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ORIGINAL ARTICLE

B-cell-Specific Moloney Murine Leukemia Virus Integration Site 1 and Fas Ligand Expression in Colorectal Cancer Progression and Prognosis

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SUMMARY

Background: **B-cell-specific Moloney murine leukemia virus integration site 1 (BMI1) and Fas ligand (FasL) are two critical stemness genes believed to play a role in the development of colorectal cancer (CRC). This study aimed to investigate the expression levels of these genes in primary CRC tumors to assess their correlation with cancer progression and prognosis.**
Methods: **The relative expression levels of BMI1 and FasL were analyzed using real-time polymerase chain reaction in 100 primary CRC tumor samples along with paired adjacent non-cancerous tissues. The association between the gene expression levels in primary tumor tissues and clinicopathological features, as well as the overall survival of patients, was evaluated.**
Results: **The primary cancerous tissues exhibited higher expression levels of BMI1 and FasL compared to their adjacent non-cancerous tissues. The relative expression levels of BMI1 and FasL were found to significantly correlate with tumor size, grade, TNM stage, metastasis ($p = 0.0001$ for all), and reduced overall survival time ($p = 0.00001$). Moreover, BMI1 and FasL emerged as independent prognostic factors in the multivariate Cox regression analysis.**
Conclusions: **The results of this study showed that elevated levels of BMI1 and FasL in the cancerous tissue of colorectal cancer patients are linked to cancer progression and poor prognosis, highlighting their significant roles in the development of CRC.**
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KEYWORDS

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INTRODUCTION

Among the most common cancers, colorectal cancer (CRC) has been identified as the second leading cause of cancer-related deaths in various countries worldwide [1]. While advancements in diagnostic and therapeutic techniques have enhanced the management of CRC, the long-term survival rate of CRC patients remains unsatisfactory, with outcomes heavily reliant on the early detection of tumors [2]. However, CRC is characterized by a high degree of tumor heterogeneity, presenting a

significant challenge for diagnostic and treatment strategies [3,4]. Hence, evaluating potential molecular biomarkers that play critical roles in the pathophysiology of CRC can have significant benefits for the diagnosis, prognosis, and treatment of the disease. In this context, genes associated with cancer stem cells (CSCs) are particularly important. CSCs represent a subset of tumor cells with multipotency and self-regeneration capabilities, making them responsible for tumor formation, invasiveness, chemotherapy resistance, and tumor recurrence. Studying these genes can provide valuable insights into understanding and targeting the mechanisms underlying CRC progression and treatment resistance [5,6].

One of the relevant genes is B-cell-specific Moloney murine leukemia virus integration site 1 (BMI1), which is part of the Polycomb group proteins. It is a proto-oncogene that encodes a RING finger protein and plays a crucial role in the formation of the Polycomb group complex 1 (PRC1), where it serves as a major component [7]. The dysregulation of BMI1 has been implicated in various cancers, including colorectal cancer, due to its involvement in processes such as cell proliferation, self-renewal, and differentiation [7]. This complex epigenetically suppresses the expression of numerous critical genes, including two significant tumor suppressor genes, p16ink4a and p14Arf, thereby contributing to the development of CSCs [8,9]. BMI is situated downstream of various signaling pathways involved in the development, maintenance, and expansion of CSCs [9], positioning it as a key mediator facilitating crosstalk among these signaling pathways [10,11]. Furthermore, it has been suggested that BMI1 and TWIST1 promote epithelial-mesenchymal transition (EMT), thereby contributing to the maintenance of cancer stemness [12]. The suppression of BMI1 in mouse breast cancer stem cells, both in vitro and in vivo, has resulted in the inhibition of tumor proliferation and progression [13]. These effects were shown to be achieved by arresting the cell cycle and inhibiting tumorsphere formation. Overexpression of BMI1 has been reported in tumors of the gastrointestinal tract, including the colon [6,14].

On the other hand, Fas ligand (FasL or CD95L), a member of the tumor necrosis factor (TNF) ligand family, encodes a type II transmembrane protein. Upon binding to its receptor, Fas, it triggers a cascade of caspases that ultimately lead to apoptosis [15]. FasL exists in a membrane-bound isoform, which can be cleaved by specific matrix metalloproteinases to generate a truncated soluble isoform [16]. While the membrane-bound isoform is known to be crucial for apoptosis and cell death, the primary established function of the soluble isoform is to promote tumorigenesis [17]. The impact of FasL in cancers is complex, and the literature does not always provide a definitive conclusion on its apoptotic or tumorigenic effects. Studies on breast cancer have suggested that the absence of Fas/FasL molecules is linked to a poorer prognosis in patients [18-20]. Similarly, low levels of Fas/FasL expression in oral squamous cell carcinoma (OSCC) cells have been linked to increased proliferation and invasiveness both *in vitro* and *in vivo*, suggesting a role in apoptosis [21].

The scenario appears to differ in colorectal cancer. Because in one study, the increased expression of FasL in human colon tumor cells did not correlate with enhanced apoptosis in tumor cells in vivo [22]. Furthermore, some studies have suggested a link between elevated FasL expression and the progression of colorectal cancer [23,24]. Yet, this Fas/FasL signaling system can regulate the expression of basal factors, especially BMI1, in CRC [26]. However other investigations failed to show that increased FasL expression in colon tumor tissues can be considered a significant prognostic factor [25].

Given the lack of sufficient and the partially inconsistent data regarding the pathophysiological and prognostic roles of BMI1 and FasL in colorectal cancer (CRC), this current research aimed to improve understanding of the association between FasL and BMI1 with clinicopathologic characteristics in the progression and prognosis of CRC.

MATERIALS AND METHODS

Study population

The study included 100 CRC patients who underwent surgical procedures at the Cancer Institute of Tehran from January 2015 to December 2019. Tissue samples from cancerous and adjacent non-cancerous areas were collected post-surgery, then frozen and stored for later analysis. Patient data were gathered from medical records, and survival was tracked for 5 years post-surgery to assess prognosis. Patients provided written consent to participate, and the study was approved by the Ethics Committee of Islamic Azad University.

Inclusion and exclusion criteria

The inclusion criteria for this study specified patients diagnosed with colorectal cancer. Patients with cancer in different organs, individuals with conditions such as colon inflammation, or those who had undergone radiotherapy or chemotherapy were excluded from the study.

Total RNA extraction and complementary DNA (C.DNA) synthesis

For total RNA extraction, 70 mg of tissue was combined with TRIzol™ reagent and homogenized according to the manufacturer's instructions. The extracted RNA samples underwent DNase treatment using the Qiagen DNase kit to eliminate any remaining DNA impurities. RNA quantity and quality were assessed by using a Nanodrop 2000 system and agarose gel electrophoresis. Following the procedures, RNA samples in RNase-free water were stored at -80°C for further analysis.

Also, total RNAs were reverse transcribed using the PrimeScript™ 1st Strand cDNA Synthesis kit from Ta-

kara, Otsu, Japan, following the manufacturer's protocol. Eventually, the resulting cDNA templates were stored at -20°C for qRT-PCR amplification.

Quantitative real-time PCR (qRT-PCR) amplification

For the qRT-PCR amplification of cDNAs, the methods were adapted from the RT2 SYBR Green qPCR Mastermix kit (Qiagen, Hilden, Germany) and carried out on an Exicycler™ 96 Quantitative Real-Time PCR machine (Bioneer, Daejeon, Korea). Each real-time PCR reaction, with a total volume of 25 µL in the wells of the plate, consisted of a 12.5 µL aliquot of SYBR Green Mastermix, 1 µL of each primer (150 mL), 1 µL of cDNA template (5 ng/mL), and 10.5 µL of RNase-free water. The real-time PCR reactions were duplicated, following the specified profile: an initial step at 95°C for 10 minutes, followed by 40 cycles at 94°C for 15 seconds and 60°C for 10 minutes. Positive and negative control reactions (one for each) were included in the qRT-PCR assay to maintain quality control. The specificity of all target amplifications was verified by analyzing the melting curves. In the study, the expression levels of FasL and BMI1 were normalized relative to the expression of the internal reference gene, beta-2-microglobulin (β2M). This normalization was done because the amplification efficiency of β2M was very close to that of FasL and BMI1. After validation by Primer Blast, the relevant primer sets for the qRT-PCR assay are presented in Table 1. Finally, the comparative normalized expression values of FasL and BMI1 in tumors relative to the paired adjacent normal tissues were calculated using the $2^{-\Delta\Delta Ct}$ method and by applying the Pfaffl model [27].

Statistical analysis

The study data were statistically analyzed using SPSS software version 21.0 (IBM Corp., Armonk, NY, USA). The relative expression levels of FasL and BMI1 in cancerous and paired adjacent non-cancerous tissues were compared using the Wilcoxon signed-rank test. For comparisons between two patient groups, the nonparametric Mann-Whitney U test was utilized. Differences among more than two groups were analyzed with the Kruskal-Wallis test. The relationship between the relative expression of BMI1 and FasL in cancerous tissues and the overall survival rate of CRC patients was assessed through Kaplan-Meier analysis and the log-rank test. Factors with independent prognostic effects were identified using univariate and multivariate Cox regression analyses. Statistical significance was determined by p-values < 0.05.

RESULTS

Relative expression of BMI1 and FasL genes in cancerous and adjacent non-cancerous tissues

The expression levels of BMI1 and FasL genes were significantly higher in cancerous tissue of colorectal cancer (CRC) patients compared to the adjacent non-cancerous tissues, as indicated in Figure 1 ($p = 0.0001$).

Relationship between the overexpression of BMI1 and FasL and clinicopathological features in CRC

The study categorized the relative expression values of BMI1 and FasL in colorectal cancer (CRC) patients based on clinicopathological features. Table 2 demonstrates a significant association between higher expression levels of BMI1 and FasL and advanced tumor TNM stage, grade, size, and metastasis. This indicates that the overexpression pattern of these genes aligns with the progression-related clinicopathological characteristics of CRC.

Relationships between the expression of BMI1 and FasL and the overall survival time of patients

The study investigated the impact of BMI1 and FasL expression on the overall survival of CRC patients. Patients were divided into high- and low-expression groups based on median expression values. Kaplan-Meier and log-rank test survival analysis revealed that higher expression levels of BMI1 and FasL were associated with shorter survival times ($p = 0.00001$, log-rank test; Figure 2). Univariate and multivariate Cox regression analyses confirmed that BMI1 and FasL expression levels, along with TNM stage, grade, and metastasis, independently predicted overall survival in CRC patients (Table 3).

DISCUSSION

Evaluations indicate that the expression of BMI1 and FasL genes may be involved in invasion, metastasis, and cancer stemness in colorectal cancer patients [26]. Consequently, this study aimed to assess the overexpression of BMI1 and FasL genes in colorectal cancer tissues compared to non-cancerous tissues. The findings revealed a correlation between advanced clinical parameters and poor prognosis. Nevertheless, the expression of these genes could serve as valuable biomarkers for prognosis, treatment, and potentially early diagnosis in this patient population.

Previous studies have evaluated the tumorigenic effects of BMI1 in colorectal cancer through both *in vitro* and *in vivo* experiments. In a recent study by Karmi et al., it was shown that the inhibition of BMI1 in colon cancer cell lines by miR-200c could effectively suppress their proliferation [28]. This tumorigenic function of BMI1 has been attributed to various mechanisms, including its role in the development and maintenance of cancer stem cells (CSCs) [6], induction of epithelial-mesenchymal

Table 1. Designed primer pair set for qRT-PCR quantification in the study.

Target gene	Primer sequence	Tm (°C)	Amplicon length (bp)
BMI1	F: 5′-ATTGTCTTTTCCGCCCGCTTC-3′	61.81	153 bp
	R: 5′-GGCATCAATGAAGTACCCTCCA-3′	60.09	
FasL	F: 5′-GCCTCCTCTTGAGCAGTCAG-3′	60.11	164 bp
	R: 5′-ACTGCTGTCCACCCAGTAGA-3′	60.18	
β2M	F: 5′-AGCAGCATCATGGAGGTTTGA-3′	59.99	187 bp
	R: 5′-TCAAACATGGAGACAGCACTCA-3′	59.90	

BMI1 - B-cell-specific Moloney murine leukemia virus integration site 1, FasL - Fas ligand, β2M - beta-2-microglobulin, F - forward primer, R - reverse primer, Tm - melting temperature.

Table 2. Association between the expression of BMI1 and FasL in cancerous tissues with clinicopathologic features of CRC patients.

Clinicopathologic features	n	BMI1 expression level (fold change)	p-value	FasL expression level (fold change)	p-value
Age (year)			0.87		0.85
≤ 60	56	6.02 (4.22 - 7.46)		3.65 (2.27 - 6.26)	
> 60	44	5.61 (4.38 - 7.42)		3.82 (2.29 - 5.91)	
Gender			0.91		0.98
Male	57	5.68 (4.17 - 7.42)		3.71 (2.42 - 5.79)	
Female	43	6.00 (4.25 - 7.50)		3.76 (2.14 - 5.96)	
Tumor size			0.0001		0.025
≤ 5 cm	53	5.00 (4.00 - 6.85)		3.11 (2.10 - 4.76)	
> 5 cm	47	6.76 (5.11 - 8.12)		4.19 (3.16 - 6.14)	
TNM stage			0.0001		0.0001
0	5	3.12 (3.01 - 3.80)		1.42 (1.12 - 1.61)	
I	7	3.99 (3.78 - 4.32)		1.61 (1.49 - 2.31)	
II	21	4.80 (3.85 - 5.78)		2.64 (2.16 - 3.30)	
III	32	6.34 (4.88 - 8.05)		3.95 (2.46 - 5.45)	
IV	35	7.33 (6.10 - 8.31)		6.14 (4.07 - 8.14)	
Tumor grade			0.0001		0.0001
I	17	3.99 (3.62 - 4.81)		2.19 (1.47 - 2.47)	
II	44	5.15 (4.23 - 6.92)		3.31 (2.22 - 4.01)	
III	39	7.35 (6.03 - 9.01)		6.03 (4.26 - 7.51)	
Metastasis			0.0001		0.0001
M0	65	5.00 (3.87 - 6.25)		2.64 (1.92 - 4.01)	
M1	35	7.50 (6.32 - 8.64)		6.44 (4.14 - 8.11)	
Lymphatic invasion			0. 1		0.13
Negative	46	6.91 (3.88 - 7.74)		3.87 (2.40 - 6.06)	
Positive	54	7.02 (3.96 - 8.03)		4.29 (2.80 - 6.93)	
Vascular invasion			0.07		0.08
Negative	56	5.15 (4.05 - 7.16)		3.31 (2.23 - 5.29)	
Positive	44	6.73 (4.69 - 7.76)		4.29 (2.80 - 6.35)	

TNM - tumor-node metastasis.

Table 3. Univariate and multivariate Cox regression analyses for determining independent predictors of overall survival in colorectal cancer.

	Univariate		Multivariate	
Clinicopathologic factors	**HR (95% CI)**	**p-value**	**HR (95% CI)**	**p-value**
Age (≤ 60 years/> 60 years)	**0.94 (0.54 - 1.62)**	**0.82**	**1.033 (0.58 - 1.84)**	**0.91**
Gender (male/female)	**1.22 (0.69 - 2.13)**	**0.49**	**1.21 (0.66 - 2.23)**	**0.53**
Tumor size (> 5 cm vs. ≤ 5cm)	**2.47 (1.41 - 4.31)**	**0.002**	**0.97 (0.44 - 2.15)**	**0.93**
TNM stage (III + IV/0 + I + II)	**23.06 (5.57 - 95.06)**	**0.0001**	**8.80 (1.77 - 43.68)**	**0.008**
Tumor grade (III/I + II)	**4.36 (2.46 - 7.71)**	**0.0001**	**1.79 (0.91 - 3.53)**	**0.09**
Distant metastasis (M1/M0)	**3.64 (2.08 - 6.35)**	**0.0001**	**1.47 (0.84 - 4.32)**	**0.07**
Lymphatic invasion (positive/negative)	**3.15 (1.70 - 5.84)**	**0.0001**	**2.53 (0.99 - 6.44)**	**0.051**
Vascular invasion (positive/negative)	**2.47 (1.39 - 4.39)**	**0.002**	**1.66 (0.61 - 2.81)**	**0.20**
BMI1 expression level (high/low)	**6.85 (3.54 - 13.26)**	**0.0001**	**3.32 (1.40 - 7.91)**	**0.007**
FasL expression level(high/low)	**6.12 (3.11 - 12.04)**	**0.0001**	**1.73 (1.09 - 3.87)**	**0.02**

CI - confidence interval, HR - hazard ratio.

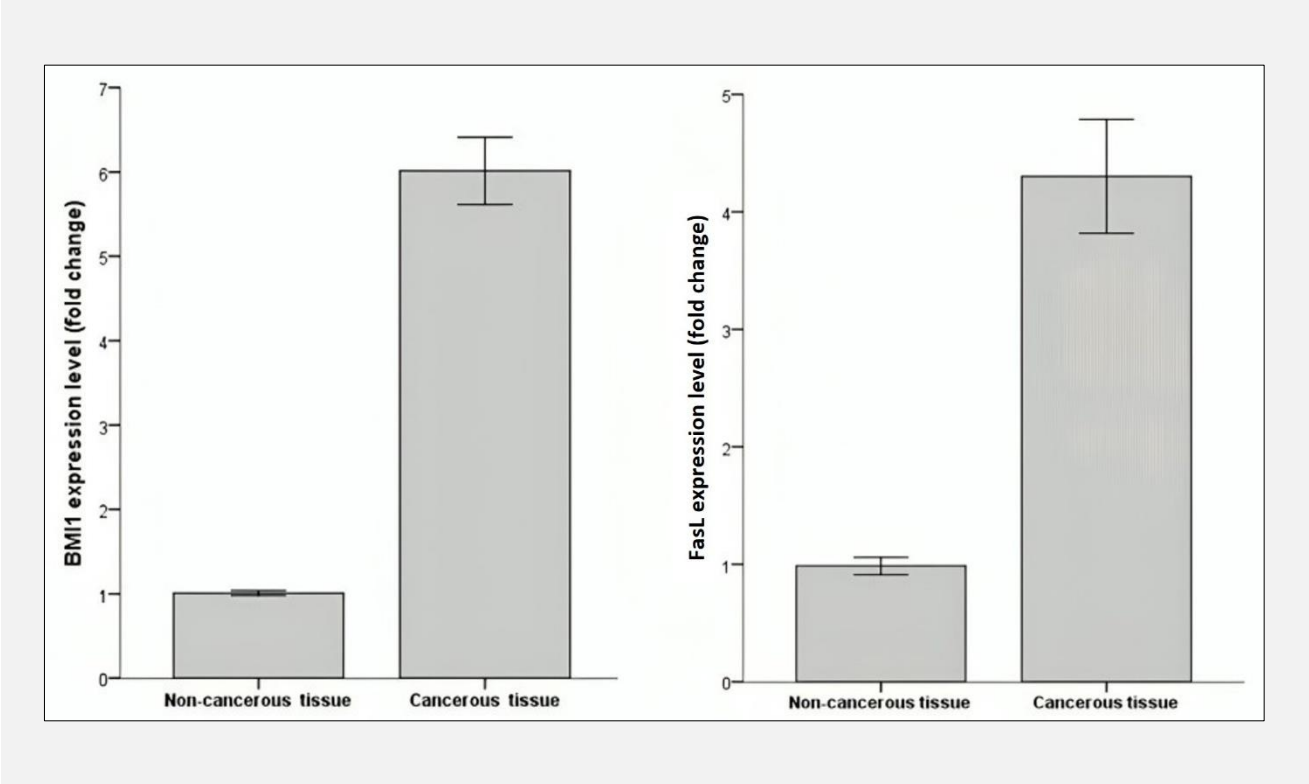

Figure 1. Figure 1 displays higher expression levels of BMI1 and FasL in colorectal cancerous tissues compared to adjacent non-cancerous tissues (p-value = 0.0001, Mann-Whitney U test).

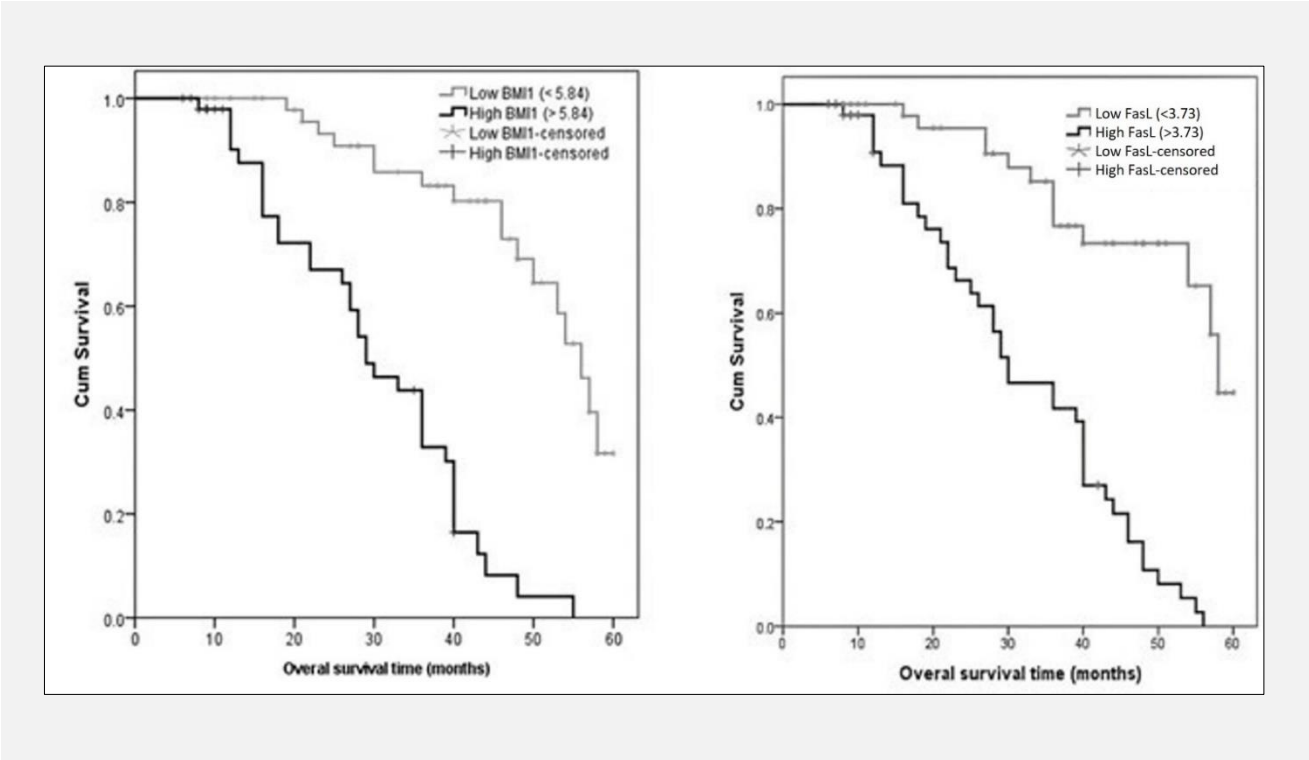

Figure 2. Kaplan-Meier survival curve analysis was performed based on the relative expression values of BMI1 and FasL in cancerous tissues of CRC patients. Elevated expression levels (above the median values) of FasL and BMI1 correlated with reduced overall survival time.

transition (EMT) [29,30], and metastasis and provoking invasion [31]. Furthermore, the overexpression of BMI1 has been linked to resistance against chemotherapy and radiation therapy [9]. Taken together, these findings highlight the promoting effects of BMI1 in various cancers, which are consistent with the results we found in CRC.

Several studies have investigated the clinical effectiveness of BMI1 expression as a classification and prognostic tool in colorectal cancer (CRC), yielding varying results. Li et al. [32] found that higher levels of BMI1 protein in colon cancer tissues were linked to shorter overall survival among patients. Additionally, an evaluation of CRC datasets demonstrated the negative prognostic impact of BMI1 overexpression [33]. However, additional studies have presented conflicting results. One study indicated that higher nuclear expression of BMI1 protein in CRC tumors was strongly linked to prolonged overall survival [34]. Conversely, another study did not observe a significant association between BMI1 protein expression in primary tumors of stage II colon cancer and patient relapse or overall survival [35]. The underlying reason for this inconsistency remains unclear and necessitates further investigation. Moreover, elevated BMI1 mRNA levels in colon cancer patients have been reported to correlate with poorer overall survival rates and clinicopathological variables [36]. The study conducted by Zhang et al. supports our findings, demonstrating that BMI1 mRNA serum levels in CRC patients can serve as an independent prognostic factor for overall survival. However, this factor may not be able to differentiate between the pathological and clinical features of CRC patients [37]. This lack of differentiation seems to be due to the use of serum samples to examine BMI1 gene expression, because serum samples cannot reflect the gradual progression of CRC. Also, this disconnect between clinical features and pathology was similarly noted in Motalebzadeh et al.'s study, albeit with the differentiation that no elevation in BMI1 mRNA levels was observed during tumor tissue analysis [38]. Yet, the sample size in this study was small and may affect the results. In any case, our discovery strengthens the hypothesis that BMI1 is upregulated in CRC, and its expression level could serve as a marker for cancer progression and poor prognosis.

Similar to BMI1, studies have reported conflicting results regarding the role of FasL expression in the pathogenesis of colorectal cancer. In the study by Pryczynicz et al., it was noted that 70% of colorectal cancer tissues displayed strong expression of the FasL protein. At the same time, the glandular epithelium also exhibited this protein's normal expression [25]. Additionally, another study illustrated that the expression of FasL protein escalates in tissues from colorectal adenoma to adenocar-

cinoma stepwise and is associated with the progression and negative outcomes in CRC patients [39].

Furthermore, in a study by Szarynska et al., it was found that patients with advanced CRC exhibit higher levels of FasL mRNA expression compared to patients in the early stages of the disease [24]. These elevated levels of FasL expression could serve as a diagnostic marker to differentiate between early and late stages of CRC [40].

In another study, it was reported that the activation of FasL in human colorectal cancer cell lines leads to the expression of BMI1. The upregulation of these genes in precancerous and cancerous colorectal tissues is linked to the progression of colorectal cancer and a poorer prognosis [26]. Recent studies support our findings regarding FasL expression in colorectal cancer. However, there is limited understanding of the interplay between FasL and BMI1 expression in colorectal cancer. Therefore, it is necessary to conduct more studies in this field to investigate the relationship between the expression of these genes and the progress and prognosis of colorectal cancer. One of the limitations of this study is the absence of information regarding adenoma or benign tissue and its association with the expression of the genes under investigation. Additionally, the small sample size in this study posed a limitation for the authors. For future research, it is recommended to conduct studies with larger sample sizes and to assess the expression of these genes in relation to benign tumors. This could provide further insights into the role of FasL and BMI1 in colorectal cancer progression.

CONCLUSION

According to our study findings, we observed increased expression of FasL and BMI1 genes in the cancerous tissue of colorectal cancer patients, while there was a correlation between advanced clinical parameters and poor prognosis. However, the heightened expression of these genes can signify disease progression and prognosis. Nevertheless, additional evidence is required to confirm the predictive value of these potential biomarkers in colorectal cancer. Further research and validation studies are crucial to determine the practical utility of these markers in clinical settings.

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Declaration of Interest:
All authors have no conflicts of interest to declare.

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