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Clinicopathological, Immunohistochemical, and *PMS2* Gene Expression Profiling of Patients with Sporadic Colorectal Cancer

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Abstract
Background: The DNA mismatch repair (MMR) system is one of the molecular pathways involved in colorectal cancer (CRC) carcinogenesis that consists of several genes, including *MLH1* (MutL homolog 1), *MSH6* (MutS homolog 6), *MSH2* (MutS homolog 2), and *MSH3* (MutS homolog 3). The protein encoded by *PMS2* (post-meiotic segregation 2) is also essential for MMR. Here, we address the correlation between immunohistochemical and transcriptional expression of PMS2 with the tumor grade and clinical stage of non-hereditary/sporadic CRC disease.
Methods: This study retrospectively analyzed 67 colorectal resections performed for 38 male and 29 female patients. Random biopsies were taken by a gastroenterologist from patients referring to three hospitals in the cities of Zanjan, Urmia and Qazvin (Iran) during 2017-2019. All specimens were examined and classified for localization of tumor, pathological stage and grade. The PMS2 protein expression was studied immunohistochemically and analysis of mRNA expression was performed in the same tissue sections.
Results: Immunohistochemistry and quantitative real-time polymerase chain reaction (PCR) analysis showed a decrease in PMS2 expression compared with paracancerous tissue ($P<0.001$), which correlated with tumor stage. In addition, reduced PMS2 expression was correlated with the tumor differentiation grade, underlining a connection between downregulation of PMS2 and progression of CRC. Comparing the PMS2 mRNA levels in different groups showed the following results: 0.92 ± 0.18 in patients with Stage I CRC tumor, 0.86 ± 0.38 in Stage II, 0.50 ± 0.29 in Stage III, and 0.47 ± 0.23 in Stage IV.
Conclusion: These findings suggest that *PMS2* may provide a potential reliable biomarker for CRC classification by combined immunohistochemical and mRNA analysis.
Keywords: Colorectal cancer, Immunohistochemistry, Mismatch repair, Neoplasm staging, *PMS2* gene
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Introduction

Colorectal cancer (CRC) is one of the most common cancers and the second leading cause of cancer-related deaths in the world.[1] Annually, over 1.23 million new cases are diagnosed with CRC (OMIM #114500) and it is estimated that about 600 000 deaths from CRC occur worldwide.[2] The disease is found to occur equally in both genders, second to breast cancer among females and third to lung and prostate for males, globally.[3]

In contrast to the familial type which is caused by germ-line mutations, sporadic CRC develops by the accumulation of a series of somatic mutations in tumor suppressor genes and oncogenes. Hereditary CRC includes hereditary nonpolyposis colon cancer (HNPCC), also known as Lynch syndrome (OMIM #120435) and familial adenomatous polyposis (FAP) (OMIM #175100). The

familial/hereditary type accounts for 10%–20% of CRC cases while 80%–90% of CRC cancers show evidence of sporadic clustering of the disease.[4]

Several acquired genetic mutations are commonly involved in the pathological processes linked to sporadic CRC risk in human populations; among which *APC* (Adenomatous polyposis coli) (OMIM #611731), *P53 (OMIM #191170)*, *DCC* (deleted in colorectal carcinoma) (OMIM #120470), *MCC* (mutated in CRCs) (OMIM #159350), and *SMAD4* (SMA- and MAD-related protein 4) (OMIM #600993) are tumor suppressors and some others such as Beta-catenin, *CTNNB1* (encoding for K-Ras β Catenin, c-myc proteins) (OMIM #116806) are oncogenes.[5] DNA mismatch repair (MMR), an important genome caretaker system, has been extensively studied and deficient DNA MMR seems to be important in cancer

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development since the major MMR genes are found to be mutated in many tumors. Given the increasing importance of the involvement of MMR abnormalities in sustaining cancer development, further consideration deserves to be given as to whether dysfunction of the DNA MMR genes is associated with CRC. This is of great importance in genetic research, particularly of non-hereditary cases which represent the most common CRC type. Characterizing hereditary versus sporadic CRC cases and the underlying molecular signatures may also provide indications regarding prognosis and risk of disease development. Initial studies in the field have shown evidence of defective MMR in CRC patients regardless of sporadic or familial origin. Further characterization suggests that components of the MMR machinery could serve as target genes for detection and genotyping of non-hereditary-sporadic CRC cancers.[6]

The DNA mismatch-repair system includes *MSH2* (MutS homolog 2), *MSH3/6* (MutS homolog 3/6), *MLH1* (MutL homolog 1) and *PMS2* (post-meiotic segregation 2).[7] Genetic as well as epigenetic changes in the MMR genes which induce MMR system failure have been increasingly recognized in the carcinogenesis process. The most commonly mutated MMR genes in human cancers are *MLH1* (OMIM: #120436), *MSH2* (OMIM #609309) and *MSH6* (OMIM #600678).[8,9] *PMS2* (OMIM #600259) also referred to as *HNPCC4*, *PMS2CL*, *PMSL2*, *MLH4*, *PMS1 homolog 2*, and MMR system component, is an essential component in eukaryotic DNA MMR[10]; however, mutations in this MMR gene are rare in the etiology of patients with a family history of CRC.[11] *PMS2* is one of five MMR genes embedded within a complex genomic locus on chromosome 7 (band p22.1). There are a number of PMS2 pseudogenes on the same chromosome, the presence of which complicate the identification and mutational analysis of *PMS2* gene.[12] Recent advances in mutation detection technologies have allowed clinical testing for PMS2 mutations; however, there are still some challenges about whether any of the mutations in *PMS2* gene are missed or miscalled as a result of the presence of pseudogenes.

Mismatch repair component PMS2 is one of several mammalian homologs of the *Escherichia coli* MutL DNA MMR gene, an enzyme that, in humans, is encoded by the *PMS2* gene comprised of 15 exons.[13] As one of a complex of proteins in the DNA MMR system, PMS2 endonuclease activity, when in complex with MLH1, is capable of incising a defective DNA strand and thus along with MSH2, MSH6 and EXO1, corrects small errors involving mispaired nucleotides to ensure the fidelity of the newly-replicated DNA strand.[14]

Recent studies in humans have linked MMR proteins with dual functions in DNA damage repair and signaling pathways inducing apoptosis in the face of excess DNA damage. Mechanistically, one of the models that can be considered for MMR signaling pathways is the hydrolysis-independent sliding clamp (SC) model. The SC model is alike to G protein molecular switch systems. Functional complexes of the MMR proteins, unlike bacterial MutS and MutL which function as homodimers, the eukaryotic MutS homolog (MSH) and MutL homolog (MLH) proteins function as heterodimers.[15] The "hydrolysis-independent SC" model suggests that the MMR proteins are signaling molecules. These MMR proteins function as "direct sensors" that contribute to signaling pathways that may either provoke DNA repair or apoptosis.[16]

Germ-line mutations in the *PMS2* gene leading to loss of MMR function appear to underlie the development of a variety of hereditary malignancies such as colon cancer, ovarian, gastric, pancreatic, endometrial and prostate cancer.[17-20] Insights into the genetic basis of colon cancer families have revealed that 10%–15% of CRCs are due to defects in MMR represent non-hereditary sporadic form of CRCs for which screening is generally recommended.[21] Although sporadic colorectal carcinogenesis is not a rare pathological entity, little is known about its cytogenetic and pathological features necessary to identify prognostic biomarkers for early diagnosis. The adverse consequences of the delay between onset and diagnosis may be a more advanced stage of disease without clinical evidence of metastatic disease.[22,23] Identification of factors associated with the clinicopathologic discrepancy could be used for assessment of disease severity. The aim of this study is to investigate the correlation of PMS2 expression and parameters of age, sex, primary tumor location and differentiation grade as well as cancer stage that would enable an earlier diagnosis to be made. In this population-based study of patients with sporadic CRC, we examined PMS2 expression by immunohistochemistry and quantitative gene expression assay with real-time polymerase chain reaction (PCR). To our knowledge, this is the first study combining immunohistochemical and real-time quantitative PCR techniques designed to elucidate the importance of PMS2 expression pattern in tumor tissue as a prognostic factor in patients with sporadic CRC.

Materials and Methods
Sample Collection
The sample size was calculated using the appropriate statistical formula and the results of the study by Karahan et al.[29] According to this mentioned study, in order to compare the rate of *PMS2* gene expression in patients suffering from CRC cancer in different stages with the healthy control group, and considering a clinical difference rate of 50% in gene expression between higher stage colon tumors and normal tissues, the standard sample size using Cochrane formula was calculated 19 in each group with 95% validity and 90% power of the test based on the results from normal group (9.7%) and stage III CRC

cancer (76.3%).

$$n = \frac{(1.96 + 1.28)^2 [0.097(1 - 0.097)] + [0.763(1 - 0.763)]}{(\frac{0.763}{2})^2} = 19$$

Sixty-seven colonoscopy tissue samples from CRC patients were collected from patients referring to three hospitals in Iran: Vali-e-Asr hospital, Imam Khomeini hospital and Velayat hospital in the cities of Zanjan, Urmia and Qazvin, respectively, from 2017 to 2019. The patients were selected randomly and a tumor tissue sample and a normal sample located 12 cm apart from the tumor site were collected from each patient.

After pathological confirmation by a hospital pathologist, the specimens were nominated for experimental studies. Written informed consent was taken from all participants.

RNA Extraction and Reverse Transcription
All biopsy tissue specimens were transferred to 1.5 mL micro-tubes containing RNAlater solution. For the storage of biopsies, the tubes were first kept at 4°C up to about 12-24 hours followed by long-term storage at -70°C. Total RNA extraction was carried out with RNX plus Sinaclon Bioscience, Karaj, Iran (Cat. #RN7713C) according to the manufacturer's instructions, and the concentration of isolated RNA was then determined using Thermo Nanodrop. To allow the isolation of RNA free from genomic DNA contamination, the RNA extracts were treated by DNase using the EN0521 (Fermentas, Germany) isolating total RNA free from genomic DNA.

After removal of genomic DNA, the preparations of RNA were subjected to reverse transcription to synthesize the complementary DNA (cDNA) using the Thermo Scientific kit RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA. Cat. #k1622).

Real-Time Polymerase Chain Reaction
We used quantitative the real-time PCR technique to quantify the amount of *PMS2* gene expression in normal and tumoral tissue samples. Gene expression was detected using SYBR Premix Ex TaqII (Takara Bio Inc., Japan). Primer pairs were designed for exons 3 and 4 using the NCBI database. Beta-actin gene was used as the internal control gene for all tissue samples. The primer sequences for *PMS2* gene were as follows: forward: 5'-GAAGTTTCAGACAATGGATGT-GG-3' and reverse primer: 5'-TAGGTCGCAAACTCTT-GAATC3'. For beta-actin: forward primer was 5'-CTTGAT-GTCACGGACGATT-3', and reverse primer was 5'-CACG-GCATTGTCACCAACT-3'.

All the reactions were performed in triplicate. The PCR products were then subjected to electrophoresis on 1.5% agarose gel for further analysis.

Immunohistochemical Analysis
In this study, immunohistochemistry (IHC) was used to assay PMS2 protein content. Immunohistochemical staining by monoclonal antibody was used to perform IHC using a rabbit tissue monoclonal antibody prepared from Aragen Company namely (Monoclonal Rabbit Anti-Human-Post-Meiotic Segmentation 2-clone EP51) (Tashkhis Baft Aragene-Dako, Tehran, Iran) that specifically detects target proteins. This antibody binds only to its own specific protein in the tissue section. Then, during the production of color by the enzyme attached to the secondary antibody, the protein expression can be investigated through a substrate capable of producing color at sites of target protein.[24]

After collecting paraffin blocks from patients' specimens, tissue sections were placed on charged slides and then immunohistochemistry was performed according to the manufacturer's protocol.

Finally, the slides were scanned using an optical camera microscope and the photos were captured at a magnification of 40x.

The analysis of protein expression by semi-quantitative method using the scoring system produced repeatable results. PMS2 expression was reported semi-quantitatively for 45 tumor samples with the Remmele score system.

Statistical Analysis
Statistical analysis was performed using SPSS software version 16 and data plots were generated using the GraphPad Prism 7 (GraphPad Software Inc., La Jolla, CA, USA). First, the data were checked for normal distribution using Kolmogorov-Smirnov test and Normal Q-Q Plot. Levene test for homogeneity of variance was conducted. An Ordinal Logistic Regression was used to assess associations of tumor stage with PMS2 protein expression intensity in CRC tissues. For normal data, analysis of variance (ANOVA) was used to compare the mean of the variables studied in different stages of the disease; to compare the mean of variables between normal and cancerous tissues, the independent *t* test was used. For variables that were not normal, we used the Mann-Whitney U test and for nominal variables, chi-square tests for large expected cell counts, otherwise replaced by Fisher exact test. To investigate the relationship between gene expression and other variables, for normal and non-normal distributions of quantitative variables, the Pearson and Spearman rank correlation coefficient tests were performed, respectively. Nonparametric Kruskal-Wallis H test was used to compare the intensity of PMS2 expression in four stages of the disease. *P* values less than 0.05 were considered as statistically significant in hypotheses.

Results
Tumor and adjacent normal tissues as control were obtained from 67 patients [38 (57%) males and 29 (43%) females] with sporadic CRC. The mean age of the patients was 61 ± 12 years (ranged 36 to 76 years). The median size of resected specimens was 0.2 × 0.2 × 0.2 cm. The surgical

specimens were divided into four groups based on the stage of the disease. These experimental groups included Stage I = 9 (13.4 %), Stage II = 23 (34.3 %), Stage III = 17 (25.4 %), and Stage IV = 18 (26.9 %) tumors and their respective control groups. We further provided full information on the tumor growth, in terms of the tumor location (in colon, cecal colon, transverse colon, sigmoid, rectum, rectosigmoid, cecum and ascending colon) and tumor differentiation grade which characterized the phenotypic properties of these cells into three subtypes: well-differentiated, moderately differentiated, and poorly differentiated. A summary of the clinicopathological parameters of the patients is shown in Table 1. Based on ANOVA results, the mean age of the patients in the four stages was not significantly different ($P = 0.108$).

The quantitative method of real-time PCR was used to investigate the molecular changes in PMS2 mRNA expression in normal and colorectal tumor tissues. The results showed that the PMS2 mRNA level in the tumor tissues was significantly decreased, indicating the association of PMS2 with malignant phenotype of CRC cells.

Tukey's post hoc test, comparing the expression of *PMS2*

gene expression at the mRNA level between the normal and tumor cells from different stages of growth, showed significantly lower PMS2 expression in all tumor stages (in Stage I; $P = 0.039$, in Stage II; $P = 0.015$, in Stage III; $P < 0.0001$ and in Stage IV; $P < 0.0001$, Figure 1). When comparing between the groups, the highest *PMS2* gene expression was observed in Stage II tumors, and a statistically significant difference compared to Stages III and IV ($P = 0.045$ and $P = 0.026$, respectively) was noted.

Paraffin blocks were cut by microtome (CUT 2040-Company Germanic) to 4 microns in thickness. After IHC staining with anti-PMS2 antibodies and optical microscopy, the following results were obtained. The DAB sedimentation analysis, which is brown on the tissue, was evaluated for at least five fields for each tumor and the intensity of tumor cell staining was scored. The intensity of PMS2 protein expression was calculated with semi-quantitative scores. The number of positive staining cells was counted per square millimeter of tissue. The average percentage of PMS2-positive stained tumor cells was determined in terms of expression and was divided into four groups: PMS2-negative expression (group 1), expression less than 1% (group 2), 1%–50% (group 3), and 51%–100% (group 4). The intensity of PMS2-staining is reported as negative expression, +1, +2, +3, indicative of weak, moderate and strong degree of expression, respectively. Also, we used the Remmele Score system, which shows the quantitative amount for protein expressions which is the product of the percentage of stained cells in the stain intensity (which could value from 0 to 9). Immunohistochemical fields of tissues with weak PMS2 protein expression were associated with colon cancer. The results in comparison with various stages show a reduction in the average percentage of expression of PMS2 protein in the tumor tissue, especially after stage II.

Figure 2 illustrates immunohistochemical staining for PMS2 protein in normal and tumoral tissues. Normal tissue samples showed high PMS2 protein expression levels and
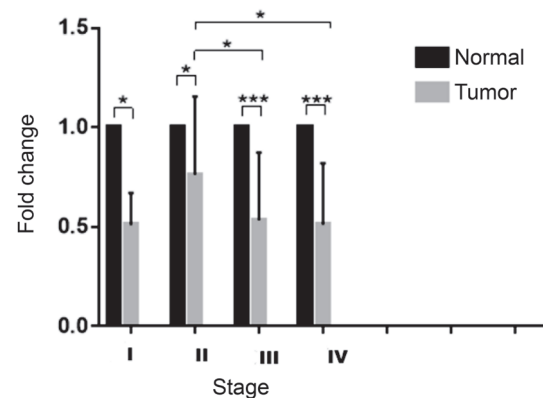
Table 1. Number of Cases According to Clinicopathological Parameters

Variables	Number of Cases	%
Sex		
Male	38	56.7
Female	29	43.3
Age (y)		
<61	27	40.3
>61	40	59.7
Location primary tumor		
Colon	7	10.3
Cecal colon	5	7.5
Transverse colon	4	6.0
Sigmoid	17	25.4
Rectum	18	26.9
Recto sigmoid	7	10.4
Cecum	3	4.5
Ascending colon	6	9.0
Differentiation		
Well	24	35.8
Moderate	24	35.6
Poor	11	16.7
Undifferentiated	8	11.9
Gene expression level in tumor		
Absent	32	45
Present	35	∘∘
Stage		
I	9	13.4
II	23	34.3
III	17	25.4
IV	18	26.9

Figure 1. Relative Expression Level of PMS2 mRNA in Different Stages of Colorectal Cancer Compared to Their Control Group. *$P<0.05$; **$P<0.01$; ***$P<0.0001$.
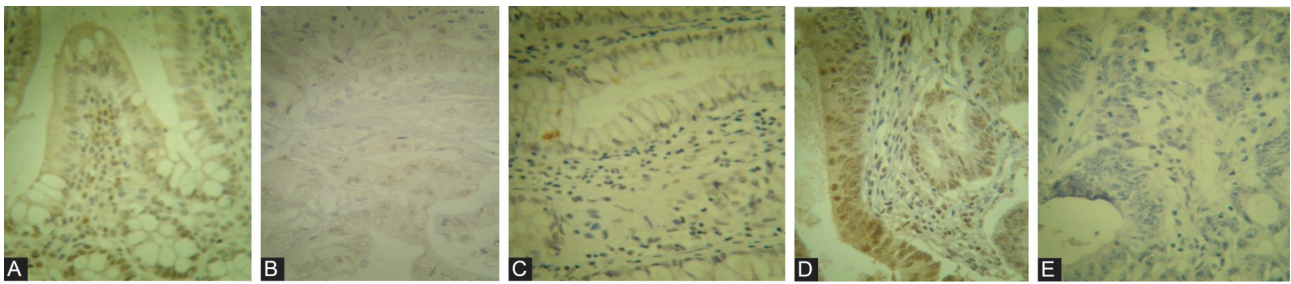
Figure 2. Immunohistochemical Staining of Colorectal Tumors for PMS2 Marker (PMS-2, x400) Visualized by DAB (brown). (A) Immunohistochemistry expression of PMS2 protein showed that normal tissue samples were positive for PMS2 protein expression (positive control). (B) Tumoral tissue with low intensity PMS2 protein expression (+) represent tumors in higher stages. (C) Tumoral tissue showing moderate intensity for PMS2 protein expression (++) which is associated with early stage cancer. (D) Tumoral tissue showing high intensity for PMS2 protein expression (+++), and (E) absence of PMS2 expression (Negative control).

tumoral tissues showed decreased or loss of PMS2 protein expression as follows: low-intensity protein expression (+, weak positive) regarded as well-differentiated, moderated-intensity protein expression (++, positive) regarded as moderately differentiated, and high-intensity protein expression (+++, strong positive) was regarded as poorly differentiated tumors. The percentage of PMS2 protein expression by immunohistochemical staining is shown in Table 2. In PMS2 staining of experimental tissue samples, 32 out of 71 tumor cases (45.1%) were negative ($P = 0.01$).

Using Fisher's Exact test, we compared the intensity of *PMS2* expression in different stages of the disease with their control group (normal tissue). Confounding adjustment was not performed due to small sample size. Tissue samples from four disease stages had lower expression of *PMS2* than the control groups (Table 3). The intensity of *PMS2* expression in four stages of the disease was significantly different ($P = 0.01$). ANOVA showed a significant difference in the percentage protein expression in different stages; with increasing the stage of CRC cancer, the intensity and percentage of PMS2 protein expression decreased significantly ($P = 0.01$).

As shown in Table 4, the odds ratio of PMS2 positive expression between the experimental groups and the

control group was significant with values of 0.1, 0.191, and 0.034 for stage IV, stage III, and stage II tumors, respectively, (all values <1). PMS2 protein expression was measured semi-quantitatively (Remmele score) using immunohistochemistry. The PMS2 median Remmele score was found to be -5.46 ± 0.95 ($P = 0.001$), -3.01 ± 1.05 ($P = 0.044$), and -3.99 ± 1.13 ($P = 0.007$) for stage II, stage III, and stage IV tumor tissues, respectively, compared to the normal group (Table 5).

In Figure 3, the Box Plot compares the intensity of expression and the percentage of protein expression between the four stages and the control group. In both plots, the median and percentiles of 25th and 75th in the control group were higher than those of different tumor stages, and stages II and IV had the lowest percentile of protein expression and intensity. Based on the nonparametric Kruskal-Wallis H Test, the distribution of protein expression percentage ($P = 0.003$) and its intensity ($P < 0.001$) were different in various stages. Figure 3 shows a significant relationship between the intensity of PMS2 protein expression and different stages of the disease ($P < 0.001$). Figure 3 demonstrates a significant relationship between the percentage of protein expression and different stages of the disease. Four stages have a lower percentage of protein expression than the normal group ($P = 0.003$). finally, Figure 3 shows the intensity of PMS2 staining in the Remmele Score system, which was significantly higher in the normal tissues than tumoral tissues ($P < 0.001$).

Table 2. Percent of the Cases According to Staining of the Immunohistochemical Marker

PMS2 expression (%)	51–100	1–50	1<
Cases (%)	29.6	23	1.4

Table 3. Amount of Protein Expression in Normal Tissue Compared to Tumor Tissue in Four Different Stages of the Diseases

Variable			Intensity			
			—	1+	2+	3+
Groups	Normal	No. (%)	6 (20.7)	0 (0.0)	5 (17.2)	18 (62.1)
	Stage I	No. (%)	2 (50.0)	0 (0.0)	2 (50.0)	0 (0.0)
	Stage II	No. (%)	13 (81.3)	0 (0.0)	3 (18.8)	0 (0.0)
	Stage III	No. (%)	5 (41.7)	1 (8.3)	4 (33.3)	2 (16.7)
	Stage IV	No. (%)	6 (60.0)	0 (0.0)	3 (30.0)	1 (10.0)
Total		No. (%)	32 (45.1)	1 (1.4)	13 (23.9)	21 (29.6)

$P = 0.01$.

Table 4. Parameter Estimates. Association Between the Intensity of Protein Expression and Different Stages of the Disease

Parameters	P value	Odds Ratio	95% Wald Confidence Interval for OR	
			Lower	Upper
Stage IV	0.002	0.100	0.022	0.443
Stage III	0.013	0.191	0.052	0.708
Stage II	< 0.001	0.034	0.007	0.157
Stage I	0.032	0.117	0.016	0.835
Normal (Reference group)	—	1	—	—

Dependent Variable: Intensity.
Model: (Threshold), Groups.

Table 5. Mean Difference of Percentage of Protein Expression and Remmele Score between Stages of the Disease

Dependent Variable	(I) Groups	(J) Groups	Mean Difference (I–J)	P Value	95% Confidence Interval	
					Lower Bound	Upper Bound
Percentage of protein expression	Stage I	Normal	-25.905	0.628	-77.72	25.91
	Stage II	Normal	-47.030	**<0.001**	-77.28	-16.78
		Stage I	-21.125	0.811	-75.43	33.18
	Stage III	Normal	-13.155	0.803	-46.50	20.19
		Stage I	12.750	0.968	-43.34	68.84
		Stage II	33.875	0.090	-3.22	70.97
	Stage IV	Normal	-27.655	0.201	-63.28	7.97
		Stage I	-1.750	1.000	-59.22	55.72
		Stage II	19.375	0.638	-19.79	58.54
		Stage III	-14.500	0.864	-56.10	27.10
Remmele score	Stage I	Normal	-3.84483	0.143	-8.4384	0.7488
	Stage II	Normal	-5.46983	**<0.001**	-8.1519	-2.7877
		Stage I	-1.62500	0.877	-6.4395	3.1895
	Stage III	Normal	-3.01149	**0.044**	-5.9677	-.0553
		Stage I	.83333	0.990	-4.1391	5.8057
		Stage II	2.45833	0.234	-.8306	5.7473
	Stage IV	Normal	-3.94483	**0.007**	-7.1032	-.7865
		Stage I	-.10000	1.000	-5.1952	4.9952
		Stage II	1.52500	0.733	-1.9468	4.9968
		Stage III	-.93333	0.954	-4.6210	2.7543

Discussion

It is well established that early detection and accurate disease progression prediction benefit from integrating molecular analysis with clinical data. An outstanding example is FAP – an inherited condition where a genotype–phenotype correlation for the *APC* gene exists.[25] As mentioned earlier, technical difficulties in analyzing the *PMS2* gene mutations due to pseudogenes is clearly consistent with a significant underreporting of *PMS2* mutations that indicates poor understanding of the CRC genetic risk.[26] Yet the immunohistochemical analysis of tumor samples carries significant clinical implications.[27] Using this method, we found that the immunohistochemical staining pattern of PMS2 in the colorectal tumors contrasts with the adjacent normal tissues. Importantly, PMS2 was differentially expressed in normal, premalignant, and malignant colorectal tissues supporting the idea that *PMS2* expression was in some way associated with tumor initiation and progression. By itself, PMS2 may have significant

impact on early CRC detection in sporadic cases and thus, will have implications for future diagnostic and treatment measures. CRC is one of the most appropriate cancer types for prevention and early diagnosis.[28] Since sporadic CRC under the age of 40 is often very rare,[29] in our study, the mean of age in the subjects was 61 ± 12 years. Multicentric sporadic CRC samples were collected and the expression of *PMS2* was measured at both protein and mRNA levels. In addition, most tumors were located in the rectum and sigmoid colon, and the majority had a moderately to well differentiated grade of cancer. The results showed that both PMS2 protein and gene expression were significantly lower in the tumor tissue than in the area considered as normal. Furthermore, in malignant tissues, the expression rate of PMS2 decreased gradually from benign colorectal tumors (stage I) to malignant CRC (stage IV), indicating that decreased *PMS2* expression is associated with stage, grade, and histologic features of the tumor.
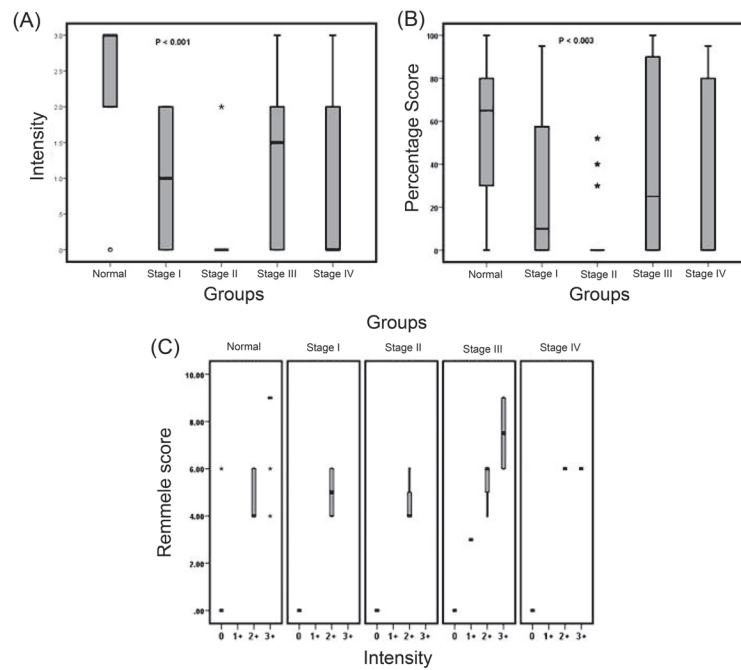
Our study is a population-based examination with

Figure 3. Association between the Intensity of PMS2 Protein Expression (A), Percentage of PMS2 Expression (B), and Remmele Score (C) with Different Stages of Colorectal Tumors (Outlier *).

a small number of participants which yielded similar results to some previous reports. For example, Ioana et al studied the expression level of MMR genes in normal, polyp and malignant tissues in sporadic CRC, using real–time quantitative reveres-transcription PCR[6] and showed that the MMR expression level in normal tissues was significantly higher than tumor tissues. Our findings characterized the *PMS2* expression patterns with molecular and immunohistochemical confirmation.

Also, Senter et al took an important step by exploring the clinical characteristics of Lynch syndrome patients who had loss of PMS2 expression by immunohistochemistry and demonstrated that in a large series of *PMS2* mutation carriers, the incidence of CRC was 5.2-folds higher compared with the general population.[30] Notably, *PMS2* expression results obtained by immunohistochemistry were concordant with its mutational analysis. Based on the available data, it seems reasonable to conclude that *PMS2* mutations appear to contribute significantly to CRCs and clinical screening recommendations for *PMS2* mutation carriers may potentially improve the disease outcome in sporadic CRC patients. In the present study, quantitative analysis in 45% of the studied tumors showed decreased expression of *PMS2* gene expression. Our results showed that there was a correlation between decreased *PMS2* gene expression and the incidence of CRC cancer. However, one of the main limitations of our research was the small number of the specimens evaluated. There is a possibility of sparse-data bias in Table 4 due to the wide confidence interval of odds ratio which is a limitation of this study because of small sample size in some groups. Moreover,

it is recommended that for future studies, both the interacting partners of MLH1 and PMS2 be evaluated to explore whether the reduction in PMS2 is independent of changes in its heterodimeric partner, MLH1, which may reveal more details on CRC cancer pathogenesis and surveillance guidelines. We suggest that each of the four major MMR markers including MLH1, MSH2, PMS2, and MSH6 should be investigated which may prove more helpful in predicting the tumor behavior.

In conclusion, low expression of *PMS2* gene in mRNA and protein levels was demonstrated in CRC. The mutations occurring in *PMS2* gene can result in genome instability. This instability is probable to lead to colon cancer. Failure to correctly repair the DNA damages may also lead to many forms of other cancers.

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