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ORIGINAL RESEARCH

Role of hMLH1 and E-Cadherin Promoter Methylation in Gastric Cancer Progression

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Abstract
Introduction Gastric cancer (GC) is one of the leading causes of cancer-related death in Iran. Genome stability is one of the main genetic issues in cancer biology which is governed via the different repair systems such as DNA mismatch repair (MMR). A clear correlation between MMR defects and tumor progression has been shown. Beside the genetic mutations, epigenetic changes also have a noticeable role in MMR defects.
Methods Here, we assessed promoter methylation status and the level of hMLH1mRNA expression as the main component of MMR system in 51 GC patients using the methylation-specific PCR and real-time PCR, respectively. Moreover, we performed a promoter methylation study of the E-cadherin gene promoter.
Results It was observed that, 12 out of 39 cases (23.5 %) had hMLH1 overexpression. Hypermethylation of hMLH1 and E-cadherin promoter regions were observed in 25.5 and 36.4 %, respectively. Although, there was no significant correlation between hMLH1 mRNA expression and clinicopathological features, there are significant correlations between E-cadherin promoter methylation and tumor stage ($p=0.028$) and location ($p=0.025$). The rate of hMLH1 promoter methylation in this study was lower than that in the other population, showing the importance of the other mechanisms, in gastric tumorigenesis.
Conclusion The results of this study indicate that DNA repair system is adversely affected by hypermethylation of hMLH1 in a fraction of gastric cancer patients. Additionally, E-cadherin hypermethylation seen in a subset of our gastric cancer patients is consistent with other reports showing correlation with aggressiveness and metastasis of gastric cancer.

Keywords Epigenetic · Mismatch Repair System · Expressional Analysis · Methylation-Specific PCR · Iran

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Introduction

Gastric cancer (GC) is one of the highest rated cancer types and the leading cause of cancer-related deaths in Iran with 10,000 new cases and 8,000 deaths annually [1]. Although it has been observed that GC incidence has a constant decline during the last several decades, it is still one of the most common cancers worldwide [2]. Despite new therapeutic advances, GC has a poor prognosis with a low rate of 5-year survival [3]. Cancer, as a complex disease which has a high rate of mortality worldwide, is affected with different factors such as genetics and environment [4]. Genome stability is one of the main genetic issues in cancer biology, which is governed via the different repair systems such as DNA mismatch repair (MMR), and it has been shown that

there is a clear relationship between MMR defect and tumor progression [5, 6]. Microsatellite instability, as a well-known mechanism in gastric and colorectal tumorigenesis, is directly related to the function of mismatch repair genes such as hMLH1 and hMSH2 [7–9]. hMLH1, as one of the main components of the MMR system, is responsible for the replacement of the mispaired nucleotides in the genome during the replication. Therefore, it is clear that every aberration in the hMLH1 function may lead to increased genetic instability in different cancer-related genes, specifically those that are involved in cell proliferation and death [10].

Beside the mutations in hMLH1, promoter hypermethylation is another important mechanism which can suppress the MMR components, P16 and E-cadherin, transcriptionally [11–13]. Epigenetic changes include a variety of chromatin modifications which are involved in different processes such as development and tumorigenesis. Therefore, to elucidate the role of hMLH1 promoter methylation in the GC progression, we assessed its methylation status and mRNA expression. Beside the hMLH1 as one of the MMR components, E-cadherin also has an important role in GC progression. E-cadherin is an epithelial transmembrane glycoprotein, involved in cell adhesion which is directly related to tumor metastasis [14]. Considering the lack of a good prognostic marker in GC, usually, it is diagnosed in the advanced stages of tumor progression, which has a clearly poor prognosis [15]. Therefore, it is essential to identify the new prognostic and diagnostic markers for GC. Although it has been observed that the gastric cancer arises through the genetic and epigenetic changes which lead to malfunction of cancer-related genes, some other reports show that genetic mutations are not frequent in gastric tumor progression [16, 17]. On the other hand, the epigenetic aberrations such as DNA methylations, specifically in the promoter regions, play a significant role in cancer [18].

Despite the high incidence of GC in Iran, there are not enough reports to clear the significant correlation between epigenetic alterations and GC in this area. In the present study, and in line with our recent study [11], the promoter methylation changes were assessed in two cancer-related genes (E-cadherin and hMLH1) through the methylation-specific PCR (MSP) in GC patients, which are well-known factors in gastric tumorigenesis. Furthermore, the mRNA expression study was performed to approve the role of promoter methylation in the transcriptional repression of mentioned genes. Finally, the correlations between methylation/expressional analyses and clinicopathological features were assessed.

Materials and Methods

Tissue Samples

Fifty one freshly microdissected normal and tumoral gastric samples were gathered via gastrectomy from the patients in Emam Reza and Omid Hospitals of Mashhad University of Medical Sciences. The samples were kept in RNAlater solution (QIAGEN, Hilden, Germany) in −20 °C prior to RNA extraction. There were two excluding criteria in the case of tissue samples: all the patients should have no chemo–radio therapeutic treatment prior to the surgery and the tumoric samples were histologically examined by a pathologist to ensure that they contain at least 70 % tumor cells.

RNA Extraction, cDNA Synthesis, and Quantitative RT-PCR

RNA extraction and cDNA synthesis were performed as described before [19]. Expressional analysis of hMLH1 was done in triplicate reactions through a comparative threshold cycle/SYBR Green method (GENET BIO, Korea) in a real-time thermal cycler (Stratagene Mx3000P, La Jolla, CA) using the primer sequences represented in Table 1. The thermal program included an initial step of 95 °C for 10 min followed by 95 °C (15 s), 52 °C (30 s), and 72 °C (30 s) for 40 cycles. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as normalizer [20]. All of the cases with an increase of more and less than 2-fold in the level of mRNA expression in the tumor samples in comparison with the normal tissues were defined as the overexpressed and underexpressed cases, respectively. The tumors with the level of mRNA expression between −2 and +2-folds were considered as normal.

DNA Modification (Bisulfite Treatment) and Methylation-Specific PCR

Sodium bisulfite modifies the unmethylated cytosine bases to uracil, without any change in methylated cytosine. Therefore, such discrimination between methylated and unmethylated alleles, prepares the opportunity to design allele (methylation)-specific PCR primers. DNA modification was performed using the EpiTect Bisulfite Kit (QIAGEN, Hilden, Germany). DNA samples were denatured via the NaOH prior to the bisulfite modification followed by phenol/chloroform purification according to the manufacturer's protocol. Specific MSP primer sets for hMLH1 and E-cadherin are represented in Table 2. The PCR reactions were performed as described before [11]. The thermal profile of MSP–PCR comprised of an initial denaturation in 95 °C for 10 min followed by 40 cycles of 94 °C for 45 s, 61 °C for 25 s, and 72 °C for 1 min. EpiTect PCR control DNA (QIAGEN, Hilden, Germany) was used as a positive control for the unmethylated and methylated DNA.

Statistical Analysis

All data were statistically analyzed using the SPSS 16.0 software (SPSS, Chicago, IL). The correlational studies between different variables were assessed by Pearson's χ^2 test and Fisher's exact tests. Moreover, the ANOVA and *t* test were

Table 1 Real-time PCR primer sequences

	Forward	Reverse
hMLH1	5-AACTGCAGTCCTTTGAGGAT-3	5-CCATCAGCTGTTTTCGTTGT-3
GAPDH	5-GGAAGGTGAAGGTCGGAGTCA-3	5-GTCATTGATGGCAACAATATCCACT-3

used to find the significant correlations between levels of mRNA expression and different clinicopathological features based on their type. All the statistical tests were defined significantly as a p value of <0.05.

Results

Study Population

Fifty one patients with GC including 16 (31.4 %) females and 35 (68.6 %) males were enrolled in this study. The mean age was 63.7±10.5 years ranged from 30 to 85 years. The mean tumor size was 6.2±2.8 cm with a range of 1 to 14 cm. Most of the tumors were located in the cardia 24 (47 %), while the others were located in the body and antrum with 16 (31.4 %) and 11 (21.6 %), respectively. Most of the cases were in stages II/III (78.4 %). Thirty-two (62.7 %) tumor samples had showed moderate differentiation. In the case of tumor depth of invasion, despite of 5 (9.8 %) cases, all tumors were in T2/3 and most of the tumors had lymph node metastasis 43 (84.3 %). Clinicopathological features of the patients are showed in Table 3.

hMLH1 Expression in Gastric Cancer Patients

The level of hMLH1 mRNA expression was assessed via the comparative real-time PCR in 51 gastric cancer patients, in which, hMLH1 expression in tumor samples was compared with the paired normal specimens. It was observed that 12 out of 39 cases (23.5 %) had hMLH1 overexpression. The mean fold changes were ranged totally between −4.3 and 5.63 (Mean ± SD, 0.22±1.99), while in normal/underexpressed and overexpressed samples were (Mean ± SD, 2.96±1.09) and (Mean ± SD, −0.62±1.32), respectively. Fold changes of all patients are depicted as a scatter plot in Fig. 1. Moreover, comparison between the levels of hMLH1 mRNA expression in normal/underexpressed and overexpressed groups is showed as box plot in Fig. 2.

Clinicopathological Features and hMLH1 mRNA Expression

Correlations between clinicopathological features and the level of mRNA expression were studied to evaluate the probable involvement of hMLH1 in gastric tumor progression and metastasis. Although, there was no significant correlation between clinicopathological features and hMLH1 overexpression, 8 out of 12 (66.7 %) overexpressed cases were moderately differentiated. Most of the overexpressed tumors were located in cardia and they were in T2 tumor depth of invasion (6 from 12 cases, 50 %). In the case of tumor types, 9 out of 12 cases (75 %) were intestinal type (p =0.631). The majority of overexpressed cases had lymph node metastasis (11 of 12 samples, 91.7 %), indicating a noticeable role of this factor in tumor metastasis. Mean age in underexpressed and overexpressed cases were 64.87± 1.04 and 59.75±1.05 years, respectively, which shows that the overexpressed tumors were meaningfully observed in younger patients. Mean size of normal/underexpressed and overexpressed tumors were 6.15±2.81 and 6.16±2.69 cm, respectively, showing that there is no difference between the tumor sizes regarding the levels of hMLH1 mRNA expression. Generally, the number of males was higher than females in this study and most of the overexpressed cases (9 out of 12 patients, 75 %) were observed in males.

Promoter Methylation Status

We assessed the promoter methylation status of hMLH1 and E-cadherin in gastric cancer patients (Table. 3 and 4). Thirteen out of 51 (25.5 %) cases showed hypermethylation in hMLH1 promoter sequence. From 33 cases with methylation status assessment of E-cadherin promoter, only 12 (36.4 %) cases were positive. As we expected, there was a significant correlation between hypermethylation in hMLH1 promoter and the level

Table 2 MSP primer sequences

	Forward	Reverse
hMLH1 (M)	ACGTAGACGTTTTATTAGGGTCGC	CCTCATCGTAACTACCCGCG
hMLH1 (U)	TTTTGATGTAGATGTTTTATTAGGGTTGT	ACCACCTCATCATAACTACCCACA
E-cadherin (M)	TTAGGTTAGAGGGTTATCGCGT	TAACTAAAAATTCACCTACCGACC
E-cadherin (U)	TAATTTTAGGTTAGAGGGTTATTGT	CACAACCAATCAACAACACA

Table 3 Clinicopathological features and hMLH1 expression and methylation

	Total	hMLH1 Overexpression	hMLH1 Promoter methylation	
			+	–
Patients	51	12	13	38
Mean age (mean ± SD)	63.7±10.5	59.8±1.1	62.8±1.4	63.4±9.5
Size (mean ± SD)	6.2±2.8	6.2±2.7	6.8±3.6	5.9±2.4
Sex				
Male	35 (68.6 %)	9 (75 %)	10 (76.9 %)	25 (65.8 %)
Female	16 (31.4 %)	3 (25 %)	3 (23.1 %)	13 (34.2 %)
Location				
Body	16 (31.4 %)	4 (33.3 %)	8 (61.5 %)	8 (21 %)
Cardia	24 (47 %)	6 (50 %)	3 (23.1 %)	21 (55.3 %)
Antrum	11 (21.6 %)	2 (16.7 %)	2 (15.4 %)	9 (23.7 %)
Grade				
P.D.	7 (13.7 %)	1 (8.3 %)	2 (15.4 %)	5 (13.2 %)
M.D.	32 (62.7 %)	8 (66.7 %)	8 (61.5 %)	24 (63.2 %)
W.D.	12 (23.6 %)	3 (25 %)	3 (23.1 %)	9 (23.6 %)
Lymph node				
Yes	43 (84.3 %)	11 (91.7 %)	11 (84.6 %)	32 (84.2 %)
No	8 (15.7 %)	1 (8.3 %)	2 (15.4 %)	6 (15.8 %)
Stage				
I	3 (5.9 %)	–	1 (7.7 %)	2 (5.3 %)
II	17 (33.3 %)	5 (41.7 %)	2 (15.4 %)	15 (39.5 %)
III	23 (45.1 %)	4 (33.3 %)	10 (76.9 %)	13 (34.2 %)
IV	8 (15.7 %)	3 (25 %)	–	8 (21 %)
Depth of tumor invasion (T)				
T2	19 (37.3 %)	6 (50 %)	4 (30.8 %)	15 (39.5 %)
T3	27 (52.9 %)	4 (33.3 %)	9 (69.2 %)	18 (47.4 %)
T4	5 (9.8 %)	2 (16.7 %)	–	5 (13.1 %)
Tumor type				
Intestinal	36 (70.6 %)	9 (75 %)	7 (53.8 %)	29 (76.3 %)
Diffuse	13 (25.5 %)	2 (16.7 %)	5 (38.5 %)	8 (21.1 %)
Mixed	2 (3.9 %)	1 (8.3 %)	1 (7.7 %)	1 (2.6 %)

of mRNA expression, in which all of the 12 overexpressed tumors had no methylated promoter sequence ($p=0.021$). However, there were 26 cases among the 39 (66.7 %) normal/underexpressed tumors without any methylation in the hMLH1 promoter, emphasizing the probable role of other mechanisms on the regulation of gene expression except the methylation. In the case of clinicopathological features and hMLH1 promoter methylation, we observed a significant correlation between the methylation status and the stage of tumor in which 10 out of 13 hMLH1 promoter hypermethylated tumors (76.9 %) were in stage III ($p=0.028$). Moreover, there was a significant correlation between methylation status and tumor location, showing that, 8 out of 13 (61.5 %) cases were located in the stomach ($p=0.025$). Beside the hMLH1, we assessed the methylation status in E-cadherin and although the numbers of cases were lower than the hMLH1, we observed a noticeable but not significant correlation between lymph node metastasis and methylation status of E-cadherin, in which 11 out of 12 (91.7 %) E-cadherin promoter hypermethylated tumors had lymph node metastasis. All the correlations between the clinicopathological features and methylation statuses are shown in Tables 3 and 4. Only two patients had methylated promoters in both of E-cadherin and hMLH1 and interestingly, one of them was the youngest patient enrolled in this study (30 years old). It seems that the promoter methylation in these genes is more frequent among the males in comparison with the females, 28.6 % of the males had hMLH1 methylation, against 18.8 % of the females. About the E-cadherin, 42.3 % of cases were hypermethylated in their promoter, while only 14.3 % of females were methylated. Finally, having examined the tumor size, we can conclude that although there was a significant correlation between tumor size and hMLH1 promoter methylation, methylated

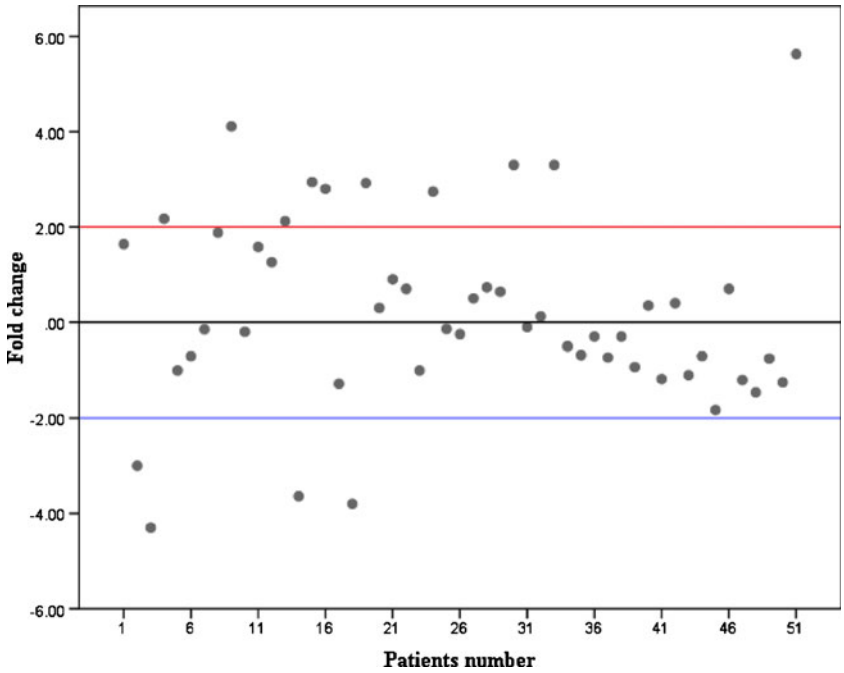

Fig. 1 Scatter plot of hMLH1 expression in GC patients

cases were bigger than the unmethylated cases (6.8±3.6 vs 5.9±2.4, p =0.402).

Discussion

This report is the second study in Iran emphasizing the role of epigenetic in gastric cancer progression. It has been shown that hMLH1 silencing is one of the most important causes of gastric cancer progression [21, 22]. Here, we examined the probable correlation between epigenetic mechanisms and gastric cancer progression. We performed a MSP analysis for promoter sequence of hMLH1 and partially, E-cadherin. Although we did not assess all of the samples in the case of E-cadherin promoter methylation, 12 out of 33 cases (36.4 %) had methylated promoters and all of the hMLH1 overexpressed tumors had no methylation in their promoters, as we expected. There was a significant correlation between the levels of hMLH1 mRNA expression and methylation status in which a lowered hMLH1 mRNA expression was observed in all of the hypermethylated cases. As it was observed, a noticeable number of normal/underexpressed cases had no methylation in the hMLH1

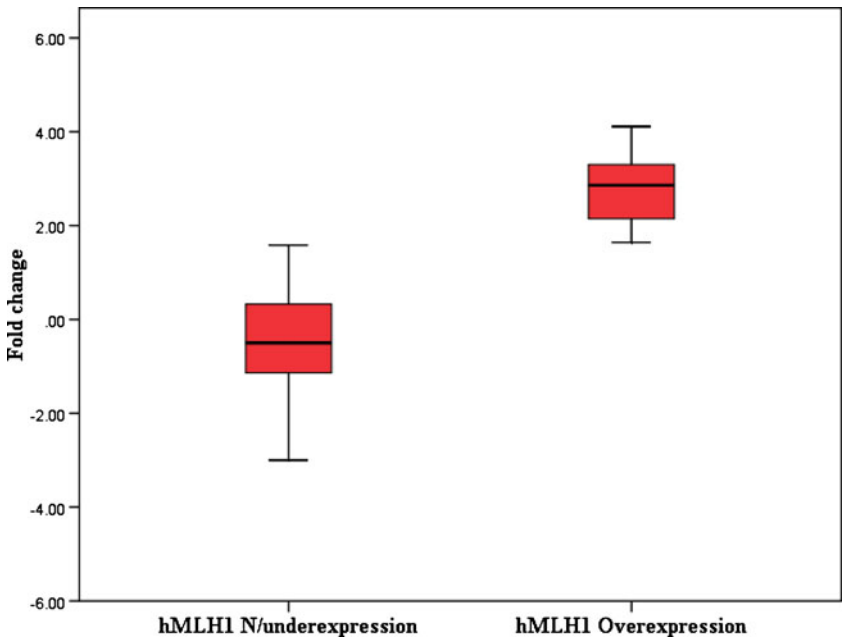

Fig. 2 Box plot illustration of hMLH1 relative quantitative mRNA expression in GC patients

Table. 4 Clinicopathological features and E-cadherin methylation

	E-cadherin promoter methylation		*p* Value
	+	–	
Patients	12	21	
Mean age (mean ± SD)	63.5±3.7	63.5±2.5	
Size (mean ± SD)	6.3±1.7	6.3±2.5	
Sex			
Male	11 (91.7 %)	15 (71.4 %)	
Female	1 (8.3 %)	6 (28.6 %)	
Location			0.025
Body	2 (16.7 %)	8 (38.1 %)	
Cardia	7 (58.3 %)	9 (42.9 %)	
Antrum	3 (25 %)	4 (19 %)	
Grade			
P.D.	2 (16.7 %)	4 (19 %)	
M.D.	7 (58.3 %)	12 (57.2 %)	
W.D.	3 (25 %)	5 (23.8 %)	
Lymph node			
Yes	11 (91.7 %)	19 (90.5 %)	
No	1 (8.3 %)	2 (9.5 %)	
Stage			0.028
I	–	–	
II	5 (41.7 %)	7 (33.3 %)	
III	5 (41.7 %)	9 (42.9 %)	
IV	2 (16.6 %)	5 (23.8 %)	
Depth of tumor invasion (T)			
T2	4 (33.3 %)	9 (42.9 %)	
T3	6 (50 %)	9 (42.9 %)	
T4	2 (16.7 %)	3 (14.2 %)	
Tumor type			
Intestinal	6 (50 %)	14 (66.7 %)	
Diffuse	6 (50 %)	6 (28.6 %)	
Mixed	–	1 (4.7 %)	

promoter sequence; therefore, it is concluded that beside the methylation, a variety of processes and factors are probably involved in hMLH1 gene silencing. Interestingly, there was no significant correlation between the levels of hMLH1 mRNA expression and clinicopathological features, whereas it has observed that there was a significant correlation between methylation status of hMLH1 and tumor stages, in which most of the promoter hypermethylated tumors were in higher stages. This can emphasize the role of hMLH1 epigenetic silencing in gastric tumor progression and shows that hMLH1 methylation plays a probable role in the advanced stages of tumor progression. Moreover, in one case, we observed a fold change of 1.26 while it was positive in the case of hMLH1 promoter sequence, indicating probable impurity of tumor tissue and presence of some normal cells within the tumor sample.

While the loss of hMLH1 expression is closely related to carcinogenesis in some cancers such as gastric, endometrial, and colorectal malignancies [23–25], overexpression of hMLH1 and/or hMSH2 is also reported in some other cancers such as some gastric cancers and sporadic endometrial cancer [26, 27]. Although the probable role of hMLH1 overexpression in tumorigenesis is not clear, there are some possible explanations about it.

First of all, it has been shown that some mutations in the MMR result to overexpression of a defected protein which has no functional ability in gastric cancer [26]. The same event is observed for p53 in a variety of cancers [28]. Second, it is believed that the MMR overexpression happened as a result of cellular adaptation in response to the accumulation of DNA mismatch errors during the tumorigenesis, especially in dividing cells [29]. Third, hMLH1 is able to induce apoptosis through inhibition of PCNA, which leads to replication arrest and induction of apoptosis [30]. Finally, hMLH1 is known as one of the caspase-3 protease substrates which lead to the production of a carboxyl terminal fragment with a pro apoptotic role, emphasizing the probable role of hMLH1 overexpression as a cellular defense mechanism against the uncontrolled cell division and malignant transformation [31].

To date, several epigenetic studies have been reported working on different markers in gastric cancer such as p16 [32, 33], E-cadherin [12, 34], and hMLH1 [33, 35]. All of these reports indicated the hypermethylation of E-cadherin up to 58.8 % of tumor cases in comparison to our report with 36.4 %. It reveals a noticeable decrease in this regard in the Iranian population, although we performed methylation assessment on a low numbers of patients. Considering the role of E-cadherin in cell attachment, we expected to see positive methylation in E-cadherin promoter in most of the metastatic cases, whereas only 11 out of 30 (36.7 %) cases with lymph node metastasis show a positive methylation. Therefore, it seems that although the E-cadherin abnormality has been reported in different malignancies and gastric cancer [36–38], it is not the only important factor in cell attachment and metastasis and there are different factors which are involved in this issue. E-cadherin hypermethylated cases were equally observed in diffused and intestinal tumor types.

Different factors such as genetic, epigenetic, and environmental are involved in gastric tumorigenesis [39]. Besides, MMR system as the main repair process is responsible for the mismatch correction during the replication and genome stability. Therefore, every MMR disorder will result in mutations in different cancer-related genes. hHML1 is one of the main components of MMR which is the target of methylation in gastric cancer [40]. Our data showed that 25.5 % of the cases had hMLH1 promoter hypermethylation, indicating a significant difference with the similar studies with almost up to 73 % [41]. Therefore, it seems that in contrast with the mentioned

studies in other countries, although the hMLH1 hypermethylation has a noticeable role on gastric cancer progression in Iranian patients, it has a lower involvement in gastric tumorigenesis in comparison with other populations; however, further studies are needed to find the accurate biology of gastric cancer in Iranian patients.

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Conflict of Interest The authors declare that they have no conflict of interest.

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