Original Article

Detecting promoter methylation pattern of apoptotic genes *Apaf1* and *Caspase8* in gastric carcinoma patients undergoing chemotherapy

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Background: DNA methylation patterns in cells dysregulation CpG island methylation of genes involved in cancer leads to increased levels of the cancer. Restoration of the apoptotic route in tumor cells of stomach in order for placing *Casp8* and *Apaf1* genes is a proper approach for new treatments of gastric cancer. The objective of the present study was to investigate the relationship between the pattern of methylation promoter in apoptotic genes of *Casp8* and *Apaf1* and gastric carcinoma in patients receiving chemotherapy.

Methods: Genomic DNA was extracted from 30 samples of FFPE tumor, normal tissues and blood samples. Hyper-methylation analysis of *Casp8* and *Apaf1* genes was conducted using MSP method; the results were analyzed through electrophoresis on agarose gel and software spss20.

Results: In this study, methylation rate of *Apaf1* gene with (P>0.05) was not significant but methylation rate of *Casp8* gene with (P<0.05) was significant. In addition, there was a significant relationship between *Apaf1* gene methylation in blood with stage (P<0.05), *Apaf1* gene methylation in tissue with stage (P<0.05) and grade (P<0.01) and between *Casp8* gene methylation in blood with age (P<0.001) of patients but no significant relationship was seen for other factors.

Conclusions: Our results suggest that epigenetic mechanisms play an important role in the pathogenesis of gastric cancer and can be utilized as prognostic biomarkers for it. Also no significant difference between *Casp8* and *Apaf1* promoter hypermethylation in blood and tissue samples indicated that methylation status of blood sample can be early and non-invasive diagnostic marker in gastric cancer.

Keywords: Gastric cancer; methylation; apoptosis; chemotherapy; *Apaf1*; *Casp8*

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Introduction

Gastric cancer is the fourth most common cancer in which is the second leading cause of death-related cancers (1,2). Its incidence varies among different regions and countries. Each year, there are approximately 934,000 new cases of gastric cancer in the world, 56% of which occur in East Asia. Among these new cases, 41% come from China and 11% from Japan (3,4). There are two main types of gastric adenocarcinoma: intestinal and diffuse. The accepted paradigm for the pathogenesis of the intestinal-type is a multi-step progress from chronic gastritis to gastric atrophy to intestinal metaplasia to dysplasia. The pathogenesis of diffuse-type gastric cancer is not yet completely understood (5).

Both environmental alternations (6) including *Helicobacter pylori* (*H. pylori*) infection (7), Epstein-Barr virus (8), diet (5) and Genetic alterations, such as *p53*, *KRAS*, *PIK3CA*,

ARID1A, *MLL3* and *MLL* mutations, as well as *PIK3CA*, *C-MET*, *ERBB4*, and *CD44* amplifications, are repeatedly found in gastric cancer, that suggest key tumorigenic events and their critical role in gastric tumor genesis (9,10). Epigenetic alterations also involved in progression of gastric cancer, including DNA methylation, post-translational modifications of histones, noncoding RNAs, and nucleosome positioning (11,12). DNA methylation which is the most common epigenetic alternation occurs at the C5 position of cytosine (5mC), mostly within CpG dinucleotide, with the DNMT enzymes using a protected mechanism, which provides a stable gene silencing mechanism and it has an important role in regulation of gene expression (13-15). DNA methylation can grant a selective growth advantage to cells when it occurs in the promoter regions of genes involved in growth regulation and DNA damage responses, that results in the development of cancer (16).

Gastric cancer's formation and progression are processes which are continuous and multiple-step (17,18). The best-known type of programmed cell death is apoptosis which plays important roles in growth and homeostasis as well as pathogenesis of many diseases (19,20). The apoptotic cascade can be triggered through two major pathways extracellular signals, such as members of the tumor necrosis factor (TNF) family can activate the receptor-mediated extrinsic pathway. Alternatively, stress signals such as DNA damage, hypoxia, and loss of survival signals may trigger the mitochondrial intrinsic pathway (21).

Various cell death receptors including TNFR1 and CD95L can trigger the caspase-8 dependent extrinsic apoptotic pathway (22,23). One of the best-defined apoptotic pathways is mediated by the death receptor CD95 (APO-1/Fas). Triggering of CD95 by its natural ligand CD95L or agonistic antibodies induces the formation of a death-inducing signaling complex (DISC) consisting of the adaptor protein Fas-associated death domain protein [FADD (MORT-1)] and procaspase-8 [FADD-like IL-1 b–converting enzyme (FLICE, Mch5)] (24,25). Two hemophilic protein interaction domains mediate the DISC formation: FADD which contains a COOH-terminal death domain (DD), and couples to the DD of the intracellular part of CD95. FADD, in addition, contains an NH2-terminal so-called death effector domain (DED), which binds to one of the DEDs of caspase-8. Further downstream, caspase-8 triggers the proteolysis activation of other caspases and cleavage of cellular substrates (21,26). *Apaf-1* gene participates in the pathway of mitochondria-mediated apoptosis. UV and ionizing radiation, hypoxia, cytochrome c is released from the mitochondria when DNA is damaged by chemotherapeutic agents, oncogenic stimuli, binds to Apaf-1 in the cytosol, and in association with dATP/ATP, facilitates a conformational change of Apaf-1 to expose its CARD domain (27). Afterwards, Apaf-1 oligomerizes through the unconcealed CARD domain and catalyzes auto activation of caspase-9, leading to the serial activation of downstream effector caspases such as caspase-3, caspase-6, and caspase-7, which results in apoptotic cell death (28).

Given the importance of *Apaf1* and *Casp8* genes in the process of apoptosis in patients receiving chemotherapy, the aim of this study was to investigate the relationship between patterns of apoptotic gene promoter methylation in gastric carcinoma in patients undergoing chemotherapy. In addition, Comparison of the promoter methylation in the blood and tissue samples in gastric cancer was performed and the impact of epigenetic in carcinogenesis was examined. Also in this study the relationship between methylation patterns of these genes with clinicopathological characteristics of patients was investigated.

Methods

Study population

Thirty samples of patient's blood and tissue that were diagnosed through clinical experiments of gastric carcinoma with average age of 61.8 years old were provided from Imam Khomeini (mercy upon him) hospital. 30 tissue samples of control individuals without any record of gastric carcinoma or related clinical symptoms and no kinship relations with patients were selected from Imam Hussein (PBH) hospital (pathological data were indicated in *Table 1*). Tissue samples were paraffinized after surgery which maintainable in laboratory temperature, blood samples were provided in the tube containing EDTA and maintained for a longtime in –20 ℃. This study was conducted at the Biological Research Center of Azad Islamic University of Zanjan and approved by the faculty of medical sciences Ethics Committee. Informed consent was taken from all the patients before entering the study and all the obtained information's from each participant was completely confidential.

Analysis of CASP8 and Apaf1 promoter methylation status

Genomic DNA was extracted from 25–30 ng of tissue using a ZS Genomic DNA™ Tissue Mini Prep Kit (Qiagen, Hilden, Germany) according to the manufacturer's

Table 1 Patient pathology information

Clinicopathological parameters	Total number (%)
Age (years)	
<50	27 (90.00)
≥50	3 (10.00)
Sex	
Male	22 (73.33)
Female	8 (26.67)
Adenocarcinoma	30 (100.00)
Tumor size (mm)	
<5	17 (56.67)
≥5	13 (43.33)
Histological grade	
Grade I	3 (10.00)
Grade II	8 (26.66)
Grade III	19 (63.33)
Metastasis	
Positive	0
Negative	30 (100.00)
Stage (PT)	
PT1	4 (13.33)
PT2	3 (10.00)
PT3	19 (63.33)
PT4	4 (13.33)
NOTE	
N0	12 (40.00)
N1	8 (26.67)
N2	4 (13.33)
N3	6 (20.00)
Differentiated	
Moderately	16 (53.33)
Poorly	7 (23.33)
Well	7 (23.33)
Type	
I	26 (86.67)
D	4 (13.33)

instructions. The DNA concentration was determined by spectrophotometry, and its integrity was checked by 1% gel electrophoresis. Bisulfite treatment was performed using an EZ DNA Methylation Gold Kit™ (Qiagen, Hilden, Germany) according to the manufacturer's instructions/ protocol. The methylation status of the promoters was detected by methylation-specific polymerase chain reaction (MSP). The methylated and unmethylated DNA sequence primers are listed in *Table 2*. PCR was performed in a total volume of 20 μL, containing 10 μL (1×). Master Mix (PCR buffer, dNTP, $MgCl_2$, Taq DNA polymerase), 6 μL DNase Free Water, 1 μL (0.5 μM) Forward primer, 1 μL (0.5 μM). Reverse primer and 2 μL (100 ng) of converted DNA. PCR cycling conditions were: initial denaturation at 95 ℃ for 5 min, followed by 35 cycles of 95 ℃ denaturation for 45 s, annealing for 45 s (primer specific temperatures are listed in *Table 2*), 72 ℃ extensions for 45 s, and final extension at 72 ℃ for 5 min. The PCR products were separated by 2.5% gel electrophoresis. If both methylated and non-methylated bands appeared in the gel represents the hemi-methylation status, if only methylated or non-methylated bands appeared, fully methylated and non-methylated situation was confirmed respectively.

Statistical analysis

Statistical analysis was carried out with the SPSS20 Statistics software. Quantitative data are presented as mean and standard deviation (SD). For testing statistical hypothesis about the independence of two variables, the chi-square test or the Fisher exact test was used. A Spearman coefficient was calculated to determine correlation. The significance level of <0.05 was selected.

Results

Characterization of clinical specimens

According to the expert diagnosis of pathological analysis, all of the patients confirmed with gastric cancer and in the process of metastasis. As shown in *Table 1*, the disease is about 73.33% men and 26.67% of women and 90% are younger than 50 years and most of intestinal type is involved. The majority of patients had tumor size less than 5 mm as well as 63.33% of patients with histological grade III and 63.33% in stage III disease, and also the majority

Table 2 Primers used for methylation-specific polymerase chain reaction

Primer	Forward sequence 5'-3'	Reverse sequence 5'-3'	Product size (bp)	Annealing temperature (℃)
Casp8 (M)	TAGGGGATTCGGAGATTGCG	CGTATATCTACATTCGAAACGA	78	59.2
Casp8 (U)	TAGGGGATTTGGAGATTGTGA	CCATATATATCTACATTCAAAACAA	81	52.7
Apaf1 (M)	GCGGCGATTTTAATTTATAGC	ATACCGAACTCGAACAACGAC	132	59.2
Apaf1 (U)	TGGTGATTTTAATTTATAGTGTTTTTTAT	CCAATACCAAACTCAAACAACAAC	87	60.4

Table 3 Comparison the results of promoter hypermethylation of *Apaf1* and *Casp8* genes in tumor and normal tissues and blood samples

Gene	Sample	Methylated, n (%)	Hemi-methylated, n (%)	Non-methylated, n (%)	OR	95%CI	P value
Apaf1	Healthy tissue (N=30)	0 (0)	26 (86.6)	4 (13.3)	1.2233	0.5960–2.5110	0.5827
Apaf1	Patients tissue (N=30)	0 (0)	29 (96.6)	1 (3.3)			
Apaf1	Patients' blood (N=30)	4 (13.3)	24 (80.0)	2 (6.6)	1.1795	0.5780–2.4073	0.6500
Casp8	Healthy tissue (N=30)	20 (66.6)	10 (33.3)	0 (0)	3.8000	0.9901–14.5845	0.0517
Casp8	Patients tissue (N=30)	27 (90.0)	3 (10.0)	0 (0)			
Casp8	Patients' blood (N=30)	29 (96.6)	1 (3.3)	0 (0)	3.1053	0.3137–30.7349	0.3326

of patients in the moderately and 40% of them are in stage Note0.

Methylation analysis

The methylation status of the promoter region of *Apaf1* and *Casp8* genes was analyzed in 60 FFPE samples (30 cancer cases and 30 normal cases) and 30 blood cancer samples. Thirty cases were analyzed for blood and tissue samples from the same patient simultaneously. Methylation frequencies of *Apaf1* and *Casp8* genes in tumor and normal tissues and blood samples have been shown in *Table 3*. According to the table, tissue samples analysis that *Apaf1* and *casp8* genes promoter in normal FFPE samples were methylated (m+/u–) in 0% and 66.6%, respectively; hemimethylated (m+/u+) in 86.6% and 33.3%, and non-methylated in 13.3% and 0%, respectively; in comparison to patient FFPE sample were methylated 0% and 90%, hemimethylated in 96.6% and 10%, and non-methylated in 3.3% and 0%, our data confirmed significant relationship between promoter hyper methylation of *casp8* gene and gastric cancer (P<0.05). It can be concluded that no significant association between promoter methylation of two genes at the same time (P>0.05).

Also according to analyze the correlation between patient's tissue and blood samples of *Apaf1* and *Casp8* genes, methylation frequency showed (0%, 13.3% and 90%, 96.6%), hemimethylation (96.6%, 80% and 10%, 3.3%), unmethylation (3.3%, 6.6% and 0%, 0%) respectively. There is no significant relationship between methylation frequency of *Apaf1* and *Casp8* genes in tissue and blood samples of patients (P=0.6 and 0.3). So evaluating the status of methylation in the blood offered as a non-invasive approach.

Clinicopathological parameters of gastric cancer were compared with the frequency of *Apaf1* and *Casp8* genes promoter methylation in *Table 4*. Our results from this analysis indicated there is a significant relationship between *Apaf1* gene methylation in blood with stage of cancer (P<0.05) and methylation of this gene in tissue with stage (P<0.05) and grade (P<0.01). In addition correlation between promoter hypermethylation of *Casp8* gene in blood and age of patients, significant association has been observed (P<0.001), but no significant relationship was seen in other pathological factors.

Discussion

DNA methylation is the most extensively and widely studied epigenetic modification in which a methyl group is added to the fifth carbon position of cytosine residue in a CpG dinucleotide. Clusters of CpG dinucleotides

Table 4 Relationship between methylation and pathological condition of *Apaf1* and *Casp8* genes

Clinical parameters	Apaf1 methylation in blood, n (%)	P value	Apaf1 methylation in tissue, n (%)	P value	Casp8 methylation in blood, n (%)	P value	Casp8 methylation in tissue, n (%)	P value
Age (years)		0.756		0.782		0.000		0.632
<50	0 (0.0)		0 (0.0)		1 (3.3)		1 (3.3)	
≥50	4 (13.3)		0 (0.0)		27 (90.0)		18 (60.0)	
Sex		0.256		0.540		0.092		0.243
Male	4 (13.3)		0 (0.0)		22 (73.3)		16 (53.3)	
Female	0 (0.0)		0 (0.0)		7 (23.3)		4 (13.3)	
Tumor size (mm)		0.784		0.226		0.393		0.494
<5	3 (10.0)		0 (0.0)		16 (53.3)		12 (40.0)	
≥5	1 (3.3)		0 (0.0)		12 (40.0)		7 (23.3)	
Histological grade		0.062		0.001		0.761		0.395
Grade I	0 (0.0)		0 (0.0)		2 (6.7)		2 (6.7)	
Grade II	2 (6.7)		0 (0.0)		8 (26.7)		6 (20.0)	
Grade III	2 (6.7)		0 (0.0)		18 (60.0)		11 (36.7)	
Stage (PT)		0.010		0.034		0.921		0.647
PT1	1 (3.3)		0 (0.0)		2 (6.7)		2 (6.7)	
PT2	0 (0.0)		0 (0.0)		3 (10.0)		2 (6.7)	
PT3	2 (6.7)		0 (0.0)		18 (60.0)		11 (36.7)	
PT4	1 (3.3)		0 (0.0)		4 (13.3)		3 (10.0)	
NOTE		0.626		0.416		0.670		0.248
N0	2 (6.7)		0 (0.0)		11 (36.7)		9 (30.0)	
N1	1 (3.3)		0 (0.0)		8 (26.6)		4 (13.3)	
N2	0 (0.0)		0 (0.0)		4 (13.3)		4 (13.3)	
N3	1 (3.3)		0 (0.0)		6 (20.0)		3 (10.0)	
Differentiated		0.649		0.183		0.636		0.062
Moderately	3 (10.0)		0 (0.0)		15 (50.0)		8 (26.7)	
Poorly	1 (3.3)		0 (0.0)		7 (23.3)		5 (16.7)	
Well	0 (0.0)		0 (0.0)		7 (23.3)		7 (23.3)	
Type		0.562		0.690		0.690		0.129
I	4 (13.3)		0 (0.0)		25 (83.3)		16 (53.3)	
D	0 (0.0)		0 (0.0)		4 (13.3)		4 (13.3)	

in CG rich regions of the genome called "CpG islands (CGI)" frequently occurs in the 5'-flanking promoter areas of genes (29). Generally, increased methylation in the promoter region of genes leads to reduced gene expression, whereas methylation in the transcribed region has a variable effect on gene expression (30,31). So, any perceptive understanding of abnormal methylation and subsequent gene silencing, such as methylation inducing factors, which is essential for cancer prediction, prevention, treatment and prognosis evaluation (32).

The empirical evidence showed that the percentage of *Apaf1* gene methylation in normal tissue and the patient and also the percentage of methylation of this gene in tissue and blood with P>0.05, there was not a significant relationship. Li and colleagues investigated the role of DNA methylation in 2003 to prevent Apaf1 protein expression in human leukemia addressed in this study promoter methylation of *Apaf1* in four cases were examined and it was found that P<0.05 was significant. There was evidence that showed *Apaf1* gene methylation detection and treatment by demethylation, regulates *Apaf1* positive expression in both protein and mRNA expression (33). In another study, Wang and associates in 2007 evaluated *Apaf1* gene expression in gastric cancer of 35 samples of cancer tissue and adjacent normal tissue samples, methylation detection was performed by MSP method. Promoter methylation rate was detected 49% in gastric cancer tissue. Methylation significantly decreased expression in 16 of 18 cancerous tissue samples (P=0.000001) (34). In another study, Huang and colleagues in 2004 studied *Apaf1* gene promoter methylation in squamous cell carcinoma of the larynx and in all 11 samples showed that the methylation of promoter regions decreased mRNA gene expression (35). But in the current research, no significant association between gene methylation of 30 patient tissue samples and 30 normal tissue samples with methylation specific PCR method does exist.

In this study we did found that the percentage of *Casp8* gene methylation in normal and pathological tissue as well as tissue between the percentage of methylation of this gene in the blood of patients with P<0.05 there was a significant relationship. Rita and colleagues in 2013 examined methylation CpG islands of *Casp8* gene in tumor tissues and adjacent non-cancerous stomach tissues. In this study, gene methylation of *Casp8* in 69 patients with gastric cancer using methylation-specific PCR was performed, the frequency of methylation in cancer and non-cancerous adjacent was (5.8%), therefore correlation between the methylation of this gene and gastric cancer does not exist (36). Skiriute and colleagues in another study in 2012 examined promoter methylation status of *Casp8* gene for 76 patients with glioblastoma using MSP, they found 56.8% methylated (37). As well as Kordi Tamandani and colleagues in a study in 2009 assess the methylation of CpG islands of *Casp8* gene using methylation specific PCR method in 80 patients with cervical cancer. The rate of methylation was 1.2% and 1.80% respectively with P>0.05 found a significant association between gene methylation of CpG sites and cervical cancer (38). In our study by MSP on tissue samples from 30 patients and 30 normal tissues showed that significant correlation between the percentage of methylation of *Casp8* gene and gastric cancer.

Pathological results of this analysis showed a significant correlation between the percentage of *Apaf1* gene methylation in blood and tumor stage (P<0.05). Communication between methylation in the context of the stage (P<0.05) and grade (P<0.01), there was a significant relationship, But relationship between other pathological characteristics such as age, sex, tumor size, grade has not been seen. There was also a significant association between *Casp8* gene methylation in blood and age (P<0.001), but there was not a significant relationship with other pathological information such as gender, tumor size, Stage, grade. Kupcinskaite-Noreikiene and colleagues in 2013 examined CpG islands methylation of *Casp8* gene in tumor tissues and adjacent non-cancerous tissues in the stomach. In this study, gene methylation in 69 patients with gastric cancer using methylation-specific PCR was performed, results confirmed that the frequency of methylation in cancer and non-cancerous adjacent was 8.5%, therefore no correlation was detected between methylation of the gene and gastric cancer , no link between the characteristics of the patients and methylation of this gene was investigated. And it was found that a significant correlation between the pathological characteristics of this disease, such as age, sex, degree of differentiation, TNM staging of gastric cancer does not exist (36).

Despite the advances in diagnosis and treatment technologies, the prognosis of gastric cancer patients is still poor, even for those who undergo complete resection of their carcinomas. Having known that DNA methylation is a potentially reversible epigenetic alteration, demethylation inhibitors are thus proposed to be potential new anticancer agents (39,40). Currently, for epigenetic drug therapies tumor suppressor genes are promising targets because many cell cycle inhibitors and tumor suppressor genes are methylated or silenced in cancer cells. The re-expression

of tumor suppressor genes is caused by demethylators which lead to their inhibition and apoptosis of cell cycle promotion (41).

In conclusion from the results of this study can be concluded that promoter methylation of *Casp8* gene is a frequent epigenetic event in gastric cancer. The results indicated that hypermethylation of this gene was involved in some clinical and pathogenesis of the disease. With few exceptions, the gender, note and tissue type of cancer, correlation has been observed. In contrast the percentage of CpG methylation of promoter region about *Apaf1* gene was not in relationship with gastric cancer. But between methylation and pathology information such as stage and grade with few exceptions significant relationship has been observed. Furthermore the methylation pattern of these genes in blood samples, emphasize that epigenetic events have the potential to be as a molecular marker for cancer and has diagnosis and prognostic value for early carcinogenesis detection of gastric cancer.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: This study was conducted at the Biological Research Center of Azad Islamic University of Zanjan and approved by the faculty of medical sciences Ethics Committee (No. IR.IAU.ZANJAN.REC.1396.58). Informed consent was taken from all the patients before entering the study and all the obtained information's from each participant was completely confidential.

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