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HEAD & NECK

Contribution of Nitric oxide synthase 3 genetic variants to nasopharyngeal carcinoma risk and progression in a Tunisian population

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Abstract

Purpose We conduct this study to evaluate the clinical and functional impact of Nitric Oxide Synthase 3 (NOS3) T-786C and G894T genetic variants on nasopharyngeal carcinoma (NPC) risk and progression in a Tunisian population.

Methods 259 NPC patients and 169 healthy controls were enrolled into our case–control study. Blood samples were genotyped by the RFLP-PCR analysis. The levels of Nitric oxide (NO) were measured by a colorimetric assay kit in the plasma of NPC patients, healthy controls and according to NOS3 genotypes. The correlation between the NOS3 variants and the clinicopathological parameters was examined.

Results We found no linkage disequilibrium between NOS3 T-786C and G894T variants. These results showed that NOS3 variants were genetically independent. In contrast to NOS3 T-786C, a significant association was found between NOS3 G894T polymorphism and NPC risk. The 894T allele decreased significantly in NPC patients and appeared as protective factor (OR = 0.65, CI 95%= 0.48–0.88, $p = 0.006$). NPC patients had significantly higher levels of plasma NO as compared to healthy controls ($p = 0.0011$). The T-786C mutation reduced the levels of plasma NO and decreased risk of lymph node metastasis in NPC patients (OR = 0.64, 95% CI = 0.43–0.96; $p = 0.03$). In contrast, NOS3 G894T polymorphism had no effects neither on NO plasma levels nor clinical parameters.

Conclusions This is the first study to associate NPC with significantly higher levels of plasma NO. NOS3-derived NO could play key roles in NPC pathogenesis. NOS3 variants differently contribute to NPC risk and progression in a Tunisian population. NOS3 G894T was associated with NPC risk. NOS3 T-786C decreased the levels of plasma NO and reduced the development of regional lymph node metastasis.

Keywords *NOS3* gene · Polymorphisms · Plasma NO · Nasopharyngeal carcinoma · Clinical parameters

Introduction

NPC is a malignant tumor arising from respiratory-type ciliated epithelium and characterized by the presence of lymphoid stroma. Unlike other head and neck cancers, NPC shows remarkable ethnic and geographic distributions [1]. The incidence of NPC is particularly high in Southern China and Southeast Asia (20–30/100,000). However, it is rare in Europe and North America (less than 1/100,000). In Tunisia, like most of the countries of the Mediterranean Area and North Africa, the prevalence of NPC is intermediate (2–5/100,000) [2]. Histologically, there are three different types of NPC classified by the World Health Organization (WHO), the keratinizing squamous cell carcinoma (WHO Type1), non-keratinizing carcinoma (WHO Type 2) and

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undifferentiated NPC type (UCNT, WHO Type 3). This latter is known as the most frequent type of NPC in the endemic regions.

NPC belongs to the head and neck cancer family with unique clinical, etiological and biological characteristics. Despite environmental factors such as Epstein–Barr virus, tobacco use, dietary habits and chemical exposure, genetic susceptibility to NPC has been clearly established [3]. Several susceptibility genes have been recognized as risk factors for NPC. Among them, *NOS3* gene (also known as endothelial *NOS* or *eNOS* gene).which is located on chromosome 7q36 and comprises 26 exons. It encodes NOS3 enzyme which catalyzes the oxidation of L-arginine to L-citrulline and nitric oxide (NO). NOS3-derived NO is an interesting lipophilic molecule that can be synthesized in most tissues and has important physiological and pathological effects. Despite its short half-life, NO participates in various biological and pathological functions including vasodilatation, immunity, inflammation and tumor pathologies [4]. In carcinogenesis, NO can be either anti-tumor or tumor promoting factor [5] and many cancer-related events such as angiogenesis, apoptosis, cell cycle, invasion, and metastasis have been reported to be regulated by NO [6]. Besides timing and location, the biological effects of NO are closely linked to its concentration which is under the control of several factors primarily genetic variations.

Many variations in the expression of the NOS3 gene and enzyme have been previously reported in human cancers [7]. Several single-nucleotide polymorphisms (SNPs) have been identified in the *NOS3* gene, however, a few of them are functional and are associated with altered NO bioavailability. Among these variants, the T-786C (rs2070744) in the NOS3 promoter region and the G894T (rs1799983) variant located in exon 7 and leads to the amino acid substitution from Glu298Asp. Both variants have been shown to be associated with various tumor pathologies such as, gastric and breast cancers [8, 9]. However, little is known regarding their involvement in NPC risk and development. In addition, the clinical impact of *NOS3* genetic variants on NPC pathogenesis has been rarely examined. So far there is no evidence whether NOS3 polymorphisms influence the levels of plasma NO in NPC patients. Therefore, we conducted a case–control study to investigate the association between *NOS3* genetic polymorphisms and the carcinogenesis of NPC in a Tunisian population. To investigate the association between *NOS3* variants and tumor progression, the gene frequencies were compared by stratification with clinicopathological parameters of NPC. Because tobacco and alcohol intake may be possible risk factors for NPC carcinogenesis, the interaction between the T-786C and G894T polymorphisms of *NOS3* gene and these exposure factors was examined. The functional impact of these variants was investigated by measuring the levels of plasma NO in NPC patients vs healthy controls and according to NOS3 genotypes.

Materials and methods

Subjects

A total of 428 subjects comprising 259 cases (NPC patients) and 169 healthy controls were recruited for this study between January 2013 and February 2018. Both patients and controls come from the same area and population living in the middle coast of Tunisia. All patients had the undifferentiated NPC histological type (type III, WHO classification) and have been recruited from the Department of Cancerology and Radiotherapy of Farhat Hached University Hospital in Sousse, Tunisia. The control subjects were gender–age matched healthy blood donors without a personal history of cancer and recruited from the Regional Center of Blood Transfusion (Farhat Hached University Hospital, Sousse, Tunisia) similar to the patient population. For NPC patients, the sex ratio was 2.03 (174 men and 85 women) and the mean age at diagnosis was 42.21 ± 15.58 (range 10–85 years). For Healthy controls, the sex ratio was 2.52 (men 121 and women 48) and the mean age at diagnosis was 42.93 ± 17.41 (range 11–92 years). This study was approved by the Tunisian National Ethical Committee and was carried out in accordance with the ethical standards of the 1964 Helsinki declarations. A written informed consent was obtained from all enrolled individuals prior to their participation.

Plasma preparation and DNA isolation

A volume of 5 mL of peripheral blood was drawn by venipuncture in EDTA-containing tubes from each participant for DNA isolation and plasma preparation. Tubes were centrifuged for 5 min at 1800*g* and plasma was removed and frozen at − 80 °C until use. The genomic DNA was isolated from peripheral blood leukocytes using a salting procedure [10]. The isolated DNA was stored at 20 °C until analysis.

Genotyping of *NOS3* G894T polymorphism

The *NOS3* G894T (Glu298Asp) polymorphism was genotyped using the restriction fragment length polymorphism of polymerase chain reaction (RFLP-PCR) analysis with the following primers: 5′-CAT GAG GCT CAG CCC CAG AAC-3′ (forward) and 5′-AGT CAATCC CTT TGG TGC TCA C-3′ (reverse) [11]. The reaction mixture (a total volume of 25 µl) contained 100 ng of genomic DNA, 2.5X PCR buffer, 1.5 mM $MgCl_2$, 200 µM dNTP, 0.2 µM each primer, and 0.6 U of Taq DNA polymerase (Amersham, Paris, France). Amplification conditions consist of an initial

incubation at 95 °C for 5 min followed by 30 cycles at 95 °C for 45 s, 61 °C for 45 s, 72 °C for 45 s. A final extension step was carried out at 72 °C for 5 min. The migration of PCR products was realized in a 2% agarose gel containing ethidium bromide. Enzymatic digestions were carried out using BanII at 37 °C for 16 h. The presence of a T at nucleotide 894 of the *eNOS* gene was indicated by the cleavage of the amplified product (206 pb) into two fragments of 119 and 78 bp. The amplified products were separated on a 3% agarose gel containing ethidium bromide.

Genotyping of *NOS3* T-786C polymorphism

The *NOS3* T-786C polymorphism was detected by RFLP-PCR using the following primers: 5′-ATG CTC CCA CCA GGG CAT CA-3′ (forward) and 5′-GTCCTT GAA TCT GAC ATT AGG G-3′ (reverse) [11]. A 237pb was amplified under the following conditions for cycles: an initial incubation at 94 °C for 7 min followed by 30 cycles at 94 °C for 30 s, 57 °C for 30 s, 72 °C for 30 s. A final extension step was carried out at 72 °C for 7 min. Digestion with NgOMIV for 16 h produced two fragments 204 pb and 33 pb. DNA fragments were separated on a 2.5% agarose gel containing ethidium bromide.

Measurement of the levels of plasma NOx

The amounts of NO in the plasma of NPC patients and healthy controls were quantified indirectly by measuring Nitrate and Nitrite levels by Nitric oxide colorimetric assay kit (Biovision, USA) according to the manufacturer's protocol. One hundred and thirty NPC patients were selected randomly from NPC group to measure the plasma levels of NO according to NOS3 genotypes. The absorbance was detected at 540 nm. Results were expressed in mM, based on standard curves. The detection limit of the assay is approximately 1 µM.

Statistical analysis

To evaluate the Hardy–Weinberg equilibrium (WHE) in both patient and control groups we used an online calculator (http://www.oege.org/software/hardy-weinberg.html). Baselines characteristics analysis and Plasma Total NOx were evaluated by Student *t* tests. Allele and genotypes frequencies were calculated by Chi-square test. To analyze the association of the T-786C and 894G/T polymorphisms of NOS3 gene with the risk of NPC, we calculated the odds ratio (OR) and confidence interval 95% (95% CI) by the Epi-Info statistical program (version 7.1.3.3). A *p* value less than 0.05 were considered statistically significant. Haplotype frequencies were performed using cubex program.

Results

Genetic analysis

The clinical and demographic characteristics of NPC patients and healthy controls are summarized in Table 1. We found no significant differences between NPC patients and control subjects in age or gender ($p = 0.65$ and 0.32, respectively).

The allelic, genotypic and haplotype distributions of the T-786C and G894T polymorphisms in NPC patients and controls were summarized in Table 2. The distribution of *NOS3* T-786C and G894T polymorphisms were in Hardy–Weinberg equilibrium (HWE) for both control and patients groups (T-786C: patients: $X2 = 0.11$; controls: $X2 = 0.63$); (G894T: patients: $X2 = 1.49$; controls: $X2 = 0.18$). For the T-786C polymorphism, no statistically significant associations were observed in both allelic (C:

Table 1 Clinical and demographic characteristics of NPC patients and controls

Characteristics	NPC patients	Controls	*p* value[a]
Sample size	259	169	
Age. years (mean ± SD)	42.21 ± 15.58	42.93 ± 17.41	0.65
Gender			
Male	173 (66.8)	121 (71.6)	
Female	85 (32.8)	48 (28.4)	0.32
Smoking status			
Smokers	92 (38.3)	–	
Non-smokers	148 (61.4)	–	
Alcoholism			
Alcoholics	29 (12)	–	
Nonalcoholics	212 (88)	–	
Tumor size			
T1–T2	107 (41.3)	–	
T3–T4	149 (57.5)	–	
Unknown	4 (1.2)	–	
Lymph node status			
N0	63 (24.3)	–	
N+	195 (75.3)	–	
Unknown	1 (0.4)	–	
Metastasis			
M0	227 (87.6)	–	
M+	29 (11.2)	–	
Unknown	3 (1.2)	–	
Stage			
I–III	67 (25.9)	–	
IV	188 (72.6)	–	
Unknown	4 (1.5)	–	

[a]Calculated by Student's *t* test (NPC Patients vs Control)

Table 2 Allelic and genotypic frequencies of the NOS3 T-786C and G894T polymorphisms in NPC patients and controls

Genotypes	Patients	Controls	OR (95%CI)	*p* value[a]
NOS3 T-786C				
Genotypes				
TT	81 (31.3)	52 (30.8)		
TC	130 (50.2)	88 (52.1)	0.94 (0.61–1.47)	0.813
CC	48 (18.5)	29 (17.1)	1.06 (0.59–1.89)	0.83
Alleles				
T	292 (56.4)	192 (56.8)		
C	226 (43.6)	146 (43.2)	1.01 (0.77–1.34)	0.9
HWE	0.11	0.63		
NOS3 G894T				
Genotypes				
GG	149 (57.5)	73 (43.2)		
GT	90 (34.7)	78 (46.1)	0.56 (0.37–0.85)	0.006
TT	20 (7.7)	18 (10.7)	0.54 (0.27–1.09)	0.08
Alleles				
G	388 (74.9)	224 (66.3)		
T	130 (25.1)	114 (33.7)	0.65 (0.48–0.88)	0.006
HWE	1.49	0.18		
Haplotypes				
TG	256 (49.4)	143 (42.3)		
CG	132 (25.5)	81 (24)	0.91 (0.64–1.28)	0.59
TT	36 (7)	49 (14.5)	0.41 (0.25–0.66)	0.00019
CT	94 (18.1)	65 (19.2)	0.8 (0.55–1.17)	0.26

[a]Calculated by $\chi 2$ test (Patients vs Controls); *CI* confidence interval, *OR* Odds ratio by logistic regression; Haplotypes frequencies using cubex program;

OR = 1.01; CI 95%= 0.77–1.34; $p = 0.9$) and genotypic frequencies (TC: OR = 0.94; CI 95%= 0.61–1.47; $p = 0.8$); (CC: OR = 1.06; CI 95%= 0.59–1.89; $p = 0.83$). However, the analysis of the G894T polymorphism showed a significant difference in allelic and genotypic frequencies between NPC patients and controls. The heterozygous genotype (GT) was significantly associated as a protector factor for NPC (OR = 0.56; CI 95%= 0.37–0.87; $p = 0.006$). Similarly, the 894T minor allele significantly decreased in NPC patients (OR = 0.65; CI 95%= 0.48–0.88; $p = 0.006$). However, there were no significant association between the homozygous rare genotype (TT) of *NOS3* G894T variant and NPC (OR = 0.54; CI 95%= 0.27–1.09; $p = 0.08$).

To evaluate the combined effect of *NOS3* T-786C and G894T polymorphisms, we conducted a haplotype analysis in NPC patients and controls subjects. The haplotype TG, formed by common alleles of T-786C and G894T, served as referent haplotype. We found a significant association for the TT haplotype formed by common alleles of the T-786C and rare allele of the G894T polymorphisms. The TT haplotype was higher in controls subjects compared to NPC patients (OR = 0.41; CI 95%= 0.25–0.66; $p = 0.00019$). Pairwise linkage disequilibrium (LD) between the T-786C and G894T polymorphisms was calculated in both patients and controls. Our analysis showed no significant LD in the two groups (patients: $D' = 0.514$; $r^2 = 0.1$ and controls: $D' = 0.2$; $r^2 = 0.04$). This result suggested that the T-786C and G894T polymorphisms of *NOS3* gene were independent.

The genotype distribution of *NOS3* T-786C and G894T polymorphisms according to clinicopathological characteristics of NPC patients were summarized in Table 3. There were no significant differences in genotype frequencies between men and women groups for both polymorphisms. Similarly, age-stratified analysis did not show any association when we compared patients less or older than 30 years of age. In stratified analysis according to tumor extension, metastatic status and TNM stage, neither T-786C nor G894T polymorphisms were associated with NPC risk. However, as shown in Table 3, the homozygous rare genotype CC of *NOS3* T-786C polymorphism was significantly associated as an independent protective factor for regional lymph node metastasis (OR = 0.36; CI 95%= 0.13–1.03; $p = 0/05$). Similarly, the presence of the -786C minor allele was significantly associated with decreased regional lymph node metastasis risk (OR = 0.64; CI 95%= 0.43–0.96; $p = 0.03$). In contrast, genotype distribution and allele frequency of the G894T polymorphisms were not significantly different between NPC patients with or without regional lymph node extension. In addition, we investigated the association between *NOS3* polymorphisms and factor exposure including smoking and alcohol consumption in NPC patients. Our results showed that the T-786C and G894T polymorphisms were not associated to smoking status (Table 3). However, when we compared patients according to alcohol consumption we found a significant correlation with the T-786C but not G894T polymorphism (CC: OR = 5.01; CI 95%= (1.63–15.42); $p = 0.005$). The multivariate analysis showed that alcohol consumption was not independently associated with the T-786C polymorphism among NPC patients after adjusting for age, gender, smoking status [TC + CC: OR = 2.81; CI 95%= (0.95–8.28)]; $p = 0.06$ (Table 4).

Plasma levels of NO

As shown in Fig. 1a, NPC patients had significantly higher levels of plasma NO as compared to healthy controls ($p = 0.0011$). The genotype-dependent effects were examined in 130 NPC patients whose clinical parameters were clearly known and established. For *NOS3* T-786C variant, results summarized in Fig. 1b showed a significant reduction of the NO plasma levels among patients caring the -786C rare allele ($p = 0.006$). However, as indicated in Fig. 1c, no significant difference was observed in the levels of plasma NO according to G894T genotypes ($p = 0.27$). To evaluate the association between NOx levels and NPC risk, we conducted

Table 3 Genotype distribution of NOS3 T-786C and G894T variants according to clinicopathological characteristics of NPC patients

Characteristics	NOS3 T-786C genotype			NOS3 G894T genotype		
	TT	TC	CC	GG	GT	TT
Age at diagnosis						
≤30	16 (24.4)	38 (57.6)	12 (18)	38 (57.6)	25 (37.9)	3 (4.5)
>30	65 (33.7)	92 (47.7)	36 (18.6)	111 (57.5)	65 (33.7)	17 (8.8)
p value[a]		0.12	0.48		0.69	0.41
OR (95% CI)		0.59 (0.30–1.15)	0.73 (0.31–1.73)	1	0.89 (0.49–1.6)	1.93 (0.53–6.98)
Gender						
Men	51 (29.5)	92 (53.2)	30 (17.3)	101 (58.4)	60 (34.7)	12 (6.9)
Women	30 (35 0.3)	38 (44.7)	17 (20)	48 (56.5)	29 (34.1)	8 (9.4)
p value[a]		0.23	0.92		0.95	0.48
OR (95% CI)	1	1.42 (0.79–2.56)	1.03 (0.49–2.19)	1	0.78 (0.56–1.72)	0.71 (0.27–1.85)
Smoking status						
Smokers	28 (30.4)	43 (46.8)	21 (22.8)	54 (58.7)	33 (35.9)	5 (5.4)
Nonsmokers	45 (30.4)	78 (52.7)	25 (16.9)	85 (57.4)	50 (33.8)	13 (8.8)
p value[a]		0.693	0.43		0.89	0.365
OR (95% CI)	1	0.88 (0.486–1.61)	1.35 (0.63–2.85)	1	1.03 (0.59–1.81)	0.60 (0.20–1.79)
Alcoholism						
Alcoholics	5 (17.2)	12 (41.4)	12 (41.4)	16 (55.2)	12 (41.4)	1 (3.4)
Non-alcoholics	69 (32.7)	109 (51.7)	33 (15.6)	124 (58.8)	70 (33.2)	17 (8)
p value[a]		0.450	0.005		0.48	0.46
OR (95% CI)	1	1.51 (0.51–4.50)	5.01 (1.63–15.42)	1	1.32 (0.59–2.96)	0.45 (0.05–3.66)
Tumor size						
T1–T2	34 (31.8)	51 (47.7)	22 (20.5)	59 (55.1)	38 (35.5)	10 (9.4)
T3–T4	47 (31.5)	77 (51.7)	25 (16.8)	89 (59.7)	50 (33.6)	10 (6.7)
p value[a]		0.75	0.59		0.61	0.38
OR (95% CI)	1	1.09 (0.62–1.92)	0.82 (0.39–1.69)	1	0.87 (0.51–1.48)	1.15 (0.65–2.02)
Lymph node status						
N0	16 (25.4)	29 (46)	18 (28.6)	37 (58.7)	19 (30.2)	7 (11.1)
N+	65 (33.3)	101 (51.8)	29 (14.9)	112 (57.4)	70 (35.9)	13 (6.7)
p value[a]		0.65	0.02		0.53	0.33
OR (95% CI)	1	0.85 (0.43–1.70)	0.39 (0.1–0.8)	1	1.21 (0.64–2.28)	0.61 (0.22–1.65)
OR(95% CI)[b]	1	0.68 (0.29–1.59)	0.36 (0.13–1.03)	–	–	–
p value[a]		0.38	0.05	–	–	–
Metastasis						
M0	72 (31.7)	114 (50.2)	41 (18.1)	132 (58.2)	77 (33.9)	18 (7.9)
M+	9 (31)	14 (48.3)	6 (20.7)	16 (55.2)	11 (37.9)	2 (6.9)
p value[a]		0.96	0.78		0.69	1
OR (95% CI)	1	0.98 (0.4–2.38)	1.17 (0.38–3.52)	1	1.17 (0.52–2.66)	0.91 (0.19–4.32)
Stage						
I–III	20 (29.9)	29 (43.3)	18 (26.8)	37 (55.2)	23 (34.3)	23 (34.3)
IV	61 (32.5)	98 (52.1)	29 (15.4)	110 (58.5)	65 (34.6)	13 (6.9)
p value[a]		0.75	0.1		0.86	0.24
OR (95% CI)				1	0.95 (0.51–1.73)	0.62 (0.23–1.68)

CI confidence interval, *OR* Odds ratio by logistic regression

[a]Calculated by $\chi 2$ test

[b]Adjusted by age, gender, smoking status, alcoholism

Table 4 Multivariate logistic regression analysis between NOS3T-786C polymorphism and NPC risk

	OR (95%CI)[e]	*p* value[e]
Age at diagnosis[a]	0.55 (0.27–1.10)	0.09
Gender[b]	1.03 (0.51–2.09)	0.91
Smoking status[c]	0.76 (0.37–1.57)	0.47
Alcoholism[d]	2.81 (0.95–8.28)	0.06

[a]≤30 versus > 30 years

[b]Women versus men

[c]Nonsmoker versus smokers

[e]under dominant model

[d]Non-alcoholics versus alcoholics

a univariate analysis. We found no significant correlations with age, gender, smoking status and alcohol consumption (data not shown).

Discussion

Many functional polymorphisms in *NOS3* gene have a significant clinical impact in malignancies and are implicated as risk factor for various cancers (Table 5). However, their roles in NPC remain unclear. In this study, we investigated the impact of the *NOS3* T-786C and G894T functional variants on the risk and progression of NPC in a Tunisian population. In accordance with previous report [12], we found no linkage disequilibrium between these polymorphisms. These findings point towards the independence of the two *NOS3* variants. The case–control study showed that *NOS3* T-786C and G894T polymorphisms differently contribute to nasopharyngeal carcinoma risk and progression in a Tunisian population. We found a significant association between *NOS3* G894T and NPC risk. The heterozygous genotype (GT) and the minor allele (894T) significantly decreased the risk of NPC. However, there was no significant association between the rare genotype (TT) and NPC probably due to lower frequency of this homozygous genotype. In addition, there was no correlation between *NOS3* G894T polymorphism and clinical phenotypes. The involvement of *NOS3* G894T polymorphism in various malignancies has been amply investigated across different populations and overall, results appeared either supporting [13] or refuting [14] a significant association. In the Tunisian populations, to our knowledge, one study was interested in *NOS3* genetic polymorphisms and NPC risk [15]. Our findings were inconsistent with this study in which authors indicated that *NOS3* G894T polymorphism significantly increased the risk of NPC. It should be noted that the authors have used different populations from the one we used. In addition, it is very well known that genetic polymorphisms often vary between

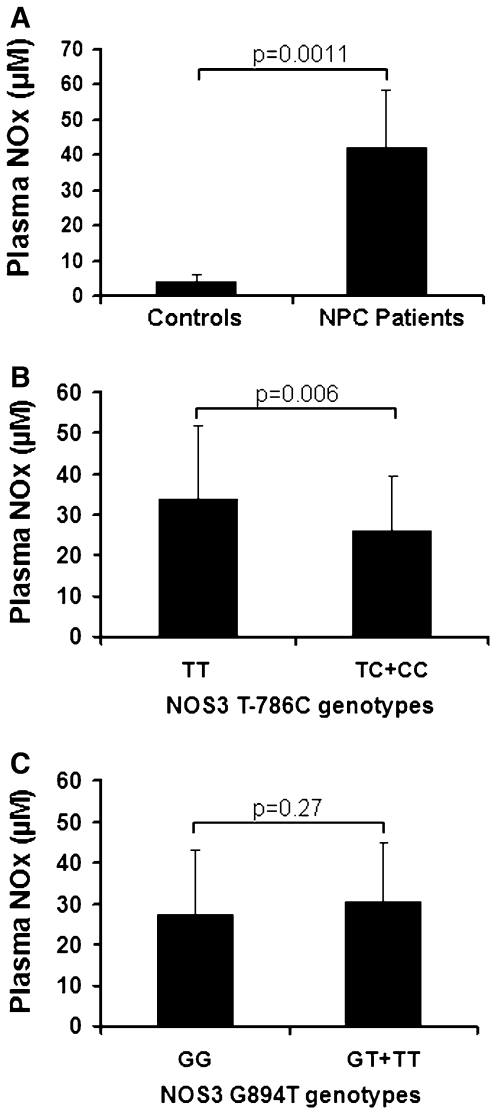

Fig. 1 Plasma levels of NO in NPC patients *vs* healthy controls, and in NPC patients according to *NOS3* genotypes. **a** The levels of plasma NO were significantly higher in the patients with NPC than in the healthy controls ($p=0.0011$). **b** The presence of the minor allele of the *NOS3* T-786C variant significantly reduced the levels of plasma NOx ($p=0.006$). **c** The presence of the T allele of the *NOS3* G894T variant had no significant effects on the levels of plasma NOx ($p=0.27$). The number (n) of genotypes with mutant allele for each NOS3 polymorphism is indicated in the figure. Results are indicated as the mean ± SD. Two-tailed Student t test between genotypes of the NOS3 polymorphisms $p<0.05$

ethnic groups. Sample sizes, ethnic heterogeneity and environmental factors may contribute to these contradictory findings. Moreover, it has been clearly reported that the Tunisian population is a mixture of many cultures and showed a very heterogeneous genetic structure [16], which could explain the difference in genetic susceptibility to NPC.

In contrast to *NOS3* G894T variant, our study showed no significant association between *NOS3* T-786C

Table 5 NOS3 variants in cancer risk and nodal localization

Polymorphisms	Cancer risk and/or lymph node involvement	References
	Association with breast cancer risk	[9, 13]
	Lymph nodal localization	[17]
	Association with gastric cancer risk	[8]
	Lymph nodal localization	Unknown
	Association with NPC risk	This work
NOS3 G894T	No lymph nodal localization	This work
	No association with prostate cancer risk	[14]
	Association with prostate cancer risk	[18]
	Lymph nodal localization	Unknown
	Association with colorectal cancer risk	[11]
	Lymph nodal localization	Unknown
	Association with beast cancer risk	[9, 13]
	No association with breast cancer risk	[19]
	Lymph nodal localization	[19]
	Association gastric cancer risk	[8]
	Lymph nodal localization	Unknown
NOS3 T-786C	No association with NPC risk	This work
	Lymph nodal localization	This work
	Association with prostate cancer risk and progression	[20, 21]
	Lymph nodal localization	Unknown
	Association with colorectal cancer risk	[11]
	Lymph nodal localization	Unknown

polymorphism and NPC risk. The clinical impact analysis of this promoter variant showed no association with gender, age, tumor extension, stage and metastatic status. However, as far as we know, this is the first study to associate *NOS3* minor allele (-786C) with decreased risk of lymph node metastasis in a Tunisian population. These results suggested that *NOS3* T-786C polymorphism could play key roles in NPC progression.

The association studies between *NOS3* T-786C promoter variant and cancer risk showed conflicting results. For example, a significant association between *NOS3* T-786C and the development of breast cancer has been previously reported [13]. However, Lee et al. [17, 19] found no overall association. Nevertheless, the authors found an association between this promoter variant and the decreased risk for invasive breast cancer with lymph node involvement. Interestingly, many evidences suggested that NO was involved in inducing lymph node metastasis [22]. Among head and neck cancers, NPC has the highest preponderance for regional lymph node metastasis [17] and NPC patients had a significantly higher expression of the lymphangiogenic factors such as VEGF-C and D [23]. Noteworthy the expression of these factors has been shown to be up-regulated by NO in various types of cancers [24, 25] and the inhibition of NOS3 activity significantly reduced the number of tumor cells arriving to the tumor-draining lymph node, as well as formation of macroscopic metastasis [23].

The haplotype analysis in NPC patients and controls subjects was conducted to evaluate the combined effects of *NOS3* T-786C and G894T polymorphisms. Our results showed that the TT haplotype composed of common alleles of the T-786C and rare allele of the G894T variants of *NOS3* gene was significantly associated with reduced risk of NPC in a Tunisian population.

Overall, genetic association studies showed conflicting results regarding the effects of *NOS*3 genetic polymorphisms in NPC. These results are not surprising since the etiology of NPC is related to complex interactions of genetic, viral, dietary and exposure factors. Among exposure factors, smoking and alcohol consumption are major risk factors for NPC carcinogenesis. In addition, adjustments for these exposure factors are essential to investigate the association between gene polymorphism and susceptibility of diseases such as NPC. Here, we reported no significant interaction between *NOS3* genetic variants and these factors in NPC patients.

NO has been shown to play a dual role in cancer biology and can either inhibit or promote tumor pathogenesis. The heterogeneous effects of NO in cancer are dependent on many factors such as the activity and localization of NOS isoforms, cellular sensitivity to NO, and duration of NO exposure. In carcinogenesis, the effects of NO are closely linked to its concentrations. In the present study, we reported for the first time that NPC patients had significantly higher levels of plasma NO as compared to healthy controls. The

high levels of plasma NO were significantly associated with metastatic status (Data not shown). Although, three isoforms of NOS are known to be involved in NO generation, the circulating levels of NO sensitively reflect endothelial NO formation [26]. Despite its role in cancer progression, the increased production of NO may have clinical relevance as a biomarker of inflammation and estimation of cancer risk. Given the crucial role of endogenous NO in inflammation-induced cancers, our results suggested that NOS3-derived NO could play key roles in the development and progression of NPC.

The levels of NO were also measured in the plasma of NPC patients according to NOS3 genotypes for both polymorphisms. Our results showed that *NOS3* G894T and T-786C exhibited different effects on NOS3 activity. For *NOS3* G894T variant, minor allele (894T) had no significant effects on the plasma levels of NO. These results suggested that *NOS3* G894T polymorphism did not affect the promoter activity and the constitutive expression of *NOS3* gene. The G894T polymorphism is located in the exon 7 of the human *NOS3* gene and it corresponds to an amino acid substitution (Glu-Asp) at codon 298 (Glu298Asp). This polymorphism was found to alter the cleavage of the NOS3 protein with the Asp (894T) variant leading to a decrease in NO generation [27]. However, other studies reported that the specific cleavage of the 298Asp allele is a methodological artifact [28]. This finding appeared reasonable since the substitution of glutamic acid to aspartic acid is conservative and remote from the catalytic site. In addition, it is known that the *NOS3* G894T polymorphism affects neither the structure nor the active site of the NOS3 enzyme [29]. Besides, genetic linkage studies have reported that *NOS3* G894T polymorphism may be in linkage disequilibrium with one or more loci present in the *NOS3* gene or in the whole genome [30]. Collectively, these data suggested that the *NOS3* G894T polymorphism did not affect the biological activity of the NOS3 isoform and the constitutive production of NO. Therefore, given the emerging key role of NO in NPC pathogenesis, further experiments are needed to explore whether G894T polymorphism could affect the expression of *NOS3* gene, the activity of NOS3 isoform and the bioavailability of NO especially in NPC patients. For *NOS3* T-786C variant, our results showed a significant decrease in the levels of plasma NO in patients carrying the -786C minor allele. It is established that the -786C minor allele binds the inhibitory transcription factor protein A1 resulting in a low *NOS3* mRNA level leading to reduced NO production [31]. Moreover, a decreased promoter activity for -786C allele carriers has been clearly reported [32]. In a previous report [33], it has been shown that the T-786C polymorphism is associated with a low *NOS3* expression.

In summary, the present study clearly reported that, (i) the T-786C and G894T variants of *NOS3* gene were genetically independent; (ii) they differently contribute to NPC pathogenesis; (iii) a significant association was found between NPC risk and G894T but not T-786C variant. The 894T minor allele decreased significantly in NPC patients and appeared as a protective factor; (iv) tobacco and alcohol consumption appeared to have no significant interactions with both NOS3 variants; (v) Significant higher levels of NO were observed for the first time in the plasma of NPC patients as compared to healthy controls; (vi) The G894T mutation had no effects on the promoter activity of the NOS3 gene; (vii) However, to our knowledge this is the first study to associate *NOS3* -786C minor allele with reduced levels of plasma NO and decreased risk of lymph node metastasis in NPC patients in Tunisian population. Patients with the CC-homozygous genotype of *NOS3* T-786C had low risk of developing lymph node metastasis than the other genotypes. The *NOS3* T-786C polymorphism may play a protective role in NPC suggesting a possible role of *NOS3* gene in NPC risk and progression. Given the highest preponderance for regional lymph node metastasis in NPC and their critical role in the generation of tumor-directed immune response and cancer prognosis, pharmacological targeting *NOS3* signalling will help in developing novel NO-based therapies for the prevention and the treatment of several lymphatic system diseases, especially NPC. Further studies should clarify the prognostic significance of *NOS3* signalling in NPC. The genetic and functional studies investigating the impact of *NOS3* variants on NPC pathogenesis were important for at least two reasons. First, they could be crucial in increasing our understanding and reducing the controversy regarding the dichotomous effects of NO, especially in tumor biology and, second, they will aid in understanding the molecular pathogenesis of NPC.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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