Colorectal cancer (CRC) is a common malignancy with a high rate of morbidity and mortality. CRC represents the second or third most common cancer worldwide and is one of the five common causes of cancer in the Iranian population.\(^1\)\(^-\)\(^3\) Due to a rapidly progressive nature and delayed clinical manifestation, CRC represents treatment challenges in affected patients. Numerous risk factors, including genetic instability and epigenetic changes have been described in the pathogenesis of CRC.\(^4\)\(^,\)\(^5\) Epigenetic alterations are defined as heritable modification in the genome resulting in altered gene expression levels without causing any change in the DNA sequence. DNA methylation in CpG islands of genes is a major epigenetic change associated with gene silencing.\(^6\)

The Wnt/β-catenin signaling pathway plays a pivotal role in cell homeostasis and inappropriate activation of this signaling pathway has been implicated in the pathogenesis of many cancers.\(^7\) Wnt inhibitory factor-1 (WIF-1), a member of secreted mediators of Wnt/β-catenin signaling pathway, acts as a direct suppressor and antagonist of this signaling pathway.\(^8\)\(^,\)\(^9\) Downregulation of WIF-1 gene by aberrant DNA methylation is a hallmark of various cancers and confers a more aggressive phenotype of the disease.\(^4\) Gene methylation is mediated by DNA methyltransferase enzymes and is dependent on 5-methyltetrahydrofolate (5mTHF). 5mTHF acts as a one-carbon donor cofactor in various
biological processes such as DNA methylation. Methylene tetrahydrofolate reductase (MTHFR) is a key regulator of 5mTHF biosynthesis and reduced activity of MTHFR enzyme strongly limits the bioavailability of 5mTHF, which may affect DNA methylation. The C677T polymorphism of the MTHFR gene was reported to be associated with 70% and 35% diminished bioactivity of MTHFR enzyme in mutant homozygous and heterozygous states, respectively, which can restrict the pool of 5mTHF in cells. Our study sought to investigate the methylation status of WIF-1 gene and its interaction with MTHFR C677T polymorphism in a group of CRC patients and a control group.

METHODS
The studied samples consisted of 50 formalin-fixed paraffin-embedded (FFPE) cancerous tissue and adjacent healthy non-affected tissues obtained from patients with CRC while conducting curative surgery between September 2015 and September 2017 in Zanjan, Iran. The sample size was calculated by OpenEpi version 2.2 software (Informer Technologies, Inc. Atlanta, USA) (free online statistical software available at: www.openepi.com). The calculated sample size based on the previously reported frequency of WIF-1 methylation was 50. The clinical features of patients with CRC including age, sex, and pathological characteristics such as tumor location, tumor size, tumor grade and stage, lymph node metastasis, and the histological type was collected using medical records. Patients were excluded from the study if they received chemotherapy or radiotherapy before surgery. The study was approved by the ethical committee of Zanjan University Medical Sciences (Ethical code: ZUMS.REC.1394.337), Zanjan, Iran.

Genomic DNA was extracted from a 5–10 µm section of FFPE tissues using an FFPE DNA extraction kit (Qiagen, Germany). The concentration and purity of extracted DNA were determined by nanodrop spectrophotometer and subsequently stored at -80 ºC. Approximately 1–2 µg of isolated DNA was treated by sodium bisulfite to convert unmethylated cytosine to uracil by an epiTect Fast DNA Bisulfite kit (Qiagen, Germany), according to the instruction of kit. Methylation-specific PCR (MSP) using two sets of specific primer for amplification of methylated and unmethylated state of the gene was used for methylation analysis, as previously described with some modification. The MSP condition included 10 µL of 2 × hot start master mix (Qiagen, Germany), 1 µL (0.5 µM) of each forward and reverse primer, 100 ng of bisulfite modified DNA, and appropriate volume of PCR grade water to a final volume of 20 µL. Methylated and unmethylated controls (EpiTect PCR Control DNA Set, Qiagen, Germany) were used in each PCR reaction. Following MSP, the amplified products were visualized on 2.5% agarose gel under UV light. The amplicon size for both the methylated and unmethylated PCR products was 199 bp.

The C677T polymorphism of the MTHFR gene was analyzed using the PCR-restriction fragment length polymorphism (PCR-RFLP) technique. The polymorphic site was amplified using specific primer, as previously described. Then, 7 µL of amplified PCR product in conjunction with 5 u Hinfi (Fermentas, Germany) restriction enzyme, 2 µL buffer, and 10.5 µL of PCR grade water was incubated at 37 ºC overnight. Then, the digested products were electrophoresed on a 3% agarose gel. The mutant allele produces 175 bp and 23 bp bands, while the wild allele appears as an undigested 198 bp band.

Categorical variables were compared using the chi-square test or Fisher’s exact test, as appropriate. Numerical data were expressed as mean±standard deviation and were analyzed by Student’s t-test. GraphPad Prism 8 was used for statistical analysis. A p-value of < 0.050 was considered significant.

RESULTS
The mean age of CRC patients with methylated and unmethylated WIF-1 gene in the cancerous tissue was 60.4±10.6 years and 58.3±12.6 years, respectively (p= 0.535). The size of tumor varied

| WIF-1 methylation status | Cancerous tissue, n = 50 n (%) | Healthy tissue, n = 50 n (%) |
|-------------------------|-------------------------------|-------------------------------|
| UU                      | 22 (44.0)                     | 46 (92.0)                     |
| UM                      | 2 (4.0)                       | 0 (0.0)                       |
| MM                      | 26 (52.0)                     | 4 (8.0)                       |

p < 0.001; χ² = 260.60
WIF-1: Wnt inhibitory factor-1; U: unmethylated; M: methylated.
between 2.5 and 12 cm. Twenty-six patients (52.0%) were male while 24 (48.0%) were female. Stage III–IV were seen in 33 (66.0%) of CRC patients, while stage I–II was present in 17 (34.0%) of CRC patients. Regarding the histological type of tumor, 80.0% had the non-mucinous type, while the remaining 20.0% were the mucinous type. Left-sided tumors were seen in 27 (54.0%) patients and right-sided tumors were seen in 23 (46.0%) patients. The promoter DNA methylation of WIF-1 gene seen in cancerous tissue (52.0%) was significantly higher than that of healthy adjacent tissue (8.0%).

### Table 2: Relationship between clinicopathological characteristics of CRC patients and the Wnt inhibitory factor-1 (WIF-1) methylation status in the cancerous tissue.

| Clinicopathological features | Number (n = 50) | WIF-1 methylation status in the cancerous tissue | p-value* |
|-----------------------------|-----------------|-----------------------------------------------|---------|
|                             | Methylated n = 28 | Unmethylated n = 22                          |         |
| Age, mean ± SD, years       | 60.4 ± 10.6      | 58.3 ± 12.6                                  | 0.535** |
| Gender                      |                 |                                               |         |
| Female/Male                 | 24/26           |                                               | 0.166   |
| Tumor size, cm              |                 |                                               |         |
| ≤ 5                         | 23              | 15                                            | 0.264   |
| > 5                         | 27              | 13                                            |         |
| Grade                       |                 |                                               |         |
| I                           | 8               | 5                                             | 0.107***|
| II                          | 34              | 16                                            |         |
| III                         | 8               | 7                                             |         |
| Tumor location              |                 |                                               |         |
| Left-sided                  | 27              | 20                                            | 0.009   |
| Right-sided                 | 23              | 8                                             |         |
| Stage                       |                 |                                               |         |
| I–II                        | 17              | 10                                            | 0.999   |
| III–IV                      | 33              | 18                                            |         |
| Histological type           |                 |                                               | 0.479   |
| Mucinous                    | 10              | 7                                             |         |
| Non-mucinous                | 40              | 21                                            |         |
| Lymph node metastasis       |                 |                                               | 0.153   |
| Positive                    | 25              | 11                                            |         |
| Negative                    | 25              | 17                                            |         |

*determined by Fisher’s exact test; **determined by Student’s t-test; ***determined by chi-square test.

between 2.5 and 12 cm. Twenty-six patients (52.0%) were male while 24 (48.0%) were female. Stage III–IV were seen in 33 (66.0%) of CRC patients, while stage I–II was present in 17 (34.0%) of CRC patients. Regarding the histological type of tumor, 80.0% had the non-mucinous type, while the remaining 20.0% were the mucinous type. Left-sided tumors were seen in 27 (54.0%) patients and right-sided tumors were seen in 23 (46.0%) patients. The promoter DNA methylation of WIF-1 gene seen in cancerous tissue (52.0%) was significantly higher than that of healthy adjacent tissue (8.0%).

### Table 3: The association between MTHFR C677T polymorphism with the methylation status of the WIF-1 gene in patients with CRC.

| MTHFR C677T polymorphism | Methylated, n = 28 n (%) | Unmethylated, n = 22 n(%) | p-value* | OR (95% CI) |
|--------------------------|--------------------------|---------------------------|----------|-------------|
| CC                       | 22 (78.6)                | 6 (27.3)                  | -        | Ref         |
| CT                       | 5 (17.9)                 | 13 (59.1)                 | < 0.001  | 9.53 (2.56–40.96) |
| TT                       | 1 (0.6)                  | 3 (13.6)                  | 0.025    | 11 (1.30–14.74) |
| CT+TT vs. CC*            | 6 (21.4)                 | 16 (72.7)                 | < 0.001  | 9.77 (2.53–34.03) |
| TT vs. CC+ CT**          | 1 (0.6)                  | 3 (13.6)                  | 0.307    | 4.26 (0.58–57.08) |
| C allele                 | 49 (87.5)                | 25 (56.8)                 | -        | Ref         |
| T allele                 | 7 (12.5)                 | 19 (43.2)                 | 0.001    | 5.32 (1.89–13.24) |

*dominant genetic model; **recessive genetic model.

WIF-1: Wnt inhibitory factor-1; CRC: colorectal cancer; OR: odds ratio; CI: confidence interval.
This polymorphism was shown to be associated with cancer susceptibility in numerous studies. Zhao et al., reported that the carrier of MTHFR C677T allele was associated with a significantly reduced risk of CRC, suggesting a protective role for minor T allele against CRC risk. Our study revealed that in carriers of the MTHFR TT and CT genotypes the frequency of unmethylated WIF-1 gene was significantly higher than in carriers of the CC genotypes. The carriage of the MTHFR TT and CT genotypes may restrict the availability of 5mTHF for methylation reaction resulting in a hypomethylated WIF-1 gene and may overwhelm the tight regulation of cells leading to cancer. Also, the interaction between genetic and epigenetic alterations may affect the development of cancer. We saw a high frequency of WIF-1 gene methylation in cancerous (52.0%) tissue compared to non-cancerous (8.0%) tissue, suggesting a role for WIF-1 gene methylation in CRC development. This epigenetic change may be used as an early diagnostic biomarker for CRC patients. The reported frequency of WIF-1 gene methylation in our study (52.0%) was higher than that of a study by Samaei et al., and was lower than that of studies by Abdelmaksoud-Dammak et al., (87.95%), Ni et al., (74%) and Patai et al., (82%). Generally, a higher WIF-1 methylation frequency was reported in tissue-based methylation assays than serum- or stool-based assays, which may explain the heterogeneity of reported results. In agreement with our result, a study by Deng et al., indicated a correlation between DNA methylation of some tumor-specific genes and tumor location. Moreover, investigating the correlation between WIF-1 gene methylation and clinicopathological features of CRC patients identified no significant association between age, gender, tumor grade, tumor stage, lymph node metastasis, and tumor histological type (p > 0.05), suggesting that WIF-1 gene silencing by DNA methylation is an early event in the evolution of CRC. This finding proposes WIF-1 DNA methylation as an early diagnostic marker not associated with the severity and progression of CRC. Similarly, Taniguchi et al., reported no significant association between clinical and pathological features of CRC patients and WIF-1 gene silencing by DNA methylation.

MTHFR is involved in the production of 5mTHF, an essential precursor for methylation reactions. The C677T polymorphism of MTHFR gene reduces the enzymatic activity of MTHFR by 70% and 35% in homozygous and heterozygous state, respectively. This polymorphism was shown to be associated with cancer susceptibility in numerous studies. Zhao et al., reported that the carrier of MTHFR C677T allele was associated with a significantly reduced risk of CRC, suggesting a protective role for minor T allele against CRC risk. Our study revealed that in carriers of the MTHFR TT and CT genotypes the frequency of unmethylated WIF-1 gene was significantly higher than in carriers of the CC genotypes. The carriage of the MTHFR TT and CT genotypes may restrict the availability of 5mTHF for methylation reaction resulting in a hypomethylated WIF-1 gene and may overwhel...
Our study had some limitations including that we did not evaluate the gene and protein expression of WIF-1, our study population was small, and was conducted retrospectively.

CONCLUSION

Our study demonstrated promoted hyper-methylation of the WIF-1 gene as a significant risk factor for development. Also, MTHFR C677T polymorphism was associated with a hypomethylated state of WIF-1 gene in carriers of TT and CT genotypes that may explain the protective role of this common polymorphism against CRC development.

Disclosure

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