Single Nucleotide Polymorphisms of *DNA Methyltransferase 1* Gene and Gastric Cancer in Iranian Patients: a Case Control Study

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Abstract

Background: Gastric cancer is one of the most common malignant tumors in Iran. Hypomethylation and/or hypermethylation of DNA have been described in Gastric cancer and is presumed to be an early event in this process.

Objective: We hypothesized that Single nucleotide polymorphisms of DNMT1 gene may be associated with the genetic susceptibility to Gastric cancer.

Methods: 200 patients and 200 controls, both with Iranian origin were studied. Three polymorphisms were genotyped by PCR-RFLP. Allele frequencies and genotypes were compared between the cases and controls. Odds ratios were calculated and the interaction between polymorphisms, age and sex were examined.

Results: There was no significant association between DNMT1 polymorphisms and Gastric cancer.

Conclusion: We could not show any association between DNMT1 polymorphisms and gastric cancer. Larger sets of polymorphisms and sample sizes are required to test the possibility of association between polymorphisms of this gene and gastric cancer.

Keywords: single nucleotide polymorphisms (SNPs), DNA methyltransferase 1 (DNMT1), gastric cancer (GC)

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Introduction

Gastric cancer is a complex disease with both environmental and genetic factors being important in pathogenesis of disorder 1. It is one of the most common cancer in Iran and in most cases occurs in most cases as a sporadic disease in which no single gene with obvious mendelian pattern of inheritance can be identified. It is hypothesized that there is a genetic background that in the presence of other known or unknown environmental factors the risk of gastric cancer in individual patients, can be determined.

The DNA methylation plays a crucial role in transcriptional regulation and chromatin remodeling in mammalian cells 2. Both The DNA hypomethylation and/or regional DNA hypermethylation have been well documented in various tumors 3. Three enzymes , DNA methyl transferase 1 (DNMT1), DNMT3a and DNMT3b 4 have been shown to possess DNA methyltransferase activity 5-9. DNMT1 is required for maintenance of DNA methylation whereas DNMT3A and DNMT3B are responsible for de novo methylation of DNA 10-12.

Over-expression of DNMT1 has been detected in several human cancers 13, 14 including gastric cancer. Mammalian DNA replication initiates from multiple replication foci targeting region (FTR) during the S phase. Two of these FTRs are located on exon 17 and 20 of the DNMT1 gene15 so the polymorphisms in FTRs region could be an important factor of DNMT1 alteration.

DNMT1 mRNA is undetectable in growth-arrested cells but is induced upon entrance into the S phase of the cell cycle. The 3'-UTR of the DNMT1 mRNA can confer a growth-dependent regulation on its own message. A 54-nucleotide highly conserved element within the 3'-UTR is necessary and sufficient to mediate this regulation 16, so any modification of 54-nucleotide highly conserved element in 3'-UTR could be an important factor that controls the role of DNMT1 gene products.

The single nucleotide polymorphisms (SNPs) are common allelic variants occurring around once for every 500 to 2000 base pairs in human genome17.It

Table 1: Primer set sequences used for amplification of four SNPs in DNMT gene.

SNP	Forward primer	Reverse primer	Tm
rs721186	*5'-CTGGTAGAATGCCTGATGGTC*	*5'- TGAACCGCTTCACAGAGGAC*	67°c
rs13784	*5'- CCCAGGGTGGTTTATAGGAGAG*	*5'-GGCTGACATGAAGCTGTTGTGCAAGGTT*	60°c
rs2228611	*5'- TATGTTGTCCAGGCTCGTCTC*	*5'- GTACTGTAAGCACGGTCACCTG*	63°c
rs11488	*5'- CACTGGGTGGTTTATAGGAGAGATTTCTT*	*5'- GA TCAAATTGTGCAGTACTTAGTGCATTC*	62°c

Table 2: Restriction enzymes and the resulting RFLP digestion products used for the detection of DNMT gene SNPs.

SNP ID	Restriction Enzyme	PCR product length	Homozygote Wild	Homozygote mutant
rs721186	AcyI	300bp	200bp,200bp	300bp
rs13784	BstAPI	210bp	210bp	27bp,183bp
rs2228611	Alw26I	260bp	232bp,28bp	108bp,124bp,28bp
rs11488	DdeI	162bp	144,28	116,18

Table 3: Distribution of genetic polymorphisms of DNMT1 among patients with gastric cancer and the control group in an Iranian population.

		Case	Control	OR(95%CI)
rs721186	Wild/Wild	99(99%)	200 (200%)	1
	Wild/Mutant	1 (1%)	0 (0 %)	1.121(0.06 -16)
rs13784	Wild/Wild	200(200%)	200 (200%)	1
	Wild/Mutant	0(0%)	0 (0%)	0.00 (0.00)
rs2228611	Wild/Wild	34(34%)	32(28%)	1
	Wild /Mutant	50(50%)	62(55%)	1.126 (0.05 - 6.30)
	Mutant/Mutant	16(16%)	18(16%)	0.836 (0.06 -12.6)
rs11488	Wild/Wild	200 (200%)	200 (200%)	0.00 (0.00)
	Wild/Mutant	0 (0%)	0 (0%)	

OR=odd ratio, CI=confidence interval.
All odds ratios were calculated for odds of Heterozygous against Homozygous genotypes.

is hypothesized that SNPs may determine the genetic susceptibility and/or resistance to different complex disorders including gastric cancer 18. Several polymorphic genes have been associated with modification of susceptibility to gastric cancer 19-21.

To our knowledge, the association between DNMT1 SNPs and risk of gastric cancer development has not been reported yet .In addition, the prevalence of DNMT1 SNPs in Iranian population has not been documented so far. In this study, we examined the hypothesis that the polymorphisms in DNMT1 gene may be associated with gastric cancer. We selected four SNPs of DNMT1 [NCBI Gene ID: 1786] and compared their frequencies between patients with gastric cancer and matched normal controls. Two of selected SNPs, rs2228611 on exon 17 and rs721186 on exon 20 , are located in the FTR region, as described above. The other two polymorphisms are located in the 3´-UTR region of DNMT1 mRNA and have been selected to detect their probable effect on growth-dependent regulatory function of DNMT1 gene.

Materials and Methods

Patient samples

The study recruited 200 patients with gastric cancer and 200 controls from the biobank of the Research Center for Gastroenterology and Liver Diseases (RCGLD) in Iran. All tumours were pathologically confirmed. Case and controls were matched by age, sex and ethnicity and were selected from the same hospital. At least one control was chosen for every case.

DNA extraction

Five millilitres of venous blood was collected in vacuum tubes containing EDTA and stored at 4°C. Genomic DNA was extracted within one week of sampling using a standard phenol-chloroform extraction method 22.

DNMT1 genotyping

The loci for the SNPs rs11488 and rs13784 on 3-UTR , rs2228611 on exon 17 and rs721186 on exon 20 (in the FTR region of the DNMT1 gene) were amplified by polymerase chain reaction (PCR) (table

Table 4: Median survival of gastric cancer patients stratified by various factors against SNP 2228611

A/A Vs A/G and G/G genotypes			
	N	**Median survival time (days)**	**P-value**
Gender			
Male	17 vs. 50	1101 vs. 785	0.849
Female	11 vs. 22	1353 vs. 790	0.446
Age at baseline (years)			
≤50	1 vs. 9	-	-
>50	22 vs. 47	496 vs. 790	0.733
Ethnicity			
Fars	8 vs. 24	647 vs. 1200	0.661
Tork	13 vs. 25	1036 vs. 427	0.432
Other	2 vs. 8	457 vs. 440	0.295
Tumor site			
Non-cardiac	21vs. 63	1036 vs. 790	
Cardiac	6 vs. 9	-	-
Differentiation			
Well and moderate	8 vs. 24	639 vs. 1334	0.319
Poor	4 vs. 18	297 vs. 616	0.216
Cigarette smoking			
Nonsmokers	17 vs. 46	1353 vs. 790	0.611
Smokers	5 vs. 7	528 vs. 828	0.220
Alcohol consumption			
Nondrinkers	22 vs. 62	496 vs. 790	0.484
Drinkers	4 vs. 3	684 vs. 340	0.889

1). The PCR was performed in a final volume of 25 µL containing 200 ng of template DNA, 2.5 µL of 10X PCR buffer, 1 U of Taq-DNA-polymerase, 200 µmol/L of dNTPs and 400 nmol/L of primers. The PCR program consisted an initial denaturation step at 95 °C for 5 min followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 55-67 °C (dependent on the loci) for 30 s, extension at 72 °C for 40 s, and a final step of elongation at 72 °C for 10 minutes.Restriction fragment length polymorphism (RFLP) was used for detection of polymorphisms. The SNPs , restriction enzymes detecting each allele and resulting RFLP digestion products are summarized in table2.

DNA sequencing analysis

DNA sequence analysis was used to confirm the results of DNMT1 genotyping by PCR-RFLP in 12 randomly selected representative patients. PCR amplicons were first resolved on 1% agarose gels and gel extraction/purification was performed (Promega PCR-clean up system Madison,WI,USA) . Purified products were used as templates in cycle sequencing reactions using dideoxynucleotide chain-termination method (BigDye Terminator V3.1 cycle sequencing kit Applied Biosystems) .All amplification products were sequenced bi-directionally . ABI PRISM 377 genetic analyzer (applied Biosystems) was used for capillary electrophoresis and data collection.

Statistical analysis

Allele frequencies were calculated by counting alleles. Goodness of fit between observed and estimated genotype frequencies according to Hardy –Weinberg equilibrium (HWE) was determined by

Table 5: Median survival of gastric cancer patients stratified by various factors

	N	Median survival time (days)	P-value
Gender			
Male	67	496	0.5
Female	33	790	
Age at baseline (years)			
≤50	31	3226	0.013
>50	69	440	
Ethnicity			
Fars	32	790	0.4
Turk	38	433	
Other	28	646	
Tumor site			
Non-cardiac	43	790	0.6
Cardiac	15	684	
Differentiation			
Well and Moderate	32	1036	0.4
Poor	30	558	
TNM pathological (stage)			
I,II	12	1036	0.3
III,IV	65	684	
Treatment			
Surgery	75	1036	0.1
Palliative therapy	25	440	
rs2228611 genotypes			
A/A	30	991	0.646
A/G and G/G	70	1065	
Cigarette smoking			
Nonsmokers	63	790	0.4
Smokers	33	684	
Alcohol consumption			
Nondrinkers	84	790	0.5
Drinkers	7	684	

chi-square test. The observed genotype frequencies were compared with those calculated from Hardy - Weinberg equilibrium theory ($p2 + 2pq + q2 = 1$ where p is frequency of the variant allele and $q = 1 - p$). In this study, we hypothesized that the presence of the polymorphic allele might be associated with a higher risk of gastric cancer. However, because it was unclear whether the polymorphic allele had a dominant, recessive or gene-dosage effect, statistical modeling was performed on relative risk of the mutant/mutant genotypes or wild/mutant genotypes against wild/wild genotypes, respectively. The ORs and 95% CIs were calculated to estimate the relative risk. All statistical tests were two-sided and performed with Statistical Package for Social Science V.10.0 (SPSS, Chicago, IL).

Result

The majority of both case and controls were men. Turk and Fars ethnic groups constituted more than 70% of study population. Sixty nine percent of cases were older than 50 compared to 57.7% of controls. Twenty four percent of cases had positive family history of cancer. The most common specified sites for gastric tumours were in Cardia or spanned over two anatomical regions of stomach.

The mean age of the patients was comparable to what in healthy controls (50±13 vs. 58±13, respectively; P= non significant). Gender distribution in both groups was similar (P = 0.980).Both groups were successfully genotyped for selected polymorphisms (Figure 1). Results for re-genotyped samples were always consistent. Results of PCR-RFLP genotyping were confirmed by direct sequencing

Table 6: Multivariable analysis of prognostic factors in cases

Variables	Risk ratio	P-value
Gender	1.2 (0.57-2.1)	0.67
Age at baseline	1.0 (0.56-2.0)	0.31
Ethnicity	2.7 (0.3-25)	0.38
SNP 2228611 genotypes (A/A vs . A/G, G/G)	1.11 (0.58-2.1)	0.28
Tumor site(Non cardiac vs. Cardiac)	1.3 (0.3-6.0)	0.15
Differentiation (Well/Moderately vs. Poorly /Differentiated)	1.7 (0.1-6.7)	0.22
Smoking	2.1 (0.1-14)	0.63
Treatment type (Surgery vs. Pallivate)	0.1 (0.01-4)	0.07

Table 7: Age and rs2228611 genotypes .

rs2228611	Age	Case	Control	OR	SI
A/A	<50	7	12	1	1.065
A/A	≥50*	23	20	OR A= 1.971	
G/*	<50	18	32	OR G = 0.964	
G/*	≥50*	52	44	OR AG= 2.025	

ORA = ((Cases with Risky Age and Wild/Wild Genotype)/ (Controls with Risky Age and Wild/Wild Genotype))/ ((Cases with Risk less Age and Wild/Wild Genotype)/ (Controls with Risk less Age and Wild/Wild Genotype))
ORG = ((Cases with Risk less Age and Mutant/ Genotype)/ (Controls with Risk less Age and Mutant/* Genotype))/ ((Cases with Risk less Age and Wild/Wild Genotype)/ (Controls with Risk less Age and Wild/Wild Genotype))*
ORGA = ((Cases with Risky Age and Mutant/ Genotype)/ (Controls with Risky Age and Mutant/* Genotype))/ ((Cases with Risk less Age and Wild/Wild Genotype)/ (Controls with Risk less Age and Wild/Wild Genotype))*
Synergy Index (SI) = ORGA/ (ORA × ORG)
**Risky Age*

(Figure 1). Genotype distribution in both groups was consistent with what expected by Hardy-Weinberg equilibrium. Distribution of DNMT1 genotypes did not correlate with gender and age in either group. Neither the rs11488 A/T and T/T genotypes nor the G/A and the A/A genotypes of rs13784 were detected in any individual. Both groups were homozygous for the G/G genotype of rs721186 except one person .For rs2228611, the frequencies of A/A, A/G and G/G genotypes in the case and controls were 34% vs. 28%,50% vs. 55% and 16% vs. 16%, respectively (Table 3).The distribution of genotypes and their respective ORs are shown in table 3.

To scrutinize the possible role of rs2228611, we studied the prognostic value of its genotypes by stratifying various clinicopathological parameters of patients (table 4). No specific factor appeared to influence the survival (table5).

Age, sex, ethnicity and stage associations of rs2228611 genotypes with risk of gastric cancer are presented in table 6.

The independent effects of rs2228611 on gastric cancer prognosis were tested in a multivariate analysis model (table5). While being stratified by smoking and family history, distribution of genotypes did not show any significant difference between two groups.

Discussion

To our knowledge, this is the first report on an association between the gastric cancer and DNMT1 polymorphisms among Iranian patients. The frequency and degree of DNA hypermethylation were increased in gastric cancer tissue compared with normal mucosa 23, 24 .The same phenomenon has been reported by other groups in the different conditions such as lung cancer, hepatocellular carcinoma, hepatitis and liver cirrhosis25, 26. An increase in DNMT1 mRNA expression level in the gastric cancer in comparison to corresponding noncancerous mucosa was detected 27. It was reported that DNMT1 mRNA overexpression correlates significantly with CpG island methylator phenotype in the gastric and colorectal cancers 28, 29.

The DNMT1 mRNA is regulated by the cell cycle process 30, 31. The DNMT1 mRNA is essentially absent in arrested cells and then increases and reaches its maximum level just before the peak of S phase . The 3'-UTR of DNMT1 confers a growth-dependent regulation on its own mRNA32 .Two of selected SNPs, rs11488 and rs13784, are located in

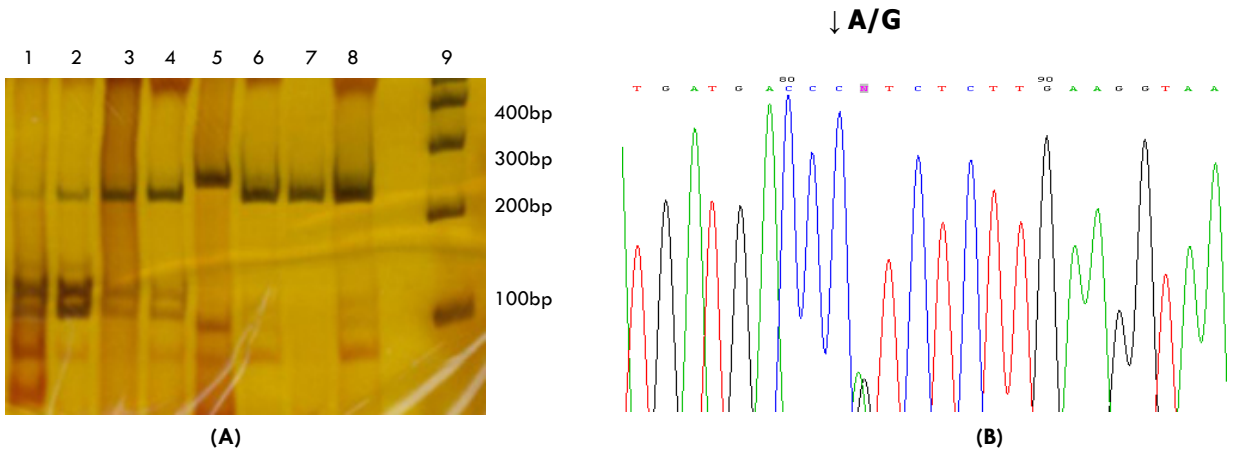

Figure 1: A) rs2228611 genotyping patterns by PCR-RFLP analysis. B) rs2228611 genotyping patterns by sequencing.

Line 5: PCR product
Line 1: RFLP product (homozygote polymorphism)
Lines 2, 3, 4, 6, 8: RFLP product (heterozygote)
Lines 7, 8: RFLP product (homozygote wild)
Line 9: Size marker200bp

a highly conserved element within the 3'-UTR that is required for mediating the down-regulation of DNMT1 mRNA in arrested cells. We observed no association between these two SNPs and the risk of gastric cancer.

Evidence for the role of DNMT1 in abnormal methylation of genomic DNA in cancers has been contradictory. Despite the assumption that the DNMT1 is responsible for most of the methylation changes observed in cancer cells, it was shown that lack of its activity was associated with a 20% decrease in overall genomic methylation 33. Mammalian DNA replication initiates from multiple sites throughout the S phase. These sites are determined both by cis-acting DNA sequences, known as replicators, and by trans-acting elements, defined by initiator proteins such as DNMT1 that bind to the replicator .The SNPs rs2228611 on exon 17 and rs721186 on exon 20 are placed in replicator recognition region on The DNMT1 gene .

As shown in table 7, older age and the rs2228611 heterozygote genotype have synergistic effect on carcinogenesis. Therefore, the individuals with age more than 50, having A/G or G/G genotypes for snp 2228611 are more susceptible to gastric cancer. This result should be interpreted cautiously due to lack of consistent findings while we were trying to measure the gene-dosage effects of this polymorphism on the etiogenesis of gastric cancer (table 3). In this study, we could not detect a significant association between the selected DNMT1 polymorphisms and gastric cancer. It is more desirable to determine the haplotype-tagging polymorphisms of this locus in our population. Genomic association studies performed by selecting and testing the hypotheses of association of the selected tagging polymorphisms with GC may provide more meaningful results. Investigating a complex disorder like gastric cancer, it is also more desirable to consider the role of confounding factors such as environmental factors. These results need to be confirmed by a larger sample size and more polymorphic markers to investigate the true effect of polymorphisms in the DNMT1 locus involved in gastric cancer pathogenesis.

In conclusion, the SNPs rs721186, rs11488 and rs13784 are not associated with the risk of GC. The rs2228611 needs to be studied in a larger sample size.

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