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Analysis of Promoter Hypermethylation of *DAPK* and *BAX* Apoptotic Genes in Iranian Gastric Cancer Patients Undergoing Chemotherapy

Somayeh Nazerian Poudineh, Golnaz Asaadi Tehrani♦

Department of Genetics, Faculty of Basic Sciences, Zanjan Branch, Islamic Azad University, Zanjan, Iran

Abstract

Background: The apoptotic route is mostly damaged in gastric cancer tumor cells. DNA methylation of promoter associated CpG islands inactivates tumor suppressor genes. The objective of the present study was to analyze the hypermethylation of death-associated protein kinase (*DAPK*) and Bcl-2-associated X protein (*BAX*) genes in individuals suffering from gastric cancer and undergoing chemotherapy.

Methods: Genomic DNA was extracted from blood samples and the tissue fixed in the paraffin of 30 patients and normal individuals. Hypermethylation investigation of *DAPK* and *BAX* genes was conducted via methylation specific PCR technique, the outcomes of which were analyzed through electrophoresis and SPSS software version 20.

Results: Methylation of both BAX and *DAPK* genes with a frequency of (28.3%, 21.7%) in blood and (23.3%, 23.3%) in tissue, respectively, had a significant relationship with gastric cancer (P<0.01). A significant relationship was also observed between the methylation of *BAX* gene in tissue and tumor type (12, 35.3% and P<0.01). No relationship was found between methylation and grade, stage, node, age, sex, and other pathologic and clinical data of the patients (P>0.05). There was a significant association between simultaneous methylation of *DAPK* and *BAX* genes in tumor and typical tissues with methylation a frequency of 40% and 95.83%, respectively (P< 0.01).

Conclusion: Methylation of the *BAX* and *DAPK* genes can be used as a biomarker in blood and an approach in the early detection of malignity and illness management. Methylation inhibitors with the potential for drug targeting of *DAPK* and *BAX* can further be employed in pharmacotherapy.

Keywords: *BAX*, *DAPK*, Apoptosis, Gastric cancer, Hypermethylation

♦**Corresponding Author:**
Golnaz Asaadi Tehrani, PhD
Department of Genetics, Faculty of Basic Sciences, Zanjan Branch, Islamic Azad University, Etemadeyeh, Moalem St, Zanjan, Iran
Tel: +9824 33420030
Fax: 4515658145
Email:
golnaz_asaadi@yahoo.com

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Introduction

Gastric tumor is the fourth most common cancer and the second leading reason of cancer-related mortality all over the world. It is estimated that more than of 930,000 new cases are being diagnosed each year and a minimum of 700,000 patients die from the disease.[1] The northern (Mazandaran and Golestan) and northwest (Ardebil) regions of Iran have the highest incidence of gastric cancer with age standardized rate (ASR) of 49.1 and 25.4 in men and women, respectively.[2]

Investigations have found that cancers that develop in the stomach area, compared to other areas in the abdomen, are malignant tumors.[3] A combination of genetic, epigenetic and environmental factors contribute to gastric cancer tumorigenesis and progression; mutations in genes associated with cell proliferation, apoptosis, invasion and metastasis form the basis of genetic events for conversion from normal to cancer cells and cancer progression. Genes such as *E-cadherin VEGF, Cyclin D P21* and *P53* are used as prognostic factors in gastric cancer. There are numerous risk factors for gastric cancer including diet of charred foods, salty diet, foods with preservatives, alcohol use, history of smoking, and *Helicobacter pylori* infection.[4]

One of the most important epigenetic mechanisms is the 5-nucleoside carbonylation of cytosine in regions containing CpG dinucleotide to produce 5-methyl cytosine. Methylated interferes with the binding of transcription factors and inhibits the expression of genes.[5-7]

DNA methylation is regulated by the DNA methyl transferase. Seemingly, increased expression of DNA methyl transferases is a common feature in a variety of cancers.[8] Methylation patterns occur during fetal development and are inherited through mitosis. These normal patterns are disrupted in the DNA of cancer cells, such that CpG is susceptible to methyl transferase activity and other regions of the DNA are hypomotylized.[9] The hypermethylation profile of the CpG varies in different genes for each type of cancer. Generally, hypermethylation of CpG islands in tumor suppressor genes, which is involved in the cell cycle, DNA repair, carcinogenic metabolism, inter-cellular interactions, cell death, and angiogenesis triggers cancer.[9, 10]

Apoptosis is one of the main types of programmed cell death. The inactivation of apoptosis related tumor silencer qualities may promote anomalous cell proliferation, ultimately leading to tumorigenesis or epigenetic hushing associated with promoter hypermethylation of CpG islands in the promoters of a large number of these qualities.[11,12]

BCL 2 protein family members such as apoptotic and antiapoptotic activator composed following the initiation of apoptosis, Bcl-2-associated X protein (Bax) and Bak, undergo a conformational change; thereby, inducing the per mobilization of the outer mitochondrial membrane, releasing several mitochondrial proteins from intermembrane space (cytochrome c, Smac/diablo, HtrA2/omi)[5] and ultimately, leading to cell suicide.[12,13]

Death-associated protein kinase (DAPK) apoptotic proteins are partly responsible for cell death. Domain kinase is a protein kinase that regulates calcium/calmadolyn performance and has ankyrin repeat and death domains. It has been observed that DAPK activities are reduced by promoter methylation in the number of cancers, including gastric cancer.[14] DAPK is involved in the number of apoptotic pathways, including downstream of CD95 (FAS) and tumor necrosis factor-alpha.[15, 16] The protein kinase and passing areas of DAPK have been specifically ensnared in cell works using refined cells transfected with DNA develops encoding *DAPK* or changed *DAPK*.[16,17] P53 activation by DNA damage or oncogenic expression has led to increased expressions of *DAPK*. P53 expression increases the expression of *DAPK* and it seems that there is a feedback cycle regulation of P53 and apoptosis in control by DAPK.[18,20] When DNA is damaged, DAPK is activated, increasing the intensity of P53 proteins from the cytosol; migration of Bad protein to mitochondria and its binding to BCL-xl separates BAX from BCL-xl, releasing cytochrome C.[20,21]

The loss of *DAPK* gene expression through promoter methylation is involved in many tumors. Studies have shown that methylation of the *DAPK* promoter may play a key role in carcinogenesis and gastric cancer progression.Studies have reported that *DAPK* promoter methylation has a relatively higher frequency in GC compared with non-malignant samples.[22-34]

Given the critical role of *BAX* and *DAPK* genes in apoptosis, the objective of the present study was to analyze the promoter hypermethylation of these genes in gastric cancer tissue samples and normal tissues, compare aberrant methylation between tissue and blood samples of patients, and demonstrate the relationships between promoter hypermethylation of the mentioned genes and clinically pathological parameters of gastric cancer patients.

Materials and methods

Characterization of clinical specimens

The present analytical cross-sectional study included 30 patients with tumor gastric carcinoma, and in parallel, 30 blood samples arbitrarily selected from the patients by surgical resection from 2014 to 2016. Specimens were provided by Imam Khomeini Hospital and 30 tissue samples of control individuals (healthy population) were selected from Imam Hussein Hospital; the pathology data is indicated in table 1. Control individuals had no record of gastric carcinoma or related clinical symptoms and had no familiar relationship with patients. Educated consent was acquired from all the patients who referred to the organization of medical sciences. The Ethics Committee of Zanjan University of Medical Sciences approved this study (reference number: ZUM.REC.1396.58). Tissue sample was paraffinized and maintained in laboratory temperature until DNA extraction. Blood samples were collected in tubes containing EDTA at -20°C for long-term storage, and histological findings were analyzed by a pathologist.

DNA extraction, bisulfite modification and methylation-specific PCR

Genomic DNA was obtained from tests utilizing a ZS Genomic DNA™ Tissue Smaller than expected Prep Pack as indicated by the manufacturer's guidelines (Qiagen, USA). DNA concentration was measured by spectrophotometric ingestion, and the A260/280 proportion and its integrity were checked by 1% gel electrophoresis.

The methylation status of *BAX* and *DAPK* qualities promoter was dictated by bisulfite treatment of DNA. Bisulfite treatment was performed utilizing an EZ DNA Methylation Gold Kit™ (Qiagen, USA) as per the manufacturer's directions/convention. The methylation status of qualities was assessed utilizing the methylation spesific polymerase chain response (MSP) technique. The methylated and unmethylated DNA succession preliminaries are recorded in table 1. PCR was performed in an aggregate volume of 20 µl, containing 10 µl(2X) Master Mix (PCR buffer, dNTP, MgCl2, Taq DNA polymerase) [Pars Tous, Iran], 6 µl DNase Free Water, 1µl(0. 5 µM) of each forward and reverse primer and 2µl(100ng) of converted DNA. MSP included 35 cycles, starting with denaturation at 95 ºC for 5 min, trailed by 35 cycles: at 95 ºC for 45 sec (denaturation), at 58ºC (*BAX* u and *DAPK* m), 56/8ºC (*BAX* m), and 60/4ºC (*DAPK* u) for 45 sec (annealing), at 72 ºC for 45 sec (extension), and a final extension for 5 min at 72 ºC. Genomic DNA untreated for bisultite alteration and water without DNA samples were incorporated as negative controls in each run. PCR items (15 µl) were settled by 2.5% TBE gel electrophoresis. In the situations that both methylated and unmethylated bands showed up in a gel, they were considered as a hemimethylation genotype. In order to confirm the accuracy of responses, each MSP reaction repeated two times.

Statistical analysis

The association of methylation frequencies of *BAX* and *DAPK* genes with gastric carcinoma (both tissue and blood samples) was evaluated with the SPSS20 statistics software. To test the

Table 1. Methylated specific PCR primers sequences

Primer	Primer sequence (5'- 3')	Product Size (bp)*	Annealing temperature
***BAX* M**	F: GAGGTAGGTGCGGTTACGTG R:AATCACGTAAAAACCCCGCT	102	56.8 °C
***BAX* U**	F:GGT GTT GTG GGG TAG TGG TT R:ACC ACC TCT CAC CAA ATC CA	118	58°C
***DAPK* M**	F:GGA TAG TCG GAT CGA GTT AAC GTC R:CCC TCC CAA ACG CCG A	98	58°C
***DAPK* U**	F:GGA GGA TAG TTG GAT TGA GTT AAT GTT R:CAA ATC CCT CCC AAA CAC CAA	106	60.4°C

M: Methylated specific PCR primers. U: Unmethylated specific PCR primers. Bax: Bcl-2-associated X protein, *DAPK*: Death-associated protein kinase. A: Adenine. T: Thymine. G: Guanine. C: Cytosine. F: Forward.R: Reverse.

theory; regarding the intendance of two factors, the Chi-square or the Fisher's exact test was utilized. Strategic relapse examination was performed to assess odd ratio (OR) and 95% confidence interval (CI). A Spearman's coefficient was calculated to determine the correlation. *P* value of <0.05 was considered as significant.

Results

DNA methylation is an important phenomenon in the regulation of gene expression. The main focus of the study was to analyze the hypermethylation of genes *DAPK* and *BAX* in 30 individuals suffering from gastric cancer undergoing chemotherapy and 30 controls in Tehran. As indicated by the UICC standard, 4 were T1, 3 were T2, 19 were T3, and 4 were T4. Lymph node metastasis was found in 18 cases. Non-lymph node metastasis was found in 12 cases. Furthermore, 7 of the cases were poorly- and 23 were moderately-, or well-differentiated. This gathering contained 22 male and 8 females; aged 34 to 78 years (mean age of 61.6 years).

BAX and DAPK genes methylation distribution

After treatment of the extracted DNA with sodium bisulfide to convert non-methylated cytosines to uracil, MSP analysis of genes by methylated and non-methylate primers showed 102, 118 bp and 98,106 bp bands, respectively.

Methylation frequencies of *BAX* and *DAPK* genes in tumor and normal tissues and blood samples are shown in table 2. According to the MSP analysis, our results showed that the *DAPK* gene was methylated in 45% of normal and 21.7% of cancerous tissues (*P*=0.0027). Also, *BAX* gene methylation frequency was observed in 43.3% and 23.3% of normal and cancerous tissues, respectively (*P*=0.0062). Data analysis showed a significant relationship between both genes methylation and gastric cancer (*P*<0.05). Our findings indicated that in patients with significantly higher frequency of methylation, gastric cancer risk was increased. Also, no significant statistical relationship was found regarding *BAX* gene methylation between blood and tissue samples (*P*=0.6735) and *DAPK* gene (*P*=0.5590); hence, it can be suggested as the introduction of blood samples as a non-invasive marker for prognosis and early detection of gastric cancer. The obtained results showed a significant relationship between the methylation analysis for normal and cancerous tissues of *BAX* and *DAPK* genes (*P*=0.0049) (Table 2).

Association of clinopathological factors

In the current research, possible association between clinicopathological characteristics of patients including age, sex, size, grade, stage, node tumor type, and methylation status of *DAPK*, and *BAX* genes were assessed. Two cancer types (I and D) were considered and using Chi-Square test, data analysis was performed, where a significant relationship was found between the type of cancerous tissue and gene methylation of *BAX* in the tissues of patients with gastric cancer (*P*=0.001). In addition, using Chi-square test proved that other clinical characteristics,

Table 2. Methylation status of *DAPK* and *BAX* genes in normal and tumor tissues and blood samples of patients

Gene	Sample (number)	Methylated Number (%)	Hemimethylated Number (%)	Non-methylated Number (%)	OR	95% CI	*P*-value
BAX	Healthy tissue (30)	26 (43.3%)	4(6.7%)	0(0%)	0.1964	0.0613 to 0.6295	0.0062
	Patients tissue (30)	14(23.3%)	16(26.7%)	0(0%)			
	Patients' blood (30)	17(28.3%)	12(20%)	1(1.7%)	0.8370	0.3657 to 1.9153	0.6735
DAPK	Healthy tissue (30)	27(45%)	2(3.3%)	1(1.7%)	0.1706	0.0538 to 0.5411	0.0027
	Patients tissue (30)	13(21.7%)	16(26.7%)	1(1.7%)			
	Patients' blood (30)	14(23.3%)	11(18.3%)	5(8.3%)	1.2564	0.5842 to 2.7020	0.5590

OR: odds ratios, CI: confidence intervals. *Bax*: Bcl-2-associated X protein, *DAPK*: Death-associated protein kinase.

including tumor size, grade, stage, node, and gender in patients with gastric cancer had no relationship with *BAX* and *DAPK* DNA methylation, meaning all these parameters acted independently (Table 3).

Discussion

In this study, we investigated the promoter hypermethylation of two apoptotic genes, *BAX* and *DAPK* in FFPE and blood samples of gastric cancer patients and normal individuals. We observed a statistically significant relationship between the hypermethylation of genes and gastric cancer in patients and normal control group. In addition, the evaluation of methylation status in blood and FFPE samples of patients showed no significant difference, suggesting serum analysis as a non-invasive diagnosis marker for gastric cancer. Also, a significant correlation was observed between simultaneous methylation of *DAPK* and *BAX* genes in tumor and typical tissues with methylation frequency, yet no other significant associations were detected between methylation status and other clinicopathological features.

In many cancers, tumor suppressor genes are inactivated, which does not necessarily lead to gene methylation.[35] Silencing of tumor-related genes by aberrant promoter methylation is implicated in the occurrence and development of cancers such as GC.[36,37] Numerous studies have reported that many tumor suppressor genes, play key roles in functions regarding to cancer prevention (DNA repair, cell adhesion, cell cycle control, and apoptosis) are silenced by the hypermethylation of their promoters during carcinogenesis.[38] Since methylation is an epigenetic change in cancer and demethylation mechanism is reversible, re-activate tumor-suppressing gene expression that leads to cell cycle arrest and apoptosis following methylation inhibitors can be used as drug treatment.[39]

The current study showed that the hypermethylation silenced tumor inhibiting genes, *BAX* and *DAPK,* which is associated with gastric cancer. Furthermore, in 2007, Zhang et al.[40] conducted the hypermethylation of tumor suppressor genes in the glandular stomach carcinogens in human cancer studies. The results showed that methylation-rich regions in the CpG promoters of many genes assumed an essential part in the pathogenesis of many human cancers, including glandular stomach. *DAPK* reduced the activity in a number of cancers, mainly related to regional hypermethylation and prevented apoptosis.

In our research, the frequency of *DAPK* gene promoter methylation in gastric cancer tissues was 21.7%, in comparison with normal tissues. Examining the promoter methylation of *DAPK* gene in 30 patients' tissue samples with gastric cancer, 13 (21.7%) and 16 (26.7%) of samples were methylated and hemimethylated, respectively. A research was conducted with Ying Li and his colleges in 2015, on promoter methylation of *DAPK* status in lung malignancy; they observed that the frequency of *DAPK* methylation was significantly higher in lung cancer than in non-malignant lung tissues. Results also showed the presence of a prognostic impact of *DAPK* gene methylation in lung cancer patients.[41] In 2011, Jabłonowski et al.[42] investigated the methylated and non-methylated promoter regions of *DAPK* in patients with non-invasive bladder cancer. Methylation was observed in 64.3% of cases, which was statistically significant; furthermore, a statistically significant

Table 3. Relationships of aberrant hypermethylation in gastric cancer with clinicopathological parameter

Clinical Parameters		*BAX* methylation in blood	*P*-value	*BAX* methylation in tissue	*P*-value	*DAPK*	*P*-value methylation in blood	*DAPK*	*P*-value methylation in tissue
Age	<50	2(6.9%)	0.469	0(0/0%)	0.157	2(6.9%)	0.316	0(0/0%)	0.418
	≥ 50	15(51.7%)		14(48.3%)		12(41.4%)		11(37.9%)	
Sex	Male	10(33.3%)	0.338	10(33.3%)	0.825	8(26.7%)	0.332	8(26.7%)	0.828
	Female	6(20.0%)		4(13.3%)		4(13.3%)		3(10.0%)	
Tumor size	< 5 mm	11(37.9%)	0.417	9(31.0%)	0.550	6(20.7%).	0.519	6(20.7%).	0.417
	≥ 5mm	6(20.7%)		5(17.2%)		6(20.7%)		5(17.2%)	
Histological grade	Grade I	1(3.4%)	0.818	0(0.0%)	0.151	1(3.4%).	0.698	1(3.4%).	0.227
	Grade II	6(20.7%)		3(10.3%)		2(6.9%)		1(3.4%)	
	Grade III	10(34.5%)		12(41.4%)		10(34.5%)		10(34.5%)	
Pathological Stage	PT1	2(7.1%)	0.642	1(3.7%)	0.797	1(3.6%)	0.445	1(3.6%).	0.334
	PT2	3(10.7%)		1(3.7%)		3(10.7%)		2(7.1%)	
	PT3	10(35.7%)		10(37.0%)		10(35.7%)		9(32.1%)	
	PT4	2(7.1%)		1(3.7%)		1(3.6%)		1(3.6%)	
Lymph Node	N0	6(20.0%)	0.581	7(23.3%)	0.145	7(23.3%).	0.502	4(13.3%).	0.179
	N1	7(23.3%)		3(10.0%)		1(3.3%)		5(16.7%)	
	N2	2(6.7%)		0(0.0%)		2(6.7%)		0(0.0%)	
	N3	3(10.0%)		4(13.3%)		(10.0%)		2(6.7%)	
Differentiation	Moderate	10(33.3%)	0.466	7(23.3%)	0.259	8(26.7%).	0.837	3(10.0%)	0.271
	Poor	4(13.3%)		5(16.7%)		2(6.7%)		4(13.3%)	
	Well	5(16.7%)		2(6.7%)		3(10.0%)		4(13.3%)	
Type	I	15(50.0%)	0.858	12(35.3%)	0.001	11(36.7%)	0.628	10(33.3%).	0.779
	D	2(6.7%)		2(5.9%)		2(6.7%)		1(3.3%)	

I: intestinal. D: diffuse N: Node *Bax*: Bcl-2-associated X protein. *DAPK*: Death-associated protein kinase

higher frequency of *DAPK* gene methylation (71.4%) was observed in patients with lower grade (G1) bladder cancer. Kupcinskaite et al.[43] examined CpG island methylation of *DAPK* gene in stomach tissues; their results confirmed 27.5% and 44.9% methylation frequency in cancerous and adjacent non-cancerous tissues, respectively, but the difference was not significant. There was no significant association between methylation status of *DAPK* gene and clinopathological characteristics such as age, sex, tumor type, and staging. Promoter methylation and experimental data were described in a research, proving the importance of *DAPK* and labeled tumor inhibitory activity of it for prediction and diagnosis of cancer.[44]

Zargar et al. [45] studied the DNA methylation promoter of *BAX* gene in breast and colorectal cancer cells, concluding that no hypermethylation of CpG islands in the *BAX* gene promoter cells was derived from breast and colorectal cancers. It seems that CpG island methylation of *BAX* gene does not play a significant role in the regulation of downstream genes in breast and colorectal cancers.

Researchers conducted by Hervouet et al.[46] on patients with glioma cells showed that methylation in the *BAX* promoter reduced or completely suppressed its expression. In addition, suppression of *BAX* by RNAi conferred resistant to ligand-mediated Fas, inducing apoptosis in cell cultures, showing the role of the mediator of methylation-intervened *BAX* extinguishing in resistance to apoptosis in GMB. A small number of GBM patients undergo *BAX* quieting, which does not allow for a powerful measurable investigation of survival. In any case, it should be noted that in a population of 27 GBM patients, patients exhibiting *BAX* deactivation via methylation had at least a survival curve.

Another study was conducted in 2016 to analyze DNA methylation in tumor and adjacent natural tissues using HPAII / MspI limited digestion and methylation specific PCR in colorectal cancer. In the PCR method, a

methylation-sensitive limiting enzyme, Extract DNA samples, was digested with HPAII and MspI methylation sensitive enzymes. Primers are located in the cut-off site of the HpaII / MspI enzyme. The present study showed there was no critical distinction in the condition of methylation for the *BAX* gene in HpaII tests.[47] Our results also indicated that *BAX* gene methylation promoter relationship between gastric cancers were statistically significant *(P*<0.01). The frequency of methylation was 14 (23.3%) and 17 (28.3%) in tissue and blood, respectively. It can be concluded that the methylation of CpG islands promoter *BAX* gene as a tumor inhibiting gene prevented from attending the intrinsic pathway of apoptosis in gastric cancer.

According to the present results, promoter methylation of the *DAPK* quality in tissue was altogether correlated with the kind of gastric cancer, yet no significant relationship was observed with other pathological factors such as age, stage, and tumor size. Narayan et al.[48] conducted a study on the methylation of the *DAPK* gene in the carcinoma of Cervix Uteri in 2003. In the CC cell, methylation frequency of *DAPK* was reported to be 25%. Pathological analysis did not show any significant relationship between tumor size, age, and stage. Liu Xiaofang et al.[49] performed studies on the relationship between P53 mutation and the multi-gene methylation, in particular *DAPK*, with pathologic information on cholangiocarcinoma. The results indicated a significant correlation with differentiation, invasion and pathologic type, but no correlation with tumor node, in our present investigation; however, there was no association among methylation with stage, grade, node, and type regarding tumor tissue.

Blood biomarkers can detect first-degree, residual, or recurrent cancers for both early detection and prevention. The identification of reliable biomarkers for an early diagnosis, predictive markers of recurrence and survival and markers of drug sensitivity and/ or resistance is urgently needed.[50] It is reasonable to hypothesize that the DNA methylation status of certain genes serves as a useful biomarker for predicting tumor behavior. Furthermore, DNA methylation biomarkers offer several advantages over genetic and serum markers.[51, 52] First, the incidence of aberrant DNA methylation of specific CGIs is higher than that observed in genetic abnormalities.[52,53] Second, the aberrant DNA methylation observed in cancer can be sensitively detected using a simple technique, methylation-specific PCR. Third, aberrant DNA methylation appears to occur in early-stage tumors, causing the loss- and/or gain-of-function of key processes and signaling properties.[54]

Several studies have reported that hypermethylated genes exist in plasma or serum GC patients. Hypermethylated genes represent the highest diagnostic value for GC detection.[55] In our research, the recurrence of *BAX* and *DAPK* methylation in tissue and blood was (21.7%, 23.3%) and (23.3%, 28%), respectively. No significant difference existed between *BAX* and *DAPK* promoter hypermethylation regarding blood and FFPE samples. The methylation status of the blood sample could be an early, non-invasion diagnostic marker for gastric cancer.

DNA methylation has a great potential to provide valuable information for understanding the malignant behavior of GC. Further investigations on the DNA methylation status, which regulates cancer initiation, proliferation, invasion, metastasis and drug resistance, will aid in designing strategies for an earlier detection and better therapeutic decision making in the setting of GC.[50]

In conclusion, our data indicated that a significant relationship between methylation status of *DAPK* and *BAX* genes with gastric malignancy in tissue and blood samples of patients.The amount of methylation of the two genes was higher in patients with gastric carcinoma in comparison with normal cases. A significant relationship was found between the simultaneous methylation of *BAX* and *DAPK* genes and gastric cancer. There was a significant association between the *BAX* quality methylation in the tissue and type of the cancer; in contrast, there was no relationship with other clinical and pathological symptoms of the patients.

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Conflict of Interest

None declared.

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