

RESEARCH COMMUNICATION

Lack of Effects of Single Nucleotide Polymorphisms of the DNA Methyltransferase 1 Gene on 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 has been described in gastric cancer and is presumed to be an early event in carcinogenesis. **Objective:** We therefore hypothesized that single nucleotide polymorphisms of the DNMT1 gene may be associated with the genetic susceptibility to gastric cancer. **Methods:** Totals of 200 patients and 200 controls, both of Iranian origin, were studied. Three polymorphisms were genotyped by PCR-RFLP and allele frequencies and genotypes were compared between the cases and controls. Odds ratios were calculated and the interactions between the polymorphisms, age and sex were examined. **Results :** There were no significant associations between the 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 for future testing of possible associations.

Key words: Single nucleotide polymorphisms - DNA methyltransferase 1 - gastric cancer - Iran

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Introduction

Gastric cancer is a complex disease with both environmental and genetic factors being important in the pathogenesis (Laird and Jaenisch, 1994). It is one of the most common cancer in Iran and in most cases occurs 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 which in the presence of other known or unknown environmental factors determines the risk of gastric cancer in individual patients.

DNA methylation plays a crucial role in transcriptional regulation and chromatin remodeling in mammalian cells (Kondo et al., 2000). Both DNA hypomethylation and/or regional DNA hypermethylation have been well documented in various tumors (Okano et al., 1998). Three enzymes, DNA methyl transferase 1 (DNMT1), DNMT3a and DNMT3b (Jones and Baylin, 2002) have been shown to possess DNA methyltransferase activity (Niederreither et al., 1998; Dincer et al., 2002; Liu et al., 2003; Skliris et al., 2003; Wang et al., 2005). DNMT1 is required for maintenance of DNA methylation whereas DNMT3A and DNMT3B are responsible for *de novo* methylation of the DNA (Okano et al., 1999; Turek-Plewa and Jagodzinski, 2005; Jair et al., 2006).

Over-expression of DNMT1 has been detected in several human cancers (Kanai et al., 2001; Saito et al., 2001) 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 gene (Araujo et al., 1999) so the polymorphisms in FTRs region could be an important factor for 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 (Detich et al., 2001), so any modification of 54-nucleotide highly conserved element in 3'-UTR could be an important factor that control the role of DNMT1 gene products.

Single nucleotide polymorphisms (SNPs) are common allelic variants occurring around once for every 500 to 2000 base pairs in the human genome (Sauer et al., 2000). It is hypothesized that SNPs may determine the genetic susceptibility and/or resistance to different complex disorders including gastric cancer (Hemminki et al., 2005). Several polymorphic genes have been associated with modification of susceptibility to gastric cancer (John

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et al., 1991; Miao et al., 2003; Stolzenberg-Solomon et al., 2003).

To our knowledge, associations between DNMT1 SNPs and risk of gastric cancer development have not been reported. In addition, the prevalence of DNMT1 SNPs in Iranian population has not been documented so far. In this study, we therefore 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 the 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 & 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 tumors 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 milliliters 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 1).

PCR reactions were 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 of 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, the restriction enzymes detecting each allele and the resulting RFLP digestion products are summarized in Table 2.

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 the cycle sequencing reactions using the dideoxynucleotide chain-termination method (BigDye Terminator V3.1 cycle sequencing kit, Applied Biosystems). All amplification products were sequenced bi-directionally. The 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 the Hardy-Weinberg equilibrium (HWE) was determined by chi-square test. The observed genotype frequencies were compared with those calculated from Hardy-Weinberg equilibrium theory ($p^2 + 2pq + q^2 = 1$ where p is the 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 the relative risk of the mutant/mutant genotypes or the wild/mutant genotypes against the 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).

Table 1. Primer Set Sequences Used for Amplification of Four SNPs in DNMT Gene

Temperature	Reverse primer	Forward primer	SNP
67°C	5'- TGAACCGCTTCACAGAGGAC	5'-CTGGTAGAATGCCTGATGGTC	rs721186
60°C	5'-GGCTGACATGAAGCTGTTGTGCAAGGTT	5'- CCCAGGGTGGTTTATAGGAGAG	rs13784
63°C	5'- GTACTGTAAGCACGGTCACCTG	5'- TATGTTGTCCAGGCTCGTCTC	rs2228611
62°C	5'- GA TCAAATTGTGCAGTACTTAGTGATTC	5'- CACTGGGTGGTTTATAGGAGAGATTCTT	rs11488

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	144bp,28bp	116bp,18bp

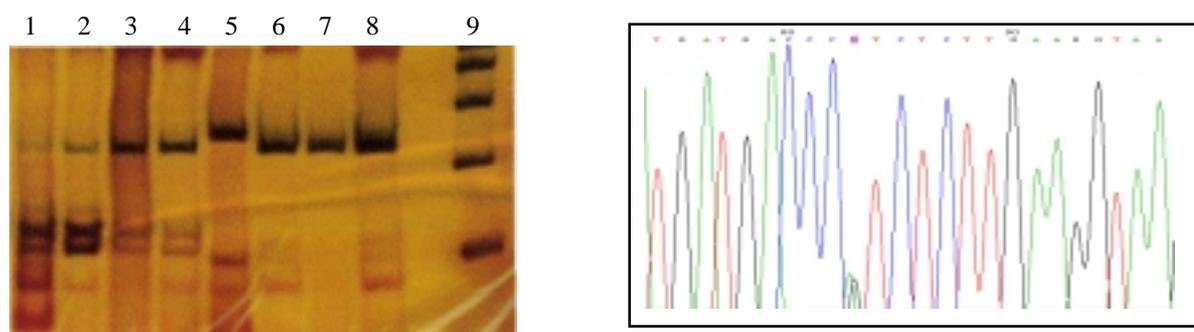


Figure 1. A) rs2228611 genotyping patterns by PCR-RFLP analysis. B) rs2228611 genotyping patterns by sequencing. Line 1, RFLP product (homozygote polymorphism); Lines 2, 3, 4, 6 and 8, RFLP products (heterozygotes); Line 5, PCR product; Lines 7, 8, RFLP product (homozygote wild); Line 9, Size markers 200bp

Table 3. Distribution of Genetic Polymorphisms of DNMT1 Among Patients with Gastric Cancer and the Control Group in an Iranian Population

		Cases	Controls	OR	(95% CI)
rs721186	Wild/Wild	99	200	1	
	Wild/Mut	1	0	1.121	(0.06-16.0)
rs13784	Wild/Wild	100	200		
	Wild/Mut	0	0		
rs2228611	Wild/Wild	34	32	1	
	Wild /Mut	50	62	1.126	(0.05-6.30)
	Mut/Mut	16	18	0.836	(0.06 -12.6)
rs11488	Wild/Wild	200	200		
	Wild/Mut	0	0		

OR, odds ratio, calculated for odds of Heterozygous against Homozygous genotypes; CI, confidence interval; Mut, mutant

Results

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 tumors were in Cardia or spanned over two anatomical regions of the stomach.

The mean age of the patients was comparable to that of healthy controls (50 ± 13 vs. 58 ± 13 , respectively; $P =$ non significant). Gender distribution in both groups was similar ($P = 0.980$). Both groups could be successfully genotyped for the selected polymorphisms (Figure 1a). The results for re-genotyped samples were always consistent. Results of PCR-RFLP genotyping were confirmed by direct sequencing (Figure 1b). The genotype distribution in both groups was consistent with that expected by Hardy-Weinberg equilibrium.

The distribution of DNMT1 genotypes did not correlate with gender and age in either groups. Neither the rs11488 A/T and T/T genotypes nor the G/A and A/A genotypes of the rs13784 were detected in any individual. Both groups were homozygous for the G/G genotype of the rs721186 except one person. For rs2228611, the frequencies of the 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).

To further scrutinize the possible role of the rs2228611, we studied the prognostic value of its genotypes by stratifying various clinicopathological parameters of the

Table 4. Median Survival (days) of Gastric Cancer Patients Stratified by Various Factors against Snp 2228611 (A/A versus A/G and G/G genotypes)

		Number	Survival	P-value
Gender	Male	17 v 50	110 v 785	0.849
	Female	11 v 22	1,353 v 790	0.446
Age at baseline (years)	≤ 50	1 v 9	-	-
	>50	22 v 47	496 v 790	0.733
	Ethnicity	Fars	8 v 24	647 v 1,200
	Tork	13 v 25	1,036 v 427	0.432
	Other	2 v 8	457 v 440	0.295
Tumor site	Non-cardiac	21 v 63	1,036 v 790	
	Cardiac	6 v 9	-	-
Differentiation grade	Well/mod	8 v 24	639 v 1334	0.319
	Poor	4 v 18	297 v 616	0.216
	Smoking	Nonsmoker	17 v 46	1,353 v 790
	Smoker	5 v 7	528 v 828	0.220
Alcohol	Nondrinker	22 v 62	496 v 790	0.484
	Drinker	4 v 3	684 v 340	0.889

patients (Table 4). No specific factor appeared to influence the survival. The independent effects of rs2228611 on gastric cancer prognosis was tested in a multivariate analysis model. When stratified by smoking and family history, distribution of genotypes did not show any significant difference between the two groups.

Discussion

To our knowledge, this is the first report on the association between gastric cancer and DNMT1 polymorphisms among Iranian patients. The frequency and degree of DNA hypermethylation was increased in gastric cancer tissue compared with the normal mucosa (Kanai et al., 1998). The same phenomenon has been reported by other groups in different conditions such as lung cancer, hepatocellular carcinoma, hepatitis and liver cirrhosis (Eguchi et al., 1997; Kanai et al., 1998; 2000). An increase in DNMT1 mRNA expression level in gastric cancer in comparison to corresponding noncancerous mucosa was detected (Kanai et al., 2001). It was reported that DNMT1 mRNA overexpression correlates significantly with CpG island methylator phenotype in gastric and colorectal cancers (Saito et al., 2001; Etoh et al., 2004).

The DNMT1 mRNA is regulated by the cell cycle

Table 5. Age and rs2228611 Genotypes

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

OR A = ((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)); OR G = ((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)); OR GA = ((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

process (Szyf et al., 1991; Robertson et al., 2000). 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 mRNA (Detich et al., 2001). Two of the selected SNPs, rs11488 and rs13784, are located in 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 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 (Rhee et al., 2000). Mammalian DNA replication initiates from multiple sites throughout 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 SNPs rs2228611 on exon 17 and rs721186 on exon 20 are placed in replicator recognition region on DNMT1 gene.

As shown in Table 5, older age and the rs2228611 heterozygote genotype have synergistic effect on carcinogenesis. Therefore individuals older than 50 years old having A/G or G/G genotypes for snp 2228611 are more susceptible to gastric cancer. This result should be interpreted cautiously due to the lack of consistent findings while we were trying to measure the gene-dosage effects of this polymorphism on etiogenesis of gastric cancer (Table 3). In this study, we could not detect a significant association between 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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