Endothelial nitric oxide synthase gene polymorphism in gastric cancer

Mide kanserinde endotelial nitrik oksit sentaz gen polimorfizmi

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Background/aims: *Nitric oxide, a labile compound synthesized by nitric oxide synthase, is a major regulator not only of physiological vascular tonus but also of the abnormal vascularity associated with tumors. Endothelial production of nitric oxide regulates blood flow and angiogenesis and reduces tumor cell adhesion to the endothelium. A high concentration of nitric oxide and its metabolites causes DNA damage during nitration, nitrosation and deamination. Both positive and negative effects on carcinogenesis and tumor growth, apoptosis, and cytotoxic mechanisms may be explained by differential susceptibility of tumor cells to nitric oxide-mediated reactions.* ***Methods:*** *In this study, three major polymorphisms (786T>C, the 27 base pair variable number of tandem repeats in intron 4, and 894G>T) of the endothelial nitric oxide synthase gene were investigated in gastric cancer and normal tissues of 50 patients with gastric cancer and in the peripheral blood of 98 healthy subjects.* ***Results:*** *We found no significant differences in intron 4a/b and 894G>T (Glu298Asp) allele and genotype frequencies between control and patient specimens. Nevertheless, the genotype and allele frequencies of 786T>C polymorphism were found to be significantly different between the healthy controls and tumor tissues.* ***Conclusions:*** *The results suggest that endothelial nitric oxide synthase 786T>C polymorphism may play a role in the development of gastric cancer.*

Key words: Endothelial nitric oxide synthase, gene polymorphism, gastric cancer

Amaç: *Nitrik oksit sentaz tarafından sentez edilen kararsız bir bileşik olan nitrik oksit sadece fizyolojik vasküler tonusu regüle etmez aynı zamanda tümörlerle ilişkili anormal vasküler tonusu da düzenler. Nitrik oksidin endotelyal üretimi kan akışını, anjiogenezi düzenler ve endotele tümör hücresi adhezyonunu azaltır. Nitrik oksidin yüksek konsantrasyonu ve metabolitleri nitrasyon, nitrozizasyon ve deaminasyon esnasında DNA hasarına yol açar. Karsinogenez, tümör büyümesi, apoptozis ve sitotoksik mekanizmalar üzerindeki hem pozitif hem negatif etkileri tümör hücrelerinin nitrik oksit aracılığı ile reaksiyonlara karşı değişken yatkınlıkları ile açıklanabilir.* ***Yöntem:*** *Bu çalışmada endotelyal nitrik oksit sentaz geninin üç farklı polimorfizmi (786T>C, intron 4'te 27 adet temel paylaşımlı ardışık değişken sayıda ve 894>T) 50 hastanın normal ve gastrik kanser dokularından alınan örneklerinde ve 98 sağlıklı insanın periferik kanlarında incelendi.* ***Bulgular:*** *İntron 4a/b ve 894G>T (Glu298Asp) alleli ve genotip frekanslarında kontrol ve hasta grupları arasında herhangi anlamlı farklılık saptanmadı. Ancak 786T>C polimorfizmi alel frekansı ve genotipte kontrol ve tümör dokuları arasında anlamlı farklılık tespit edilmiştir.* ***Sonuç:*** *Sonuçlar endotelyal nitrik oksit sentaz 786T>C polimorfizminin gastrik kanser oluşumunda rol oynayabileceğini göstermektedir.*

Anahtar kelimeler: Endotelial nitrik oksit sentaz, gen polimorfizmi, mide kanseri

INTRODUCTION

Gastric cancer is one of the leading causes of cancer-related deaths worldwide (1). Gastric cancer incidence shows large geographic differences, with relatively low rates in most Western countries, including the United States and United Kingdom, and with relatively high rates in Japan, Korea, China (2), and also in Turkey (World Health Organization Global InfoBase Online Reports: The impact of cancer).

Approximately 5% of the total gastric cancer burden is hereditary and shows a classical hereditary etiology, such as germline mutations of the E-cadherin *(CDH1)* gene (3). It is known that both familial and sporadic gastric cancers are products of

**Presented in: EPHAR, Federation of Pharmacology Societes Congress, July 13-17, 2008, Manchester*

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Manuscript received: 21.12.2009 **Accepted:** 25.05.2010

doi: 10.4318/tjg.2010.0118

multiple genetic and epigenetic alterations that transform normal gastric epithelial cells into malignant neoplasms. During the last few years, many attempts have been made in order to better define the genetic profile of gastric tumors, with the aim to improve early diagnosis, prognostic stratification and eventually cure (4).

One such factor may be nitric oxide (NO), which is a short-lived biomolecule with various biological functions (5). There has been growing evidence that NO acts as a carcinogen. For instance, high concentration of NO and its metabolites, such as peroxynitrite and NO_2, causes DNA damage in the course of nitration, nitrosation and deamination. NO also inhibits DNA ligase activity, resulting in the accumulation of DNA breaks (6), and promotes tumor angiogenesis and metastasis (7). As a result, the contradictory roles of NO in carcinogenesis remain unresolved.

NO is produced from L-arginine by the enzymes nitric oxide synthase (NOS). There are three different isoforms of NOS, which can be divided into two functional classes. One group is the constitutive class, which includes eNOS (endothelial-NOS) and nNOS (neuronal-NOS), and the other group consists of the inducible form called iNOS (inducible-NOS) (5). It has been reported that NOS isoforms are present in human tumor cell lines and solid tumor tissues.

eNOS is a Ca^{2+}-dependent enzyme and was first found in vascular endothelial cells. Expression of eNOS has been found in other types of cells, such as airway epithelial, neuronal and certain tumor cells. It has been shown that eNOS regulates blood pressure, platelet aggregation, leukocyte adherence, and vascular smooth muscle cell mitogenesis and angiogenesis (8).

The gene encoding eNOS is located on 7q35-q36, spanning 21 kb of genomic DNA. It contains 26 exons and 25 introns encoding a mRNA of 4052 nucleotides. Translation initiation and termination codons are present on exon 2 and exon 26, respectively (9). To date, more than 168 polymorphisms have been identified in the eNOS gene (7). Only three of these polymorphisms, namely 894G>T, intron 4a/b, and -786T>C, seem to be functional, and they have been investigated for their association with the risk of several diseases, predominantly cardiovascular diseases (10,11). However, the number of studies reporting the association of these three polymorphisms with cancer is limited (7, 12, 13).

The aim of this work was to investigate the correlation of three functional eNOS gene polymorphisms (894G>T, intron 4a/b polymorphism, -786C>T) and gastric cancer growth.

MATERIALS AND METHODS

DNA Samples

DNA samples used in the study were obtained from the DNA bank of the Department of Medical Genetics, Ankara University Faculty of Medicine. All samples both in control and patient groups had been provided by persons who previously joined research projects held by the department with their written informed consent, indicating permission for anonymous use of their DNA samples in other research studies. Ninety-eight healthy subjects and 50 patients with gastric cancer (50 adenocarcinoma) were included in the study as control and patient groups, respectively. In the control group, the samples were obtained from peripheral blood, while in the patient group, healthy and tumor tissues were used as DNA source for each individual.

Tumor samples were collected with informed consent from 50 patients with gastric adenocarcinoma who underwent surgical resection of their tumors at the Department of Surgical Oncology, Faculty of Medicine, Ankara University between October 2004 and January 2006. The patient group included 28 men and 22 women, with mean ages of 59.27±11.93 years and 59.74±12.22 years, respectively. Tumor samples collected after surgery were immediately rinsed with phosphate-buffered saline (PBS) and stored at 80°C until DNA extraction.

Determination of eNOS Genotypes

Glu298Asp polymorphism: 0.1 μg genomic DNA, 20 pmol of each primer (sense: 5'-AAG GCA GGA GAC AGT GGA TGG A-3'; antisense: 5'-CCC AGT CCA TCC CTT TGG TGC TCA-3'), 0.2 mM of each dNTP, 10 mM Tris, 50 mM KCl, 1.5 mM $MgCl_2$, and 1.5 U Taq DNA polymerase (Fermentas GmbH, Germany) were mixed in 50 μl total volume. Polymerase chain reaction (PCR) conditions were as follows: initial denaturation at 94°C for 2 minutes (min) and then 35 cycles of 94°C for 30 seconds (s), 61°C for 30 s and 72°C for 30 s followed by a final extension at 72°C for 5 min. Restriction digestion was performed in a total volume of 25 μl reaction mixture containing 10 μl PCR product and 2 U *Ban*II restriction endonuclease (Roche Diagnostics GmbH, Germany). Reaction mixtures were incubated at 37°C overnight and the genoty-

pes were determined by agarose gel electrophoresis of restriction digests in 2% agarose gel containing 0.5 μg/ml ethidium bromide (17).

Intron 4a/b polymorphism: 0.1 μg genomic DNA, 20 pmol of each primer (sense: 5'-GGG AAC CTC AGC CCA GTA GTG AA-3'; antisense: 5'-TCT CTT AGT GCT GTG GTC AC-3'), 0.2 mM of each dNTP, 10 mM Tris, 50 mM KCl, 1.5 mM $MgCl_2$, and 1.5 U Taq DNA polymerase (Fermentas GmbH, Germany) were mixed in 50 μl total volume. PCR conditions were as follows: initial denaturation at 94°C for 2 min and then 35 cycles of 94°C for 1 min, 54°C for 40 s and 72°C for 1 min followed by a final extension at 72°C for 5 min. The genotypes were determined by agarose gel electrophoresis of PCR products in 2.5% agarose gel containing 0.5 μg/ml ethidium bromide (17).

-786T→C polymorphism: 0.1 μg genomic DNA, 20 pmol of each primer (sense: 5'-ATG CTC CCA CCA GGG CAT CA-3'; antisense: 5'-GTC CTT GAG TCT GAC ATT AGG G-3'), 0.2 mM of each dNTP, 10 mM Tris, 50 mM KCl, 1.5 mM MgCl2, and 1.5 U Taq DNA polymerase (Fermentas GmbH, Germany) were mixed in 50 μl total volume. PCR conditions were as follows: initial denaturation at 94°C for 7 min and then 30 cycles of 94°C for 30 s, 57°C for 30 s and 72°C for 30 s followed by a final extension at 72°C for 7 min. Restriction digestion was performed in a total volume of 25 μl reaction mixture containing 10 μl PCR product and 2 U *Msp*I restriction endonuclease (Roche Diagnostics GmbH, Germany). Reaction mixtures were incubated at 37°C overnight and the genotypes were determined by agarose gel electrophoresis of restriction digests in 2.5% agarose gel containing 0.5 μg/ml ethidium bromide (18, 19).

Statistical Analysis

In statistical analysis, allele frequencies were tested with chi-square test, and genotype frequencies were tested with Wilcoxon signed rank test. P values less than 0.05 were taken to indicate a statistically significant difference.

RESULTS

The allele and genotype distributions of the eNOS 894G>T, intron 4a/b, and -786T>C in the cases and controls are summarized in Table 1. In the case of the 894G>T polymorphism, 18 of 46 tumor samples had the GG genotype, 26 had the GT genotype and 2 had the TT genotype; the number of GG, GT, and GT genotypes in the normal gastric mucosa samples were 19, 25, and 2, respectively. Twenty-six of 98 control subjects showed GG genotype, 59 showed GT genotype and 13 showed TT genotype for this polymorphism (Table 1). There was no significant difference between normal gastric tissue and gastric cancer tissue (p=0.093 chi-square test). There was also no significant difference between the control group and normal gastric tissue (p=0.093, chi-square test) and between the control group and gastric cancer tissue (p=0.129, chi-square test).

The intron 4a/b polymorphism had the following distributions: 1 of the 46 gastric tumor samples had the aa genotype, 10 had the ab genotype and 35 had the bb genotype; 4 of the 98 control subjects had the aa genotype, 28 had the ab genotype and 66 had the bb genotype (Table 1). There was no significant difference between normal gastric tissue and gastric cancer tissue and the control group (p=0.543, chi-square test).

The -786T>C polymorphism showed the following genotypic distribution: 16 TT, 22 TC, 12CC in 50 tumor samples; 31 TT, 12 TC, 7 CC in 50 normal gastric mucosa samples; and 65 TT, 17 TC, 9 CC in 91 healthy control subjects (Table 1). There was a significant difference between normal and tumor tissues by the means of allele and genotype frequencies (p=0.001: McNemar test, p=0.001: chi-square test, respectively). However, no significant difference between normal gastric tissue from patients and controls was detected (p=0.510, chi-square test) with respect to their allele and genotype frequencies (Figures 1 A, B).

There was a significant difference in the analysis of TT-TC (n=15) and TC-CC (n=5) transformation between normal and tumor tissue (p=0.001, Wilcoxon signed rank test).

DISCUSSION

In this case-control study, we investigated the association between eNOS gene 786T>C, intron 4a/b, and 984G>T polymorphisms and sporadic gastric cancer growth. Our results showed that neither 984G>T nor intron 4a/b polymorphisms were related with gastric cancer. However, T>C substitution at the position of -786 was found to be significantly higher in gastric tumor tissues when compared with the normal gastric mucosa of the same patients and healthy control individuals.

Gastric cancer is a multifactorial disorder in which genetic and environmental interactions have an

Table 1. The allele and genotype distributions of the eNOS 8946>T, intron 4a/b and -786>C

	TNM	Histopathology	Histologic Stage	eNOS T786C		eNOS 4a/b		XPD 751Lys		
				N	T	N	T	N	T	K
1	T3N2M0	ADENOCA	I	TT	TT	b/a	b/a	L/L	L/L	L/G
2	T3N2M0	ADENOCA	I	TT	TC	b/b	b/b	G/G	G/G	L/G
3	T3N2M1(peritoneal)	ADENOCA	III	TT	TT	b/b	b/b	L/L	L/L	L/G
4	T3N2M0	ADENOCA	I	TT	TT	b/a	b/a	L/G	L/G	L/L
5	T1N0M0	ADENOCA	I	TT	TC	b/b	b/b	L/G	L/G	L/L
6	T3N2M0	ADENOCA	III	TC	TC	b/b	b/b	L/L	L/L	G/G
7	T3N2M0	ADENOCA	III	TT	TC	b/a	b/a	L/L	L/L	L/G
8	T3N1M0	ADENOCA	II	TC	TC	b/b	b/b	L/L	L/L	L/G
9	T3N0M0	ADENOCA	III	TC	TC	b/b	b/b	L/G	L/G	L/G
10	T2N0M0	ADENOCA	I	**TC**	**CC**	b/b	b/b	L/G	L/G	L/L
11	T3N1M0	ADENOCA	I	TC	TC	b/b	b/b	L/G	L/G	L/L
12	T2N0M0	ADENOCA	II	TT	TT	b/b	b/b	L/L	L/L	L/G
13	T3N1M0	POOR DIF ADENOCA	III	**TC**	**CC**	b/b	b/b	L/G	L/G	L/L
14	T3N2M0	ADENOCA	III	TT	TC	a/a	a/a	L/L	L/L	L/G
15	T3N1M0	POOR DIF ADENOCA	III	TT	TC	b/a	b/a	L/G	L/G	L/G
16	T3N1M0	POOR DIF ADENOCA	III	TT	TC	b/b	b/b	L/L	L/L	L/L
17	T4N1M0	ADENOCA	I	CC	CC	b/b	b/b	L/G	L/G	G/G
18	T3N1M0	ADENOCA	I	TC	TC	b/b	b/b	L/L	L/L	L/G
19	T3N1M0	ADENOCA	I	TT	TT	b/a	b/a	L/G	L/G	
20	T3N3M0	Signet ring	III	CC	CC	b/b	b/b	L/G	L/G	L/L
21	T3N2M0	ADENOCA	I	TT	TT	b/b	b/b	L/L	L/L	L/G
22	T1N0M0	ADENOCA	I	TT	TT	b/b	b/b	L/L	L/L	G/G
23	T3N2M0	Signet ring		**TC**	**CC**	b/b	b/b	L/G	L/G	L/G
24	T2NIM0	ADENOCA	II	TT	TT	b/b	b/b	L/L	L/G	L/G
25	T2N0M0	ADENOCA	I	TT	TC	b/a	b/a	L/L	L/L	L/G
26	T1N0M0	ADENOCA	I	TT	TT	b/b	b/b	L/G	L/G	L/G
27	T3N2M0	Signet ring	III	TT	TT	b/a	b/a	L/L	L/L	L/G
28	T3N3M0	Signet ring	III	TT	TT	b/b	b/b	L/G	L/G	L/L
29	T3N3M0	Signet ring	III	CC	CC	b/a	b/a	L/G	L/G	L/G
30	T3N2M0	ADENOCA	III	CC	CC	b/b	b/b	L/G	L/G	G/G
31	T3N1M0	ADENOCA	II	CC	CC	b/b	b/b	L/L	L/L	L/G
32	T3N1M0	ADENOCA	I	**TC**	**CC**	b/b	b/b	L/L	L/L	L/G
33	T3N1M0	ADENOCA	III	TT	TC	b/b	b/b	L/L	L/L	L/L
34	T3N0M0	ADENOCA	I	TT	TC	b/b	b/b	L/G	L/G	L/G
35	T1N0M0	ADENOCA	II	TT	TT	b/b	b/b	L/G	L/G	G/G
36	T1N0M0	ADENOCA	III	TT	TC	b/b	b/b	L/G	L/G	L/L
37	T2N0M0	ADENOCA	I	TT	TC	b/a	b/a	L/G	L/G	G/G
38	T3N1M0	ADENOCA	I	CC	CC	b/b	b/b	L/L	L/L	L/G
39	T3N1M0	MUCINOUS ADENOCA	III	**TC**	**CC**	b/b	b/b	G/G	G/G	L/G
40	T3N1M0	MUCINOUS ADENOCA	III	TT	TT	b/b	b/b	L/G	L/G	G/G
41	T3N3M0	ADENOCA	III	TC	TC	b/b	b/b	L/L	L/L	L/G
42	T3N1M0	ADENOCA (III)	III	TC	TC	b/b	b/b	L/L	L/L	G/G
43	T2N1M0	ADENOCA (II)	II	TT	TC	b/b	b/b	L/G	L/G	L/L
44	T1N0M0	ADENOCA	I	TT	TT	b/b	b/b	L/L	L/L	L/L
45	T3N0M0	MUCINOUS ADENOCA	III	CC	CC	b/b	b/b	L/L	L/L	L/L
46	T3N2M0	MUCINOUS ADENOCA	II	TT	TT	b/a	b/a	L/G	L/G	G/G
47	T3N3M0	ADENOCA	II	TT	TT	b/b	b/b	L/G	L/G	L/G
48	T3N0M0	MUCINOUS ADENOCA	III	TT	TC	b/b	b/b	G/G	G/G	
49	T4N0M0	ADENOCA	II	TT	TC	b/b	b/b	L/G	L/G	L/G
50	T2NIM0	ADENOCA	I	TT	TC	b/a	b/a	L/G	L/G	L/L

Adenoca: Adenocarcinoma. Poor dif: Poorly differentiated.

important role in its development and progression. Identification of these genetic risk factors is expected to enhance our understanding of the molecular basis of gastric cancer. The studies performed during recent years showed that there are many genes and environmental factors responsible in the etiology of gastric cancer. The most frequently studied genes include CDH1, APC, CTNNB1, VEGF, hMLH1, hMSH3, and hMSH6 (1, 2, 4). The gene encoding eNOS, which catalyzes the formation of NO from L-arginine, seems to be another candidate gene participating in the gastric carcinogenesis.

In view of the physiological and pathophysiological importance of NO, the potential of eNOS in the pathogenesis of various human diseases has been examined using its polymorphic variants as potential disease markers (10). However, the number of studies concerning the role of NO/eNOS in cancer is limited and controversial results have been reported.

In some tumor tissues, NO has been found to enhance tumor angiogenesis and induce vasodilatation, thus accelerating tumor growth. In other tumors, including gastric and colon cancer, a decreased amount of NOS protein was demonstrated by immunohistochemistry, and there is a possible relationship between loss of NO and carcinogenesis (20). Therefore, it seems that NO can act as both a pro- and anti-tumorigenic factor (21).

Wang L et al. (8) showed that there was a significantly higher eNOS expression in both primary tumors and metastatic lymph nodes than in normal gastric mucosa and that eNOS expression correlates with the angiogenic phenotype of gastric cancer and predicts poor prognosis. In contrast, in the study of Wang YZ et al. (20), it was shown that gastric cancer cells had decreased eNOS expression when compared with the normal gastric mucosa samples. Koh et al. (6) also reported greatly reduced eNOS immunoreactivity in gastric tumor epithelial cells.

Lu et al. (7) reported that the polymorphism -786T>C in the eNOS promoter was found to be associated with increased risk of breast cancer. It has been reported that induced overexpression of the eNOS gene in MCF-7 cells can elevate the basal NO level, stimulate apoptosis, and diminish cancer cell motility and invasion (22). Moreover, it has been shown that NO can induce the apoptotic genes in lung endothelial cells (23).

Supporting these findings, we found that the CC genotype for the -786T>C polymorphism was significantly associated with gastric cancer. The genotype and allele frequencies of 786T-C polymorphism were found to be significantly different between the healthy controls and tumor tissues. Surprisingly, the normal tissues obtained from the patients showed almost the same frequency distributions as did the controls. However, the frequencies of the alleles and the genotypes showed significant differences between normal and tumor tissues obtained from the patients.

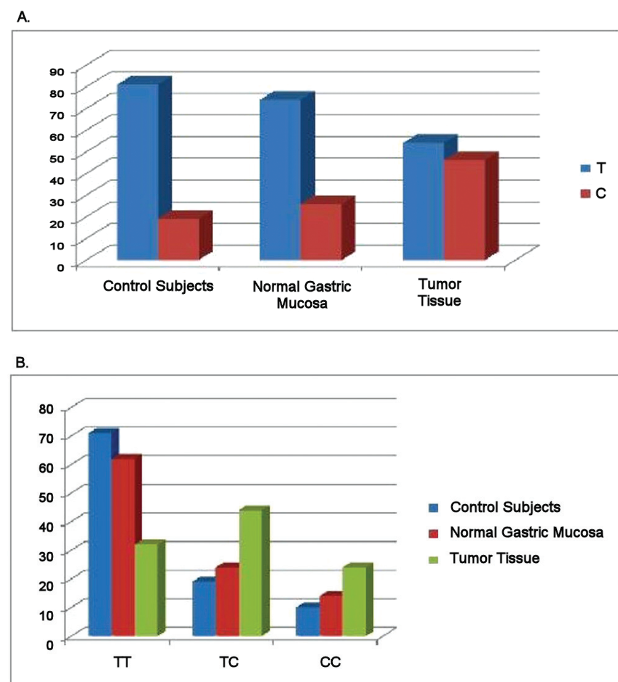

Figure 1. A. Allele and **B.** Genotype frequencies of -786T>C polymorphisms in the patient and control groups.

Most interestingly, the transformation rates of the genotypes were difficult to interpret. When samples from normal and tumor tissues were tested, 20 patients showed transformation from T to C alleles. Fifteen of the patients with TC genotypes in tumor tissues had TT genotypes in their normal tissues, whereas 5 of the patients with CC genotypes in tumor tissues had TC genotypes in their normal tissues. Whether this transformation arose before or after the carcinogenesis is not clear. If it takes place before the formation of the tumor, then it can be postulated that this mutation is carcinogenic itself. On the contrary, if this transformation occurs after the tumor has formed, then we can speculate that this change is related with the angiogenic capacity, and therefore, with the faith of the tumor.

Effects of polymorphisms of the eNOS gene on plasma NO concentrations have been recently reported. The mutant allele of the -786T>C polymorphism in the promoter region of the eNOS gene has been associated with reduced promoter activity and synthesis of NO (11). Dosenko et al. (24) have also shown that eNOS gene expression with CC genotype is significantly lower than in the case of TT and TC allelic variants, and the enzyme activity is significantly decreased. We suggest that the CC genotype could result in the reduced NO production by means of decreased eNOS level and lead to abrogation of its anti-tumorigenic role in our gastric cancer samples.

Intron 4a/b and the 894G>T variant in exon 7 of the eNOS gene cause an amino acid change (Glu298>Asp), but we did not find any association between this polymorphism and gastric cancer. Lu et al. (7) obtained similar results, in which there was no association between 894G>T polymorphism and risk of sporadic breast cancer in young non-Hispanic white females. Likewise, although the intron 4a/b VNTR polymorphism of the eNOS gene has been found to be associated with reduced enzyme activity, we found no association between this polymorphism and gastric cancer.

To the best of our knowledge, this is the first study investigating the association between the eNOS polymorphisms and gastric cancer. There are three possible eNOS gene polymorphism sites in humans. We found that CC genotype for the -786T>C polymorphism was significantly associated with gastric cancer. The genotype and allele frequencies of 786T-C polymorphism were found to be significantly different between the healthy controls and tumor tissues, and this probably shows the angiogenic capacity of the tumor tissue, which could be responsible for tumor growth. NO has been found to promote angiogenesis and vasodilatation in some tumor tissues. However, in gastric and colon cancers, the amount of NOS protein has been reported to be lower when compared to normal tissues. In the first case, the effects on angiogenesis and local hemodynamics are considered to be accelerating tumor growth, while in the latter, the decrease in the amount of NO was thought to be related with carcinogenesis. Our results suggest that -786T>C polymorphism is a risk factor for gastric cancer, and eNOS seems to be the protective molecule against gastric tumor formation. However, larger studies are needed to verify these findings.

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