Hypermethylated miR-424 in Colorectal Cancer Subsequently Upregulates VEGF

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Abstract

Purpose

Colorectal cancer (CRC) is the second leading cause of death from cancer in adults. Recent advances have shown that cancer cells can have some epigenetic changes involved in all stages of cancer. It has also been shown that miR-424 acts as gene expression regulators in many biological processes, including angiogenesis with mediators such as VEGF. In the current study, to identify the potential role of miR-424 in colorectal cancer progression, methylation status of miR-424 promoter region and its expression level have been evaluated. Besides, the correlation between VEGF level and miR-424 expression level has been assessed.

Methods

Methylation status miR-424 promoter was assessed using methylation-specific polymerase chain reaction (MSP). The expression level of miR-424 in human colorectal cancer tissue was analyzed by quantitative PCR. HCT116 cell line was selected to evaluate the correlation between the miR-424 expression level and the promoter's methylation status. VEGF expression, one out of mir-424 targets involved in angiogenesis and cancer progression, was measured by western blot analysis in the pairs of cancer tissues and their adjacent tissues.

Results

Our results have revealed that the promoter region of miR-424 is methylated in cancer cells compared to normal cells, leading to down-regulation of miR-424 in the colorectal cancer tissues compared to the normal tissues. Also, we found that the expression protein's level of VEGF in the tumor cells increased compared with normal tissues.

Conclusion

The present study suggests that hypermethylation downregulates miR-424. VEGF expression is upregulated with decreased miR-424 in colorectal cancer, which results in cancer progression.

Introduction

Colorectal cancer (CRC) in developed nations is considered the second leading cause of death in adults [1]. Despite the decreasing global incidence and mortality, CRC remains a main public health issue. Even though CRC patients' five-year survival rate at an early stage is about 90 percent, about 40 percent of the disease is diagnosed at this stage. The five-year survival rate drops to 12.5% when cancer has metastasis to distant organs [2]. Therefore, it is vital to understand CRC's molecular mechanisms to identify therapeutic approaches to improve treatment. Growing evidence has proven that miRNAs play a significant role in cancer development, differentiation, and metastasis, functioning as tumor oncogenes or suppressors [3,2,4].

One of the significant parts of non-coding RNAs is miRNAs. They are a large class of small molecules (20-22 nucleotides) regulating gene expression in several biological and physiological processes. Generally, it has been shown that impairment in the regulation of miRNA expression may affect tumor suppressor genes or oncogenes, leading to the development of several solid tumors such as lung, chest, glioma, and CRC [5-7]. Several studies have confirmed miRNA profiling's ability to identify cancer's origin, determine the prognostic features of tumors, and respond to specific and selective treatment in cancers, including CRC [8,9].

Recent advances in epigenetics have shown that cancer cells are affected by abnormal epigenetic alterations in cancer stages. These epigenetic alterations suppress gene expression through the activation or inhibition of DNA regulatory proteins. Studies have shown that some tumor suppressor genes (TSGs) in various cancers have been muted due to abnormal hypermethylation of the promoter. It has also been shown that some miRNAs, such as TSG, have an abnormal pattern of hypermetylation in their promoter, which leads to reducing the TSG activity and over-expressing the oncogenic targets [10-13]. In this regard, several studies have reported that miRNA-424 expression pattern has changed in colorectal cancer [15], and it has been shown that this miRNA inhibits the angiogenesis and the growth of the cancerous tissue by targeting the Vascular endothelial growth factor (VEGF) protein [14,15].

Over the decades, some miRNAs' vital role in cancer patients has been confirmed. Studies have also revealed the differential expression of some miRNAs in CRC patients compared with healthy individuals. This study aimed to investigate the promoter methylation status, expression level of miR-424, and its target VEGF expression in colorectal cancer subjects.

Materials And Methods

Tissue specimens

All fresh frozen colorectal cancer tissues and non-tumor tissues were collected from patients who underwent curative surgical resection at the Department of Surgery from the Imam hospital, Tehran, Iran. None of the patients received preoperative treatment such as radiation therapy or chemotherapy. Adjacent normal tissues without any tumor or necrosis were used as control samples. All samples were examined by a skilled pathologist to confirm the macroscopic and histological characteristics. After collection, samples were stored at -80°C before extraction of nucleic acids and proteins. This study was approved by the Human Research Ethics Committee of Tehran University of Medical Sciences (IR.TUMS.MEDICINE.REC.1395.997) and conducted in accordance with the Declaration of Helsinki.

Cell culture

The human colon cancer cell line (HCT116) was selected for analysis due to low expression levels of miR-424. Cells were purchased from the Pasteur Institute of Iran. All Cells were grown in Dulbecco's modified Eagle's medium (Gibco, Germany) with 10% FBS (Fetal bovine serum), 1% penicillin/streptomycin, and incubated at 37°C with 5% CO2. Cell line HCT-115 was treated with 5-aza-

2'deoxycytidine (5AZA-CdR). The epigenetic modifier 5AZA-CdR is a chemical analog of cytosine, and it is known that act as a direct inhibitor of methyltransferase activity, which decreases methylation in newly synthesized DNA.

DNA Extraction and Methylation Specific PCR Analysis

Genomic DNA was extracted from the normal and CRC tissues with the EpiTect Lyse All Lysis Kit (QIAGEN, Germany). The extracted DNA was treated with sodium bisulfite with the EpiTect Fast DNA Bisulfite Kit (QIAGEN, Germany). The quality and quantity of DNA were examined via electrophoresis on 1.5% agarose gel and quantified spectrophotometrically. The extracted DNA was stored at -20°C till DNA modification and MSP. The promoter methylation status of the miR-424 was assessed using a methylation-specific polymerase chain reaction (MSP). MethPrimer software (available from https://www.urogene.org/) was used to design the two sets of primers, as follows: methylated miR-424 forward 5 _ $GTGAGGCG \top G \top ATA \top \ \top \ \top C_3$, and reverse 5'_CCGAACTACAACCCTACTACGTA -3, $\text{and } unmethylatedmiR - 424f \text{ or } ward5$ _GTGAGGTGTTGTTATATTTTTTT_3, and reverse 5'_CCCCAAACTACAACCCTACTACATA_3. Temperature conditions of amplification consisted of initial denaturation of 95°C for 5 minutes, followed by 30 cycles (95°C; 45 seconds, 60°C; 30 seconds and 72°C; 30seconds) and a final extension (72°C; 10 minutes). The PCR products were verified on 1.5% agarose gel, containing 1X of RedSafe™ Nucleic Acid Staining Solution (iNtRON, South Korea).

RNA Extraction and Quantitative Real-time PCR

To analyze the expression of miR-424 in colorectal cancer, RT-PCR analysis was performed in 25 normal tissue and colorectal cancer tissues. Total RNA from tissues and cell lines was extracted by (RNeasy Micro Kit. Concentration and integrity of the extracted RNA were measured by Nanodrop (Thermo Fisher, USA) and 1% agarose gel electrophoresis, respectively. cDNA synthesis was carried out by 200 ng of RNA, using miScript II RT Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions, in a total volume of 20 µ L reaction mixture. Real-time PCR was performed by using the miScript SYBR® Green PCR Kit (Qiagen). Expression of U6 was used as an internal control gene. Gene expression was normalized to internal controls, and fold changes were calculated using $2^{-\Delta\Delta CT}$ method.

Western Blotting

HCT116 cells were treated with 5AZA-CdR, and all cells were harvested in RIPA Lysis buffer (0.25 M Tris/HCl, 0.75 N NaCl, 2.5% SDS, 0.1% Triton). Protein lysates were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes, according to the manufacturer's protocol. PBS containing 5% skimmed milk and 0.1% Tween-20 was used to block non-specific membrane binding. Membranes were incubated with primary rabbit monoclonal antibodies against VEGF (Vascular endothelial growth factor) and detected by chemiluminescence detection system. Beta-actin was used as a protein loading

control. Changes in VEGF and Beta-actin protein levels were quantified by scanning densitometry. ImageJ was used to determine the profiles of each lane of the gels.

Statistical Analysis

All values were expressed as means ± SEM. To analyzing the expression levels of the selected gene, ΔΔCt method was carried out. All statistical analyses were done by GraphPad Prism 7.01 (GraphPad Software, Inc., San Diego, CA, USA). Statistical analysis was performed by a 2-tailed Student's t-test and one-way analysis of variance (ANOVA). P-values less than 0.05 were considered statistically significant.

Results

Detection of Methylation in miR-424 promoter

To identify promoters CpG islands, methylation status analysis of the miR-424 promoter in CRC cells and normal cells predicted by MethPrimer (http://www.urogene.org/methprimer/index1.html) (Figure 1). The methylation status of the CpG sites was then examined by MSP (methylation-specific polymerase chain reaction). Our results have shown that methylation patterns are different between colorectal cancer tissue and normal tissue cells so that the CpG sites of miR-424 promoter region were observed to be methylated in cancer cells compared to normal cells. (Figure 2).

miR-424 is down-regulated in CRC tissues

Our data have shown that expression of miR-424 decreased in the colorectal cancer tissues compared to the normal tissues ($p<0.005$). Figure 3 have depicted the relative expression of miR-424 between adjacent cells and tumor cells.

Upregulated miR-424 expression in HCT-116 following treatment with 5-azacytidine

Concerning the identification of whether methylation regulates miR-424 in colorectal cancer, we measured mir-424 expression before and after treatment of HCT-116 with 5-azacytidine. HC116 cell lines were treated with 1 μmol/L, 5 μmol/L, and 10 μmol/L 5-aza-dC for 72 hours, and then the expression of miR-424 was measured by RT-PCR (Figure 4). After the 5-aza-dC treatment, the expression of miR-424 was significantly higher in the 5-aza-CdR-treated group compared with that in the control group ($p < 0.05$).

VEGF is upregulated in tumor tissue

Expression of VEGF and β-Actin protein was measured by western blot analysis in two pairs of cancer tissues and adjacent tissues. The results have revealed that the expression level of VEGF in the tumor cells increased compared with adjacent normal tissues (Fig 5). We also found that in the 5-aza-CdR treatment with dosage 5 μmol/L, 10 μmol/L the expression protein's VEGF level was low, whereas those in the control group were high (Fig 6).

Discussion

The present study was conducted to determine the methylation of miR-424 promoter and its effect on the expression level of this miRNA and VEGF target proteins in the HCT116 cell line and human cancer tissues in colorectal cancer patients. Our results have revealed that the promoter region of miR-424 is methylated in cancer cells compared to normal cells that lead to down-regulation of miR-424 in the colorectal cancer tissues compared to the normal tissues ($p<0.005$). Besides, we also found that the expression protein's level of VEGF (as tumor progression marker) in the tumor cells increased compared with normal tissues. It has also been demonstrated that miR-424 and VEGF expression significantly increase following 5-aza-dC treatment in the different doses ($p < 0.05$).

Numerous studies have reported that miRNA expression, which is abnormal in many tumors, seems to play a vital role in cancer. However, in these studies, the mechanism of expression modification is not fully clarified. The mechanisms that may be causative in this regard include DNA methylation and histone changes, transcription factors regulation, gene mutations, and single nucleotide polymorphisms (SNPs). The role of epigenetic changes in the etiology of human diseases has attracted a lot of attention. As one of the most important mechanisms of epigenetic changes, DNA methylation has been reported as one of the important factors in determining the change of expressed genes in the tumorigenic phenomenon[16-20]. During cancer, the expression of many tumor suppressor genes decreases with methylation of their CpG Islands.

Inconsistent with our study, Jin et al., in 2017, showed that miR-424 expression in CRC cancer tissues increased in comparison with adjacent tissues [21]. They also observed that this miR expression in cell lines A549 and H1975 was significantly reduced compared to normal cellular cells of the colorectal epithelium. Our results showed a significant reduction of miR-424 expression in cancerous tissues than in healthy tissues. In 2018, Fang and colleagues reported that miR-424 decreased expression of colorectal tissue and reduced expression in the HCT116 cell line compared to normal epithelial cells, which is consistent with our study[22]. Contrary to the results of the current study, Zhang and colleagues reported in 2017 that miR-424 contributes to the growth and invasion of lung cancer. Their investigation in cell line and animal model proposed mechanism of TNFAIP1inhibition by increasing the expression of miR-424. TNFAIP1 plays a pivotal role in reducing the proliferation, invasion, and growth of cancer cells, and reducing its expression leads to more cancers. The study of Zhang may indicate that miR-424 is present in tissues with various roles[23].

To study the effect of methylation on the expression level of miR-424, we have used 5AZA-CdR (as an inhibitor of DNA Methyltransferaz enzyme) on the HCT-116 cell line at concentrations of 1 5 and10mM. Our data have shown that by increasing the dose of 5AZA-CdR, the inhibition resulted in a decrease in the dose level of methyl-transesterified DNA enzyme and eventually resulted in a significant increase in miR-424 expression. Contrary to our study results, Pallasch et al. observed that treatment of cells isolated from patients with chronic lymphocytic leukemia by 5AZA-CdR had no effect on the expression of miR-

424 [24]. This may indicate that miR-424 expression may be regulated in other ways, including histone changes and non-coding RNA expressions.

Several studies have examined the relationship between miR-424 and DNMT1. In a study, the expression level of DNMT1 in breast cancer was increased, and its expression affected the miR-424 promoter methylation, resulting in decreased miR-424 expression [25]. In a study by Wu et al. in 2015, it was found that there was a significant and inverse correlation between mir-424 and DNMT in bladder cancer [26]. They report that expression of miR-424 increases with decreasing DNMT1 levels. It seems that the role of DNMT in the growth and invasion of cancerous tissues is essential. They also suggest that miR-424 could be considered as one of the tumor biomarkers in predicting bladder cancer.

Many studies have emphasized the role of angiogenesis and VEGF in cancer progression. Many studies reported that the tumors producing VEGF have angiogenesis and metastasis more than tumors that do not produce VEGF. These studies show that VEGF increases angiogenesis in colorectal cancer and induces the metastatic process. MiRs can perform various tasks because of their characteristics that result in the regulation after transcription of their target genes. In 2011, Jorganes reported that miR-424 can reduce the level of angiogenesis by regulating and suppressing the expression of effective genes in angiogenesis such as VEGF, VEGFR-2, and FGFR-1 [27]. At first, using bioinformatics approaches, they predicted that miR-424 plays an important role in the angiogenesis process. At the end of their study, they concluded that increased expression levels of miR-424 gene resulted in decreased reproduction and migration. For this reason, in this study, VEGF was measured as a cancer progression marker in HCT-116 cell line (before and after treatment with 5AZA-CdR), cancerous and healthy tissue. As shown in Western Blot results, a significant increase in the level of VEGF protein in the healthy tissues can indicate that in these tissues, due to reduced expression of miR-424, the VEGF mRNA's degradation is reduced, and subsequently, VEGF protein is increased. This significant increase was also observed at the cellular level, resulting in a decrease in VEGF protein level after treatment with 5 and 10 mM 5AZA-CdR.

Conclusion

Our data presented here clearly demonstrate that MiR-424 is down-regulated in colorectal cancer tissues. Promoter hypermethylation is an important mechanism involved in colorectal cancer. Consequently, it seems that CpG island hypermethylation in the promoter region of miR-424 gene is a pivotal reason for its downregulation. However, it is not the only reason for miRNA-424 regulation.

Declarations

Authors' Contributions

EK and PP: concept, ideas, and writing

ZNGH and SHSH: performed experiments and biostatistics

All authors have read and agree with this paper.

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Compliance with Ethical Standards

Conflict of interest

The authors declare that they have no conflict of interest.

Statement of Ethics

This work complies with the guidelines of the World Medical Association, Declaration of Helsinki. This work was approved by the Human Research Ethics Committee of Tehran University of Medical Sciences (IR.TUMS.MEDICINE.REC.1395.997)

Conflict of interest

The authors declared no potential conflicts of interest.

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Figures

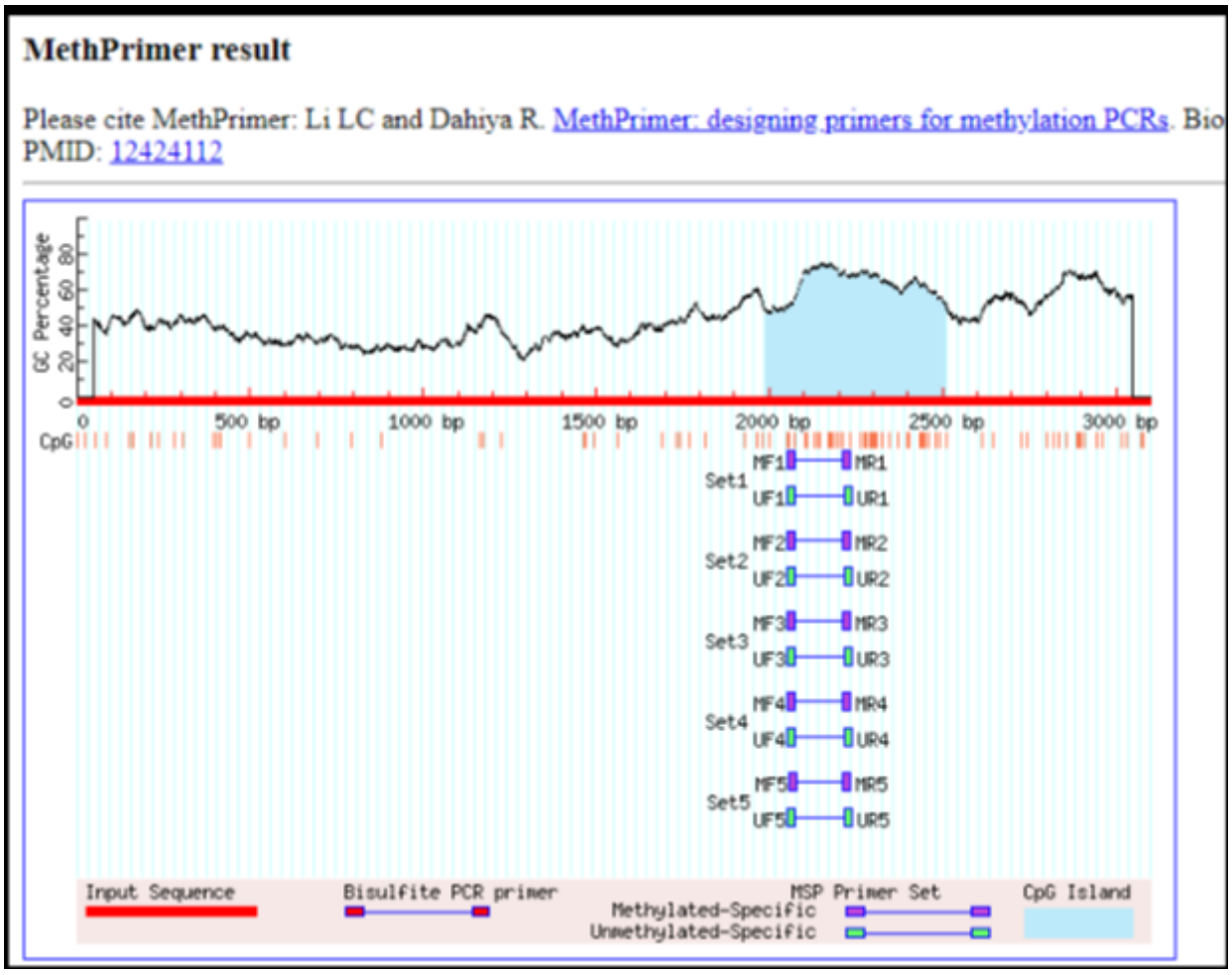

Figure 1

Prediction of methylation status of the miR-424 promoter in CRC cells and normal cells by MethPrimer

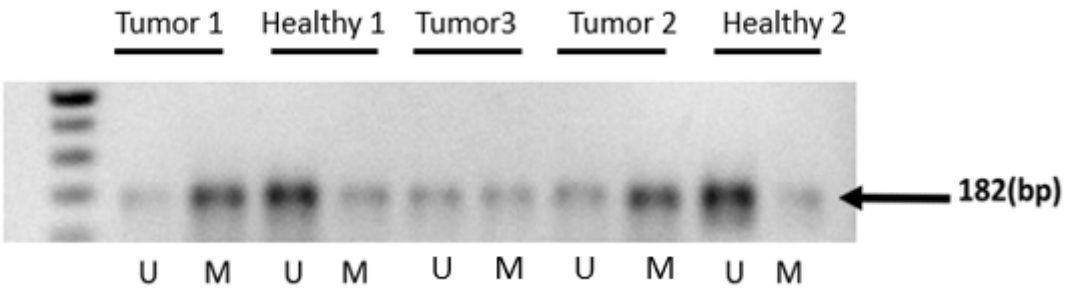

Figure 2

Increased miR-424 promoter methylation in cancer tissue. Electrophoresis Pattern of DNA methylation analysis of miR-424 using Methylation-Specific PCR (MS-PCR). Sample 1: fully unmethylated DNA [U], and 3: fully methylated DNA [M]. (The size of amplicons for the methylated and unmethylated products was 182 bp)

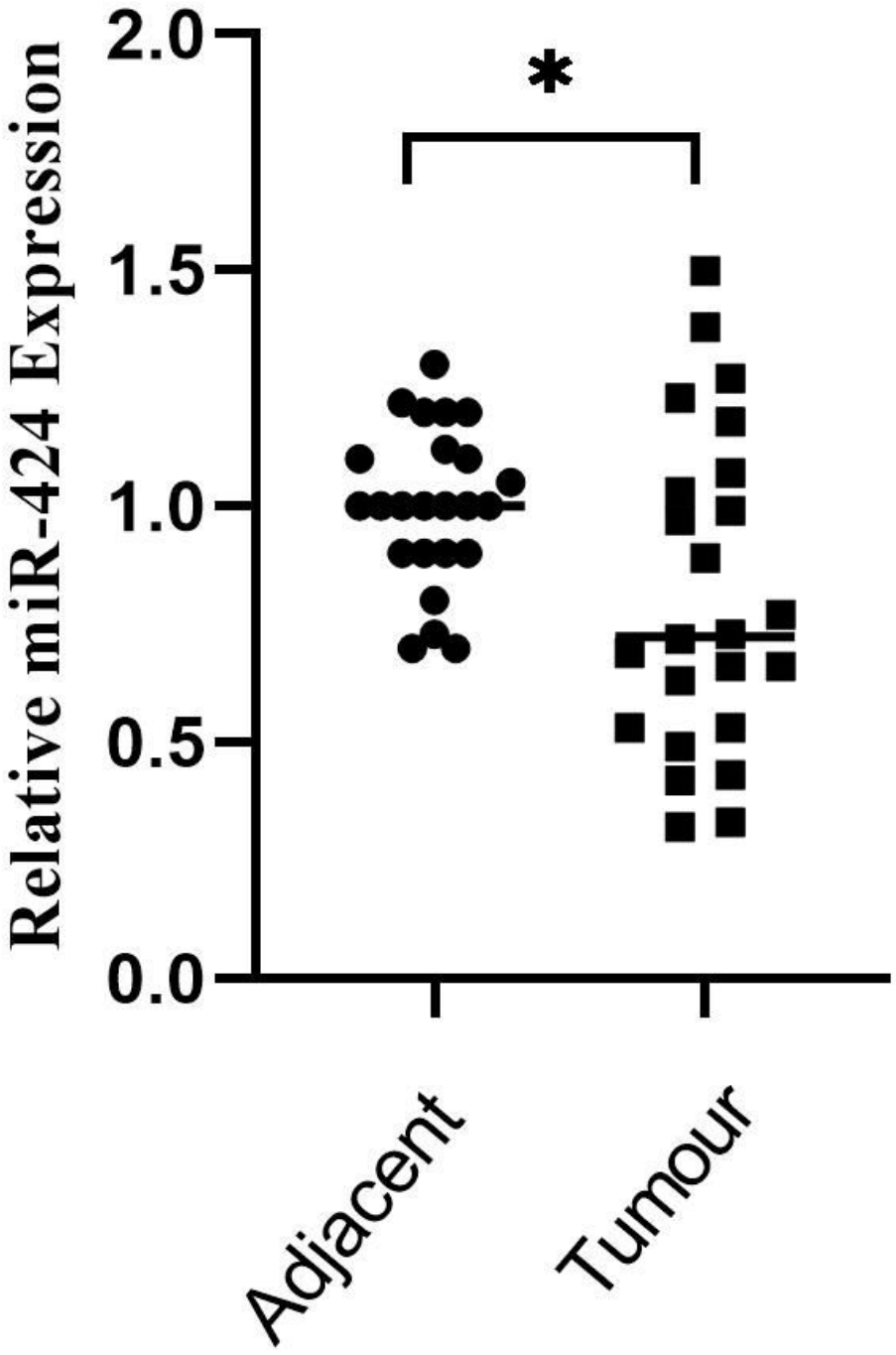

Figure 3

miR-424 downregulation in the expression analysis of miR-424 in 25 pairs of Colorectal cancer and adjacent tissues (n =25). miR-424 expression is downregulated in CRC tissues. (*=p < 0.05).

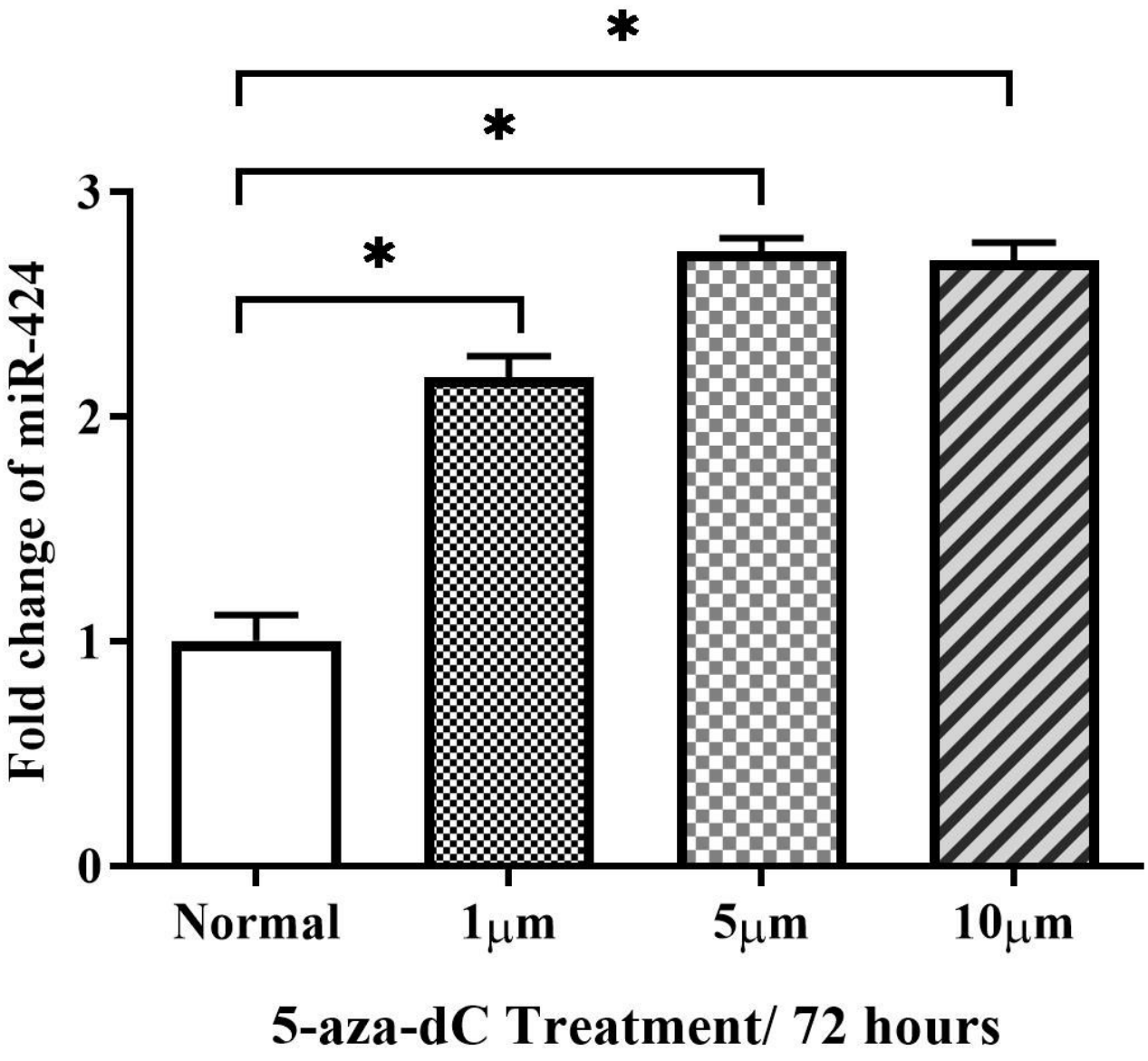

Figure 4

miR-424 upregulation expression levels in the cell lines treated with 5AZA-CdR. The levels of miR-424 expression were significantly upregulated in the 5AZA-CdR -treated cell line compared with the untreated control cell line (*=$p < 0.05$).

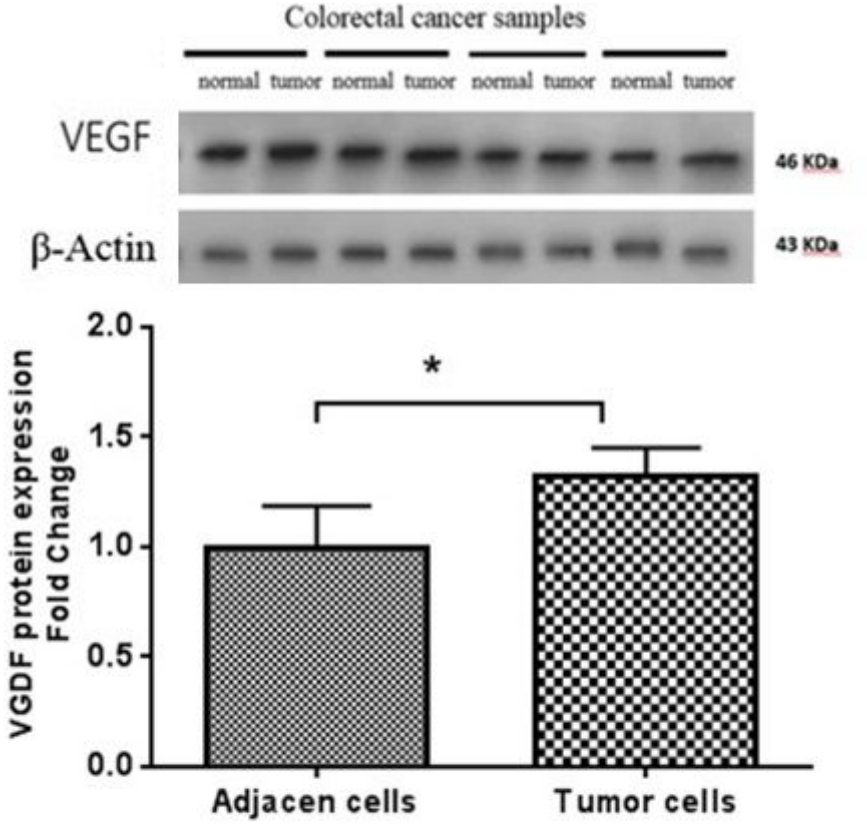

Figure 5

VEGF upregulation in cancer tissues. Expression of VEGF and β-Actin protein was measured in two pairs of cancer tissues and adjacent tissues. (*=$p < 0.05$).

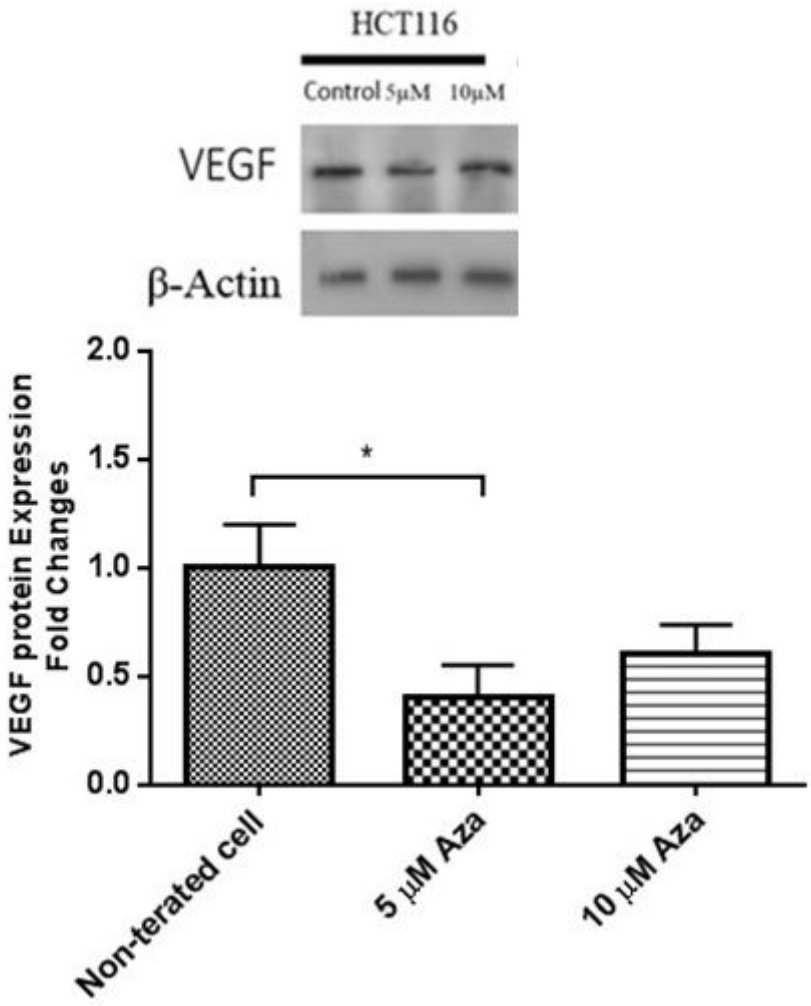

Figure 6

VEGF upregulation in HCT116 cell line following treatment with 5AZA-CdR. Expression of VEGF and β-Actin protein was measured in HCT116 cell line. (*=$p < 0.05$).