RESEARCH ARTICLE

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Investigating the Impact of Curcumin on the Methylation of *DNMT1, CDH1, SMG1*, and *WT1* Genes in the MIAPaCa2 Cell Line Using High-Resolution Melting Analysis Compared to Methylation-Specific PCR

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Abstract

Background: Curcumin, a natural compound extracted from turmeric, has shown potential in modulating epigenetic mechanisms, including DNA methylation, which is critical in gene regulation and cancer progression. This study investigates the impact of curcumin on the methylation of *DNMT1, CDH1, SMG1*, and *WT1* genes in the MIAPaCa2 pancreatic cancer cell line. **Methods:** MIAPaCa-2 pancreatic cancer cells were cultured and treated with varying concentrations of curcumin (2.5, 10, 20, 40, and 80 μM) for 24, 48, and 72 hours. DNA was extracted and subjected to bisulfite conversion to analyze methylation. The methylation status of *DNMT1*, *CDH1*, *SMG1*, and *WT1* promoters was assessed using methylation-specific PCR (MSP) and methylation-sensitive high-resolution melting (MS-HRM) analysis. MSP involved amplifying methylated and unmethylated alleles, while MS-HRM provided quantitative methylation analysis. Standard curves and controls were used to ensure accuracy and validate the results. **Results:** MSP analysis revealed that *DNMT1*, initially hemi-methylated in control cells, exhibited decreased methylation levels across all concentrations of curcumin (2.5 to 80 μM), whereas *CDH1* remained consistently unmethylated before and after treatment. MS-HRM employed a standard curve method to quantify methylation, showing that *DNMT1* methylation decreased from approximately 50% in control cells to about 20% after exposure to 80 μM curcumin. Meanwhile, *CDH1* maintained its unmethylated state throughout. The methylation status of *SMG1* was inconclusive in this study, while *WT1* initially showed 70% unmethylation, reducing after curcumin treatment. **Conclusion:** These findings underscore the differential effects of curcumin on DNA methylation patterns in pancreatic cancer-related genes, highlighting its potential as a modulator of epigenetic mechanisms in cancer therapy.

Keywords: Curcumin- *DNMT1- CDH1- SMG1- WT1* genes

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Introduction

Pancreatic cancer (PC) is one of the deadliest cancers worldwide, often referred to as the "silent murderer" due to its asymptomatic nature in early stages. It is the fourth leading cause of cancer-related deaths globally, [1, 2]. with projections indicating it will become the second leading cause by 2030. The prognosis for PC is grim, with a 5-year survival rate of less than 5%, primarily because the disease is usually diagnosed at a metastatic stage [3-5]. The prognosis for PC is grim, with a 5-year survival rate of less than 5%, primarily because the disease is usually diagnosed at a metastatic stage [6].The onset and progression of cancer are driven by genetic and epigenetic factors. Epigenetic modifications, such as DNA methylation, play a crucial role in regulating gene expression without altering the nucleotide sequence. DNA methylation, facilitated by DNA methyltransferase enzymes, [7, 8] including *DNMT1* (DNA methyltransferase 1) can lead to either the activation of proto-oncogenes through global hypomethylation or the silencing of tumor suppressor genes via hypermethylation of promoter regions [9, 10].

The *CDH1* (Cadherin-1) gene encodes *E-cadherin*, a protein vital for cell-to-cell adhesion and a negative regulator of the WNT signaling pathway [11]. Reduced expression of *E-cadherin* is associated with various malignancies, including pancreatic cancer, where it contributes to metastasis [12, 13]. *SMG1*(Serine/threonine-protein kinase) of the ATM family, regulates p53 and nonsense-mediated mRNA decay (NMD). Its

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dysfunction is implicated in tumorigenesis and is linked to advanced stages of pancreatic cancer [14]. *WT1* (Wilms' tumor 1), a Wilms tumor-related protein, is a nuclear protein associated with malignancies. The expression level of WTAP(Wilms' tumor 1-associating protein), a related protein, is closely linked to the metastatic phase of cancers. [15, 16] Curcumin, a polyphenolic compound from Curcuma longa, exhibits antiproliferative, apoptotic, anti-inflammatory, antioxidant, and antitumoral properties. It modulates various proteins and enzymes, including inflammatory cytokines and transcription factors [17]. DNA methylation serves as a promising biomarker to distinguish tumor cells from normal tissues. Techniques like MSP (methylation-specific PCR) and MS-HRM (methylation-sensitive high-resolution melting) are widely used to assess DNA methylation [18]. MSP is a sensitive, simple, and cost-effective method but lacks quantitative accuracy. MS-HRM, on the other hand, is a robust, reliable, and faster technique that offers quantitative analysis [19, 20]. This study aims to evaluate the impact of curcumin on the methylation of *DNMT1*, *CDH1*, *SMG1*, and *WT1* genes in the MIAPaCa2 pancreatic cancer cell line using MSP and MS-HRM.

Materials and Methods

Cell Culture

The MIAPaCa-2 cell line (an established human pancreatic ductal adenocarcinoma (PDAC) cell line, contains circular and spindle-shaped adherent cells, as well as, round floating cells) was obtained from the Pasteur Institute of Iran and cultured in RPMI 1640 medium (Gibco), supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% antibiotic (100 U/ml penicillin, 10 mg/ml streptomycin). Cells were maintained at 37°C in a 5% CO_2 atmosphere. The cells were plated in cell culture plates, each containing $6x10^5$ cells, for all concentrations and controls. Curcumin, dissolved in 99% ethanol, was added to prepare dilutions of 2.5, 10, 20, 40, and 80 μM. Cells were harvested and isolated from the medium after 24, 48, and 72 hours of treatment.

DNA Isolation and Bisulfite Conversion

DNA was isolated from cells treated with curcumin at 24, 48, and 72 hours using a DNA extraction kit (GeneALL, Korea). The quantity and quality of DNA were assessed using a Nanodrop spectrophotometer (260/280 ratio). DNA samples with a 260/280 ratio of 1.8 and 200 ng of genomic DNA were treated with sodium bisulfite using the EpiJET Bisulfite Conversion Kit (Thermo Scientific) according to the manufacturer's protocol. Bisulfite conversion transforms unmethylated cytosines to uracil, while methylated cytosines remain unchanged. During PCR, uracil is replaced by thymine. The methylation status of the *DNMT1* and *CDH1* promoters was analyzed by MSP and MS-HRM, while the *SMG1* and *WT1* promoters were analyzed by MS-HRM.

Analysis of DNMT1, CDH1 Promoter Methylation by MSP

Treated DNA was subjected to MSP using primers designed to amplify methylated and unmethylated alleles of the promoters (Table 1). PCR reactions were carried out in a 20 μl volume containing 10 μl Hot Start 2x Master Mix Blue (Amplicon), 0.5 μM of each primer, and 3 μl treated DNA. PCR amplification for *DNMT1* was performed with an initial denaturation at 95°C for 4 minutes, followed by 40 cycles of 95°C for 20 seconds, 50°C for 20 seconds, and 72°C for 40 seconds, with a final extension at 72°C for 7 minutes. For *CDH1*, the cycles were: initial denaturation at 95°C for 4 minutes, 40 cycles of 95°C for 17 seconds, 57°C for 20 seconds, and 72°C for 35 seconds, followed by a final extension at 72°C for 7 minutes. MSP was conducted on an ABI thermocycler (Applied Biosystems). Positive (100% methylated) and negative (0% methylated) controls were used. The results were visualized on a 1.5% agarose gel with a 100-bp ladder and stained with ethidium

Table 1. MSP Primers for the PCR Amplification of *DNMT1, CDH1, SMG1* , and *WT1* Promoter Regions

Gene	Methylation (M)/unmethylated (U) set	Primer sequence
DNMT1	M-Forward	5′-TTAGTAAATCGTGGAGTTTGGAC-3′
	M-Reverse	5′-AACGATAAACGAAAACGACG-3′
	U-Forward	5′-AGTAAATTGTGGAGTTTGGAT-3′
	U-Reverse	5′- AAAAAACAATAAACAAAAACAACATCT-3′
CDH1	M-Forward	5′-GGTGAATTTTTAGTTAATTAGCGGTAC-3′
	M-Reverse	5′- CATAACTAACCGAAAACGCCG-3′
	U-Forward	5-GGTAGGTGAATTTTTAGTTAATTAGTGGTA-3′
	U-Reverse	5′- ACCCATAACTAACCAAAAACACCA-3′
SMG1	M-Forward	5′-GTAGCGTACGTGAATTTAAGG-3′
	M-Reverse	5′-AACAAAAAATCTCCACT-3′
	U-Forward	5′-GGTGTATGTTTTAAAGGGTATGT-3′
	U-Reverse	5′-AACAAAAAATCTCCACTACTACAAC-3′
WT1	M-Forward	5′-GTTAGGCGTCGTCGAGGTTA-3′
	M-Reverse	5′-AAAACGCAAAATCCAACACC-3′
	U-Forward	5′-TGGGATTTGGGTGGTATTTG-3′
	U-Reverse	5′-CACCAACACCCACTACACCA-3′

bromide under UV transillumination.

Analysis of DNMT1, SMG1, CDH1, and WT1 Promoter Methylation by MS-HRM

MS-HRM was performed to assess the methylation status of the *DNMT1* and *CDH1* gene promoters. CpG islands in the MGMT promoter were identified using UCSC genome browser tools. Primers for both methylated and unmethylated DNA were designed using Bisearch software and the NCBI site. PCR amplification and HRM analyses were performed using the Rotor-Gene Q instrument (QIAGEN). Each PCR was performed in a 20 µl volume containing 4 µl 5x Hot FIREPol HRM Mix (No ROX), 0.5 µl of each primer, and 3 µl of bisulfite-converted DNA. PCR conditions for *DNMT1* were: 95°C for 12 minutes, followed by 47 cycles of 95°C for 19 seconds, 50°C for 23 seconds, and 72°C for 42 seconds. For *CDH1*, *SMG1* and *WT1*conditions were: 95°C for 12 minutes, followed by 48 cycles of 95°C for 18 seconds, 56°C for 23 seconds, and 72°C for 37 seconds, with continuous acquisition from 60°C to 90°C. Each reaction was performed in technical duplicates. The melting procedure was performed at 95°C for 10 seconds, 60°C for 1 minute, followed by a temperature gradient of 0.025°C per second from 60°C to 95°C [21].

Assessment of Sodium Bisulfite Conversion and MS-HRM Linearity

Real-time PCR (5x Hot FIREPol HRM Mix with ROX) and HRM technology were used to assess changes in CpG methylation status. Data were analyzed using Rotor-Gene Q software. All samples were compared with a 100% bisulfite-converted positive control (DNA EpiTect MethyLight PCR kit Qiagen) and a standard curve of serial dilutions (1:10 and 1:5 internal dilutions) to validate methylation status. Standard methylation levels of 100%, 50%, 30%, 10%, and 5% were prepared by diluting fully methylated with unmethylated DNA. Methylation levels of samples were assessed by comparing PCR product melting profiles with those of known standards. PCR amplification was performed in a total volume of 20 µl containing 4 µl 5x Hot FIREPol HRM Mix (with ROX), 0.5 µl of each primer, and 3 µl of bisulfite-converted DNA. Data were interpreted based on the guidelines of Smith et al.

Results

Methyl-specific PCR (MSP)

In this study, the methylation profiles for 4 genes were analyzed. we analyzed the methylation profiles of *DNMT1* and *CDH1* genes using MSP. The analysis was performed in triplicate at 24, 48, and 72 hours after curcumin treatment. Two primer pairs were used to amplify methylated and unmethylated alleles of *DNMT1* and *CDH1* promoters. Gel electrophoresis (Figures 1 and 2) showed that the *DNMT1* gene was hemi-methylated in control cells. Following treatment with curcumin (2.5 to 80 µM), methylation of the *DNMT1* promoter decreased across all concentrations. The *CDH1* promoter remained unmethylated throughout the experiment.

L: Ladder (100 bp)
M: Methylate
U: Unmethylated

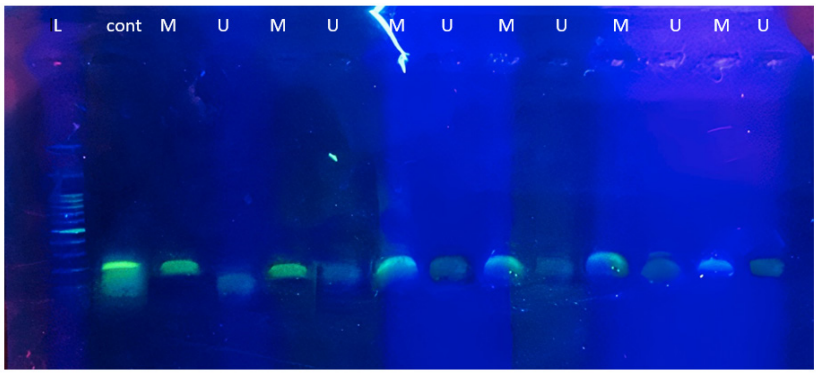

Figure 1. Gel Electrophoresis Images Showing PCR Products of Methylated and Unmethylated *DNMT1* and *CDH1* Genes before and after Curcumin Treatment

L: Ladder (100 bp)
M: Methylate
U: Unmethylated

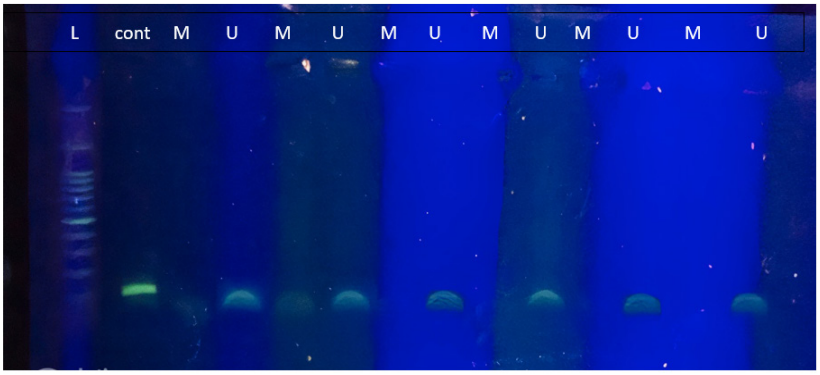

Figure 2. Gel Electrophoresis Images Showing PCR Products of Methylated and Unmethylated *DNMT1* and *CDH1* Genes before and after Curcumin Treatment

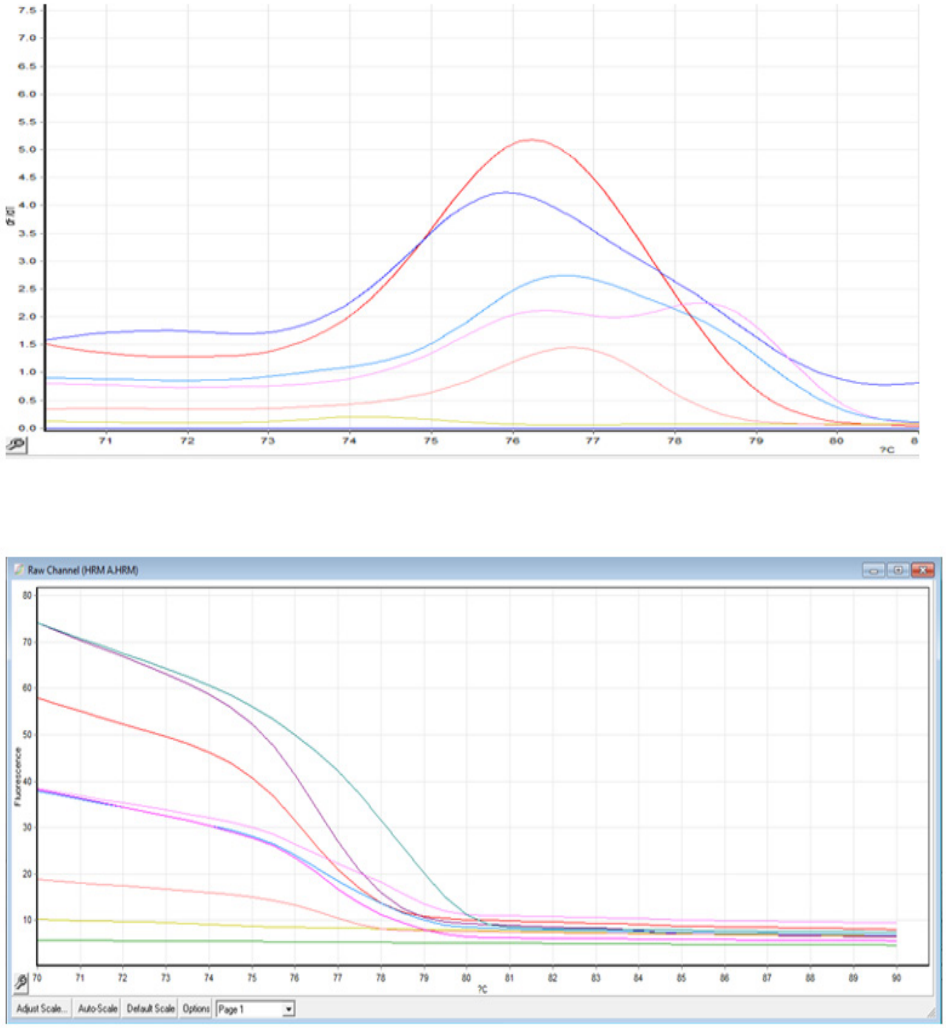

Figure 3. Standard Curve Derived from HRM Analysis, Illustrating the Relationship between Methylation Levels and HRM Curves

MS-HRM

MS-HRM analysis employed a standard curve method with known methylation standards (100%, 75%, 50%, 25%, 10%, and 0%). The standard curve demonstrated excellent linearity and fit (Figure 3). For *DNMT1*, the initial methylation rate in control cells was approximately 50%, decreasing to about 20% after treatment with 80 μM curcumin. The *CDH1* promoter showed consistent unmethylation before and after treatment. The methylation status of *SMG1* was not clearly determined in this study. However, promoter methylation of the *SMG1* gene, as well as the *CDH1* gene, was unmethylated in control cells.

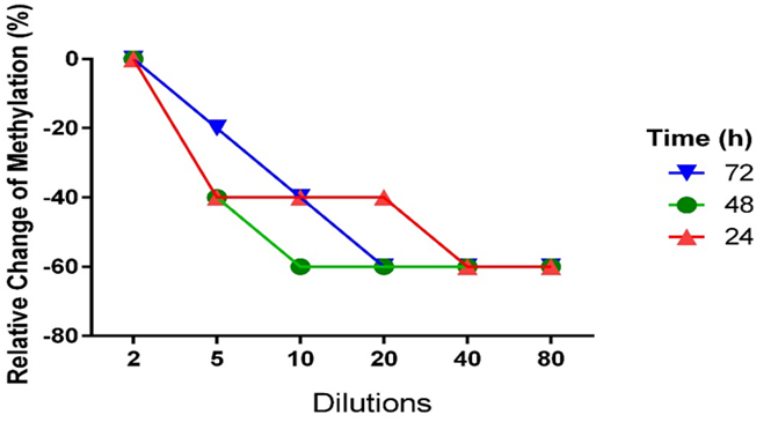

Figure 4. Relative Changes in Methylation (%) of *DNMT1, CDH1, SMG1*, and *WT1* Genes after Treatment with Curcumin

Initially unmethylated by about 70%, the *WT1* promoter showed reduced unmethylation after treatment with 80 μM curcumin. The relative change of methylation (%) is shown in Figure 4.

Discussion

Pancreatic cancer is notorious for its high mortality rates and limited treatment options[22], with Gemcitabine and Erlotinib being among the few therapies offering marginal survival benefits for advanced cases [23]. Since curcumin has antioxidant and anti-inflammatory effects and inhibits inflammatory cytokines such as TNF-α, interleukins (IL-1, IL-2, IL-6, IL-8, IL-12) and chemokines, it can be used as a cancer preventive and therapeutic compound [24]. In the present research, the methylation status of 4 genes and the effect of curcumin on pancreatic cancer cell line on 4 genes, including *DNMT1*, *CDH1*, *SMG1*, and *WT1* by two methods of MSP and HRM were studied. Epigenetic mechanisms contain improper DNA methylation, as one of the most usual molecular changes identified with the extension of human cancer [25]. Aberrant and abnormal methylation plays a role in the development of many cancers and is used as a biomarker to identify cancer [26]. In a study that looked at Alu and LINE-1 methylation and 583 volunteers from 1999 to 2012, it was found that Alu and LINE-1 methylation could be biomarkers for cancer diagnosis [27]. Respecting the methylation status of *DNMT1* genes, as one of the most important genes in methylation, results revealed about 50% methylated *DNMT1* and after treatment with curcumin, methylation rates decreased. On the other hand, in many studies, curcumin has manifested the effect of DNA hypomethylation and caused the expression of inactive gene promoters [28]. The results of this study in line with other studies demonstrated that curcumin is involved in reducing the methylation of *DNMT1*, and decreased methylation of this gene can increase the re-expression of the tumor-suppressor genes, e.g., BRCA1. In addition, the methylation and suppression of the expression of oncogene γ synuclein (SNCG) observed in many different malignant diseases, including pancreas cancer [29, 30].Concerning the methylation status of *CDH1* by MSP and HRM method, it was found that *CDH1* was unmethylated, and in the MSP method, no acceptable results were seen. MSP is a labor-intensive method that is often used in a non-quantitative way [31] and is the most widely used method for the analysis of DNA methylation in the majority of laboratories, particularly in those that are moderately equipped in developing countries [32]. However, in the HRM method, it became clear that *CDH1* is not methylated in this cell line. The percentage of a particular methylated allele is assessed by comparing profiles of methylated and unmethylated controls [33, 34]. About the two important genes in carcinogenesis, i.e., *SMG1* and *WT1* results indicated that these genes in the miapaca2 cell line are unmethylated in cancer, the somatic template of DNA methylation in cells is altered. These conversions contain increased CpG island methylation,which mediates tumor suppressor gene silencing, and genomic DNA hypomethylation, which can lead to instability of the genome [35]. The results of the present research found the activity of curcumin hypomethylation as well as activation of genes that were inactivated by methylation, including cadherins, p16, protein acidic, and rich in cysteine in PANC-1 and MIAPaCa-2 cell lines of pancreatic cancer [36]. Although the expansion and progress of the therapeutic target in cancer control and suppression has made a lot of progress, pancreatic cancer remains one of the most prevalent and lethal cancers [37].

Curcumin is anti-proliferative, angiogenesis, and restrains oxidative stress due to the induction of apoptosis [38]. Some studies observed synthetic curcumin analogs EF31 and UBS109 inhibited the growth of the pancreatic tumor by activating suppressor microRNAs and attenuating the expression of histone methyltransferase [39].The interesting thing about curcumin is that it is taken with food and as reported by some research it is safe[40]. Other studies showed that curcumin alone can be used against pancreatic adenocarcinoma, although the combination of this agent with other drugs of chemotherapy can be more efficient. Epelbaume et al. [41] reported that the curcumin and gemcitabine in patients with advanced pancreatic cancer have a therapeutic effect [42, 43]. In the future, cancer treatment goes to personalized medicine and targets the specific genes for identifying people susceptible to cancer and treatment of patients with certain biomarkers. Curcumin with its effect on methylation and suppression of metastasis can be used as an impressive compound in the treatment of all types of cancers, including pancreatic cancer [44, 45]. In the future, with the advancement of personalized medicine and the study of the genetic profile of each cancer patient and the study of individual mutations, the use of these auxiliary compounds (curcumin) along with other effective drugs can greatly contribute to the recovery process of these patients and the greater effectiveness of chemotherapy drugs [46].

In conclusion, the study demonstrated that curcumin plays a role in reducing methylation, thereby promoting hypomethylation and enhancing the expression of tumor suppressors. Given the incurable and fatal nature of pancreatic cancer, combining curcumin with chemotherapy drugs could potentially aid in treatment and help manage disease progression.

Author Contribution Statement

S.M., BP., M.A.; Conceptualization, Methodology, Software. N.K.; Data curation, Writing- Original draft preparation, and Supervision. M.A.K., A.T; Visualization, Investigation. S.M.; Software, Validation. S.M., M.A.; Writing- Reviewing and Editing. All authors read and approved the final manuscript

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Ethics approval

This project has a code of ethics : (1399.303) by Research Deputy of Qazvin University of Medical Sciences

Consent for publication

I confirm that the manuscript has been read and approved for submission by all the named authors.

Availability of data and material

All data generated or analysed during this study are included in this published article

Conflicts of interest

All authors declare that they have no conflict of interest.

Abbreviation

MSP: methyl specific PCR
HRM: high resolution melting
DNMT1: DNA methyltransferase 1
CDH1: Cadherin-1
SMG1 : Serine/threonine-protein kinase
WT1: Wilms' tumor 1
WTAP: (Wilms' tumor 1-associating protein)

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