncancerous tissues were collected from patients who underwent treatment at The First People's Hospital of Yunnan Province, the Affiliated Hospital of Kunming University of Science and Technology. All patients signed informed consent in line with the Declaration of Helsinki. All collected tissue samples were snap-frozen in liquid nitrogen and stored at -80 °C for subsequent analyses. The Ethics Committee of The First People's Hospital of Yunnan Province, the Affiliated Hospital of Kunming University of Science and Technology approved our study. None of these patients have received anti-cancer treatments.

4.2. Cell lines, cell culture, and cell transfection

Three human CRC cell lines (HCT116, SW480 and HT29) and one normal colon cell line (NCM460) were purchased from American Type Culture Collection (Manassas, VA, USA). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS, Thermo Fisher Scientific, Inc.) in a 37 °C humidified incubator containing 5 % CO_2 and 95 % air.

miR-761 mimic, miR-761 inhibitor, and negative control miRNA (miR-crtl) were synthesized by RiboBio (Guangzhou, China). A pcDNA3.1 vector containing the open reading frame (ORF) of HDAC1 was purchased from GenScript (Nanjing, Jiangsu, China). Transfection was conducted using Lipofectamine 2000 (Thermo Fisher Scientific, Inc.) according to the provided protocol.

4.3. RNA extraction and quantitative real-time PCR (qRT-PCR)

RNA was isolated from tissues and cells with Trizol reagent (Beyotime, Haimen, Jiangsu, China) according to the manufacturer's instructions. EasyScript First-Strand cDNA Synthesis SuperMix (Transgen, Beijing, China) was used to synthesize cDNA from the isolated RNA. qRT-PCR was conducted at an ABI 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using TransStart Top Green qPCR SuperMix (Transgen). Primers were synthesized by GenScript: miR-761; Forward: 5'-ACAGCAGGCACAGAC-3'; Reverse, 5′-GAGCAGGCTGGAGAA-3′; U6 snRNA; Forward, 5'-GCTTCGGCAGCACATATACTAAAAT-3'; Reverse, 5'-CGCTTCACGAATTTGCGTGTCAT-3'. Fold changes were analyzed using relative quantification method ($2^{-\Delta\Delta Ct}$).

4.4. Western blotting

Protein was extracted from tissues and cells using ProteinExt Mammalian Total Protein Extraction Kit (Transgen) and analyzed with Easy II Protein Quantitative Kit (Transgen). Same amount of protein sample was isolated with SDS-PAGE and transferred to PVDF membrane (Beyotime). After blocked with non-fat milk, the membranes were incubated with primary antibodies (anti-HDAC1: ab109411; anti-GAPDH: ab181602; Abcam, Cambridge, MA, USA). Subsequently, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies (ab205718, Abcam). Finally, the enhanced chemiluminescence kit (Beyotime) was employed to detect the protein signals in the membrane. Relative expression level was normalized to GAPDH and analyzed using Image J v1.42 software (NIH, Bethesda, MD, USA).

4.5. Cell proliferation assay

Cells were seeded at the density of 2,000 cells/well in 96-well plates. 10 µl CCK8 solution (Beyotime) was added to each well at 0, 24, 48, and 72 h after transfection, and further incubated for 2 h at the above-mentioned condition. The absorbance was measured at 450 nm with microplate reader (Thermo Fisher Scientific, Inc.).

4.6. Colony formation assay

Cells were plated in 6-well plate (1,000 cells/well) and incubated for 14 days in the above-mentioned condition. The colonies were fixed with 80% methanol and stained with crystal violet (Beyotime) for 30 s. The number of colonies were counted under microscope.

4.7. Cell invasion assay

Cell invasion was analyzed using Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) coated 8 μm transwell chambers (Corning, New York, NY, USA). 10,000 cells in FBS-free DMEM were added to the upper chamber. The lower chamber was filled with DMEM plus FBS. After incubation for 24 h, the cells on the surface of the membrane was removed by cotton-tipped swab. Invaded cells were fixed in 90% methanol and stained with 0.1% crystal violet (Beyotime). Images were observed under inverted microscope (Olympus Corp., Tokyo, Japan) to count the invasive cell numbers.

4.8. Luciferase assay

The targets of miR-761 were analyzed using TargetScan (http://www.targetscan.org/vert_72/). The wild-type (wt) and mutant (mut) 3'-UTR of HDAC1 predicted to interact with miR-761 was inserted into pmirGLO (Promega, Madison, WI, USA). Cells were co-transfected with HDAC1-wt or HDAC1-mut and miR-761 mimic or miR-crtl. Luciferase activity normalized to firefly luciferase activity was measured with dual-luciferase reporter assay system (Promega) after 48 h transfection based on the manufacturer's protocol.

4.9. Statistical analysis

Data were analyzed using SPSS version 19.0 (IBM Corp., Armonk, NY, USA) and presented as the mean ± standard deviation. Difference in two groups was analyzed by Student's t-test, while differences among three or above groups were analyzed using one-way ANOVA and Tukey post-hoc test. Chi-square test was used to analyze the correlation between miR-761 and clinicopathological parameters. P-value lower than 0.05 was considered as statistically significant.

This work was supported by Yunnan Medical Leaders Foundation (No. L-2017016) and Yunnan Applied Basic Research Foundation (No. 2017FE468(-245)).

Conflicts of interest: There is no conflict of interests.

References

Balliu M, Cellai C, Lulli M, Laurenzana A, Torre E, Vannucchi AM, Paoletti F (2016) HDAC1 controls CIP2A transcription in human colorectal cancer cells. Oncotarget 7: 25862-25871.

Chen W, Zheng R, Baade PD, Zhang S, Zeng H, Bray F, Jemal A, Yu XQ, He J (2016) Cancer statistics in China, 2015. CA Cancer J Clin 66: 115-132.

Drusco A, Croce CM (2017) MicroRNAs and Cancer: A Long Story for Short RNAs. Adv Cancer Res 135: 1-24.

Edwards BK, Ward E, Kohler BA, Eheman C, Zauber AG, Anderson RN, Jemal A, Schymura MJ, Lansdorp-Vogelaar I, Seeff LC, van Ballegooijen M, Goede SL, Ries LA (2010) Annual report to the nation on the status of cancer, 1975-2006, featuring colorectal cancer trends and impact of interventions (risk factors, screening, and treatment) to reduce future rates. Cancer116: 544-573.

Farazi TA, Hoell JI, Morozov P, Tuschl T (2013) MicroRNAs in human cancer. Adv Exp Med Biol 774: 1-20.

Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray F (2015) Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer 136: E359-86.

Grunstein M (1997) Histone acetylation in chromatin structure and transcription. Nature 389: 349-352.

Gupta GP, Massague J (2006) Cancer metastasis: building a framework. Cell 127: 679-695.

Hanahan D, Weinberg RA (2000) The hallmarks of cancer. Cell 100: 57-70.

Hanahan D, Weinberg RA (2011) Hallmarks of cancer: the next generation. Cell 144: 646-674.

Kanwal R, Gupta S (2012) Epigenetic modifications in cancer. Clin Genet 81: 303-311.

Lane AA, Chabner BA (2009) Histone deacetylase inhibitors in cancer therapy. J Clin Oncol 27: 5459-5468.

Liu X, Yu Y, Zhang J, Lu C, Wang L, Liu P, Song H (2013) HDAC1 Silencing in Ovarian Cancer Enhances the Chemotherapy Response. Cell Physiol Biochem 48: 1505-1518.

Li H, Wang X, Zhang C, Cheng Y, Yu M, Zhao K, Ge W, Cai A, Zhang Y, Han F, Hu Y (2018) HDAC1-induced epigenetic silencing of ASPP2 promotes cell motility, tumour growth and drug resistance in renal cell carcinoma. Cancer Lett 432: 121-131.

Okugawa Y, Grady WM, Goel A (2015) Epigenetic Alterations in Colorectal Cancer: Emerging Biomarkers. Gastroenterology 149: 1204-1225.

Sung JJ, Ng SC, Chan FK, Chiu HM, Kim HS, Matsuda T, Ng SS, Lau JY, Zheng S, Adler S, Reddy N, Yeoh KG, Tsoi KK, Ching JY, Kuipers EJ, Rabeneck L, Young GP, Steele RJ, Lieberman D, Goh KL; Asia Pacific Working Group (2015) An updated Asia Pacific Consensus Recommendations on colorectal cancer screening. Gut 64: 121-132.

Shi C, Zhang Z (2016) miR-761 inhibits tumor progression by targeting MSI1 in ovarian carcinoma. Tumour Biol 37: 5437-5443.

Siegel RL, Miller KD, Fedewa SA, Ahnen DJ, Meester RGS, Barzi A, Jemal A (2017) Colorectal cancer statistics, 2017. CA Cancer J Clin 67: 177-193.

Ververis K, Hiong A, Karagiannis TC, Licciardi PV (2013) Histone deacetylase inhibitors (HDACIs): multitargeted anticancer agents. Biologics 7: 47-60.

Wu MY, Fu J, Xiao X, Wu J, Wu RC (2014) MiR-34a regulates therapy resistance by targeting HDAC1 and HDAC7 in breast cancer. Cancer Lett 354: 311-319.

Zhou X, Zhang L, Zheng B, Yan Y, Zhang Y, Xie H, Zhou L, Zheng S, Wang W (2016) MicroRNA-761 is upregulated in hepatocellular carcinoma and regulates tumorigenesis by targeting Mitofusin-2. Cancer Sci 107: 424-432.

Zhang X, Zhang X, Yu B, Hu R, Hao L (2017) Oncogene LSD1 is epigenetically suppressed by miR-137 overexpression in human non-small cell lung cancer. Biochimie 137: 12-19.