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Research Paper

Association of endothelial nitric oxide synthase (*eNOS*) polymorphisms with *EGFR*-mutated lung adenocarcinoma in Taiwan

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Abstract

EGFR mutation of Non-small cell lung cancers (NSCLC) was predominantly seen in Asian population and it was considered as a predictor of responsiveness. Eendothelial nitric oxide synthase (eNOS) plays a vital role in chronic inflammation and carcinogenesis. In this study, we aimed to explore the association between the genetic polymorphisms of *eNOS* (-786T/C and 894 G/T) and EGFR mutation in patients with lung adenocarcinoma. A total of 277 patients with diagnosed lung adenocarcinoma were recruited between years 2012 and 2015. All study subjects underwent the analysis of *eNOS* genetic variants (-786 T/C and 894 G/T) using real-time polymerase chain reaction (PCR) genotyping. Our results showed that, among the 277 patients, variant types (GT + TT) of *eNOS* 894 G/T polymorphism were significantly positively correlated with EGFR mutation type, specifically exon 19 in-frame deletion. With the subgroup of EGFR L858R mutation, variant genotypes (GT + TT) of *eNOS* 894 G/T were significantly associated with lymph node invasion. Moreover, in silico analysis indicated that *eNOS* 894 G/T altered the eNOS expression. In conclusion, our study showed that *eNOS* 894 G/T variants were significantly associated with EGFR mutation types of lung adenocarcinoma, specifically exon 19 in-frame deletion. This may be utilized as a prediction of tumor invasiveness and therapy responsiveness.

Key words: Adenocarcinoma; Lung cancer; Endothelial nitric oxide synthase (*eNOS*) gene; Polymorphism; Epidermal growth factor receptor (*EGFR*)

Introduction

Lung cancer is the leading cause of cancer mortality in the United States, but also throughout the world. In the United States, lung cancer is estimated to be diagnosed in approximately 234,000 people annually and causes approximately 154,000 deaths [1]. In Taiwan, lung cancer was the top three incidence (13,312 people) of cancer diagnosed in year 2015 and the top one cancer mortality (9,232 deaths) based on the latest report published in Dec 2017. With advancements in early detection and treatment, likelihood of long-term survival is increasing. But still, high mortality was expected since diagnosed.

Survival rates of females were much higher than males (i.e. 5-year survival rate: male – 14.3%, female – 28.4%). These data of survival rates were the lowest among the top ten incidences of cancers in Taiwan.

About 80 percent of all lung cancers are classified as non-small cell lung cancer (NSCLC), and the rest are mostly small cell lung cancer (SCLC). This distinction is necessary for appropriate staging, treatment, and prognosis. Adenocarcinoma, classified as a NSCLC, is the most common type of lung cancer recently and accounting for about 50% of all lung cancers. Patients with advanced lung adenocarcinoma and other NSCLC should have their tumors assessed for the presence of a driver mutation [2]. At present, there are more and more documented genotypes with approved targeted therapies, such as epidermal growth factor receptor (*EGFR*) mutation, ROS1 translocation, anaplastic lymphoma kinase (*ALK*) translocation, BRAF mutation, HER2 mutation, MET abnormalities, RET translocation, etc. There are also some genotypes with ongoing clinical trials.

Mutations in *EGFR* tyrosine kinase are noted in about 15% of NSCLC adenocarcinoma in the Unites States [3] and more often in women and non-smokers. In Asian population, the incidence of EGFR mutation is extremely higher, up to 62% [4]. Advanced NSCLCs that contains characteristic mutations in *EGFR* are highly sensitive to EGFR-TKIs (EGFR-tyrosine kinase inhibitors), and hence, considered as a predictor of responsiveness.

Nitric oxide (NO), a small free radical, is involved in various physiologic and pathophysiologic processes, including immunity, neurotransmission and carcinogenesis [5, 6]. NO is synthesized from L-arginine, NADPH and oxygen by NO synthase (NOS) [7]. Endothelial NOS (eNOS), one of the major isoform of NOS, is constitutively expressed and is therefore also referred to as constitutive NOS (cNOS). Several studies have shown that NO can both promote and inhibit tumor metastasis and progression at different concentration [7]. Moreover, eNOS can regulate the proinflammatory molecules expression [8], such as cyclooxygenase-2 or nuclear factor-κB (NF-κB) [9].

Several researches have reported that single nucleotide polymorphisms (SNPs) of *eNOS* may regulate gene transcription, thus, affecting the causing the protein levels and activity of eNOS [10]. The *eNOS* gene is located on chromosome 7q36.1. Among the known polymorphisms, there were three extensively studied genetic polymorphisms that could modify its transcription and endogenous NO production: a single nucleotide polymorphism (SNP) −786T > C (rs2070744) in the promoter region, a 27-bp variable nucleotide tandem repeat (VNTR) in intron 4, and another SNP 894G > T (rs1799983) in exon 7 [11, 12]. Numerous studies have investigated how the *eNOS* polymorphisms influencing the risk of carcinogenesis [13-18], including breast cancer, prostate cancer and colorectal cancer, but no conclusive results were obtained [14]. So far, there are very scarce studies about the relationship between lung cancer and *eNOS* gene polymorphisms. In this study, we aimed to explore the association between the genetic polymorphisms of *eNOS* (-786T/C and 894 G/T) and *EGFR* mutation in patients with lung adenocarcinoma.

Material and Methods

Study Subjects

We recruited 277 patients diagnosed of lung adenocarcinoma at Cheng-Ching General Hospital in Taichung, Taiwan from years 2012 to 2015. This study protocol was approved by the Institutional Review Board of Cheng-Ching General Hospital (No. HP120009; 22 September 2012). Informed written consent was obtained from each individual before initiation of the study. For all lung adenocarcinoma patients, the tumor-frozen specimen and paraffin-embedded tissues were collected for EGFR gene sequencing and whole-blood specimens collected from all participants were placed in sterile tubes containing ethylenediaminetetraacetic acid (EDTA) for eNOS genotyping. The clinical information of the patients was staged at the time of diagnosis following the tumor/node/metastasis staging system of the AJCC.

Study Variables

The main endpoint of this study was the prevalence of *EGFR* mutation among Taiwanese patients with lung adenocarcinoma. Independent variable of this study was *eNOS* polymorphisms (-786 T/C, 894 G/T, and combination genotypes -786 T/C / 894 G/T). The relative variables obtained from each participant's medical record included demographics (age, gender, cigarette smoking status) and clinicopathogical characteristics of disease (AJCC classification and differentiation).

DNA Extraction and *EGFR* gene sequencing

The DNA was extracted from tumor-frozen specimen and paraffin-embedded tissues using QIAamp DNA Tissue kits (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Exons 18-21 of the *EGFR* gene were amplified using polymerase chain reaction (PCR) and then DNA sequencing reaction and subjected to electrophoresis using the ABI PRISM 3130XL System (Applied

Biosystems, Foster City, CA, USA) as previously described [19].

Genomic DNA Extraction and *eNOS* Genotyping

The genomic DNA was extracted from the whole blood leukocyte samples using QIAamp DNA blood mini kits (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The *eNOS* polymorphisms −786 T/C (C_15903863_10) and 894 G/T (C_3219460_20) have previously been found to modify its transcription and endogenous NO production and were determined by real-time polymerase chain reaction (PCR) genotyping using the ABI StepOne™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA).

Statistical Analysis

Categorical variables, including demographics, cigarette smoking status, tumor characteristics and *eNOS* genotypes polymorphisms were summarized as number and percentage stratified by *EGFR* mutation status; continuous variables were also expressed as mean and standard deviation. Genotype-Tissue Expression (GTEx) database were used to identify correlations between SNPs and the transformed fibroblasts cell gene expression levels. The distributions of demographics, clinical characteristics and genotype frequencies among lung adenocarcinoma patients, as well as clinicopathological characteristics in different genotypes, were analyzed with a χ2-test. The odds ratio with corresponding 95% confidence interval (CI) and p-values of the associations between the genotype frequencies and risk of *EGFR* mutation, as well as their relationships with the clinicopathologic characteristics, were estimated using multiple logistic regression models after controlling for other covariates. P values less than 0.05 were statistically considered as significant. All statistical analyses were conducted using SAS statistical software (SAS Institute Inc., Cary, NC, USA).

Results

Characteristics of Study Subjects

This study included 125 males and 152 females for analysis, with a mean age of 65 years. The baseline demographics and clinical characteristic of recruited participants were summarized in Table 1. There were 109 (39.4%) and 168 (60.6%) patients in the *EGFR* wild-type and mutation type groups, respectively. These two groups differed significantly in the aspects of gender, cigarette smoking status, and tumor differentiation (p-value ≤0.001). The *EGFR* mutation group, as compared with subjects in the *EGFR* wild-type group, were predominantly female (64.3% vs. 40.4%, respectively), non-smoker (77.4% vs. 45.0%), and more well-differentiated (12.5% vs. 7.3%) and moderately-differentiated (81.5% vs. 72.5%) tumors.

Table 1. Baseline demographics and clinical characteristics of 277 patients with lung adenocarcinoma by EGFR mutation status

Variable	Wild type (N=109)	EGFR mutation (N=168)	p-value
Age, n (%)			
<30	1 (0.9%)	1 (0.6%)	p=0.656
30-39	3 (2.8%)	2 (1.2%)	
40-49	10 (9.2%)	16 (9.5%)	
50-59	21 (19.2%)	44 (26.2%)	
60-69	26 (23.9%)	31 (18.5%)	
≥70	48 (44.0%)	74 (44.0%)	
Mean ± SD	65.45 ± 13.34	65.69 ± 13.58	p=0.885
Gender, n (%)			
Male	65 (59.6%)	60 (35.7%)	p<0.001
Female	44 (40.4%)	108 (64.3%)	
Cigarette smoking, n (%)			
Non-smoker	49 (45.0%)	130 (77.4%)	p<0.001
Smoker	60 (55.0%)	38 (22.6%)	
PPK ± SD	46.34 ± 28.41	19.94 ± 23.83	p<0.001
Tumor AJCC staging, n (%)			
IA	10 (9.2%)	17 (10.1%)	p=0.557
IB	9 (8.3%)	23 (13.7%)	
IIA	5 (4.6%)	7 (4.2%)	
IIB	1 (0.9%)	0 (0%)	
IIIA	10 (9.2%)	11 (6.5%)	
IIIB	17 (15.6%)	19 (11.3%)	
IV	57 (52.2%)	91 (54.2%)	
Tumor differentiation, n (%)			
Good	8 (7.3%)	21 (12.5%)	p=0.001
Moderate	79 (72.5%)	137 (81.5%)	
Poor	22 (20.2%)	10 (6.0%)	

Distribution of eNOS Genotypes of Study Subjects and Its Association with EGFR Mutation

The distribution frequency of two *eNOS* polymorphisms (-786 T/C, rs2070744 and 894 G/T, rs1799983) and haplotypes of patients with lung adenocarcinoma was shown in Table 2. The alleles with the highest distribution frequency for -786 T/C and 894 G/T among recruited patients were homozygous T/T and homozygous G/G for both *EGFR* wild-type and mutation type groups, respectively.

The associations between each genotype of *eNOS* polymorphisms and the risk of *EGFR* mutation of lung adenocarcinoma were summarized in Table 2. For -786 T/C polymorphisms, there was no statistically significant association demonstrated between variant types (TC, CC, and TC+CC) and EGFR mutation type. For 894 G/T polymorphisms, GT + TT genotypes were significantly highly associated with EGFR mutation type (Adjusted odds ratio (AOR) = 2.617, 95% confidential interval (CI) = 1.067-6.416). For combination genotypes of -786 T/C

and 894 G/T, no statistical significant relationship was seen between different haplotypes (T/G, C/T, and others) and EGFR mutation type.

Association between eNOS Polymorphisms and EGFR Hotspot Mutations among Lung Adenocarcinoma Patients

Table 3 demonstrated the association between the polymorphisms of *eNOS* and the *EGFR* hotspot mutation. Two hotspot mutations were analyzed in our study, namely L858R and exon 19 in-frame deletions. Again, no significant association was detected between -786 T/C polymorphisms and either hotspot mutations of *EGFR*. On the other hand, significant associations were observed between exon 19 in-frame deletion and GT genotype (OR = 2.619, 95% CI = 1.242-5.524) and GT + TT genotypes (OR = 2.857, 95% CI = 1.368-5.967) of 894 G/T polymorphisms. No statistically significant relationship was noted between 894 G/T genotypes and L858R mutation of *EGFR*.

Subgroup Analysis of EGFR L858R Mutation Based on Polymorphic Genotypes of eNOS 894 G/T

The AJCC Tumor, Node, Metastasis (TNM) staging system [20] is an internationally accepted system to describe the extent of tumor. It combines features of the tumor into disease stage groups that correlate with survival and are linked to recommendations for treatment, as well as an indicator of prognosis. To further investigate any relationship between clinicopathological characteristics of *EGFR* L858R mutation and *eNOS* 894 G/T polymorphism, we performed a subgroup analysis of L858R mutation, as shown in Table 4. In general, as compared with wild type of GG, GT + TT genotypes were related with a more aggressive disease in regards to AJCC staging, "TNM" classification, and tumor differentiation. Unfortunately, they didn't reach statistically significant difference except the lymph node status ("N" classification, p=0.008). This finding indicated that GT + TT genotypes of *eNOS* 894 G/T were highly associated with lymph node invasion of lung adenocarcinoma.

Table 2. Distribution frequency of *eNOS* genotypes of patients with lung adenocarcinoma and multiple logistic regression analysis of its association with EGFR mutation status

Genotypes SNP	Wild type (N=109) n (%)	Mutation type (N=168) n (%)	OR (95% CI)	AOR (95% CI)
***eNOS* -786T/C (rs2070744)**				
TT	84 (77.1%)	135 (80.4%)	1.00	1.00
TC	21 (19.3%)	31 (18.5%)	0.919 (0.495-1.703)	1.327 (0.592-2.973)
CC	4 (3.6%)	2 (1.1%)	0.311 (0.056-1.736)	0.684 (0.067-6.928)
TC+CC	25 (22.9%)	33 (19.6%)	0.821 (0.457-1.477)	1.243 (0.577-2.680)
***eNOS* 894 G/T (rs1799983)**				
GG	95 (87.2%)	130 (77.4%)	1.00	1.00
GT	14 (12.8%)	36 (21.4%)	1.879 (0.960-3.678)	2.391 (0.967-5.914)
TT	0 (0%)	2 (1.2%)	---	---
GT+TT	14 (12.8%)	38 (22.6%)	**1.984 (1.018-3.866)**	**2.617 (1.067-6.416)**
***eNOS* -786T/C /894 G/T**				
TT/GG	71 (65.1%)	101 (60.1%)	1.00	1.00
Others	37 (33.9%)	63 (37.5%)	1.197 (0.721-1.987)	1.176 (0.685-2.020)
CC/TT	1 (0.9%)	4 (2.4%)	2.812 (0.308-25.690)	1.745 (0.187-16.301)
Others + CC/TT	38 (34.9%)	67 (39.9%)	1.239 (0.751-2.045)	1.195 (0.701-2.039)

The AORs with 95% CIs were estimated by multiple logistic regression models after controlling for age and smoking.

Note: Bold text indicated a significant association with p-value <0.05.

Abbreviations: SNP, single nucleotide polymorphism; AOR, adjusted odds ratio; CI, confidence interval.

Table 3. The associations between the polymorphisms of *eNOS* and the EGFR hotspot mutations in patients with lung adenocarcinoma

Genotypes	Wild type	L858R		Exon 19 in-frame deletion	
	(N=109) n (%)	(N=78) n (%)	OR (95% CI)	(N=81) n (%)	OR (95% CI)
***eNOS* -786T/C (rs2070744)**					
TT	84 (77.1%)	58 (74.4%)	1.00	68 (84.0%)	1.00
TC	21 (19.3%)	18 (23.1%)	1.241 (0.609-2.532)	13 (16.0%)	0.765 (0.357-1.638)
CC	4 (3.6%)	2 (2.5%)	0.724 (0.128-4.085)	0 (0%)	---
TC+CC	25 (22.9%)	20 (25.6%)	1.159 (0.589-2.279)	13 (16.0%)	0.642 (0.306-1.350)
***eNOS* 894 G/T (rs1799983)**					
GG	95 (87.2%)	66 (84.6%)	1.00	57 (70.4%)	1.00
GT	14 (12.8%)	12 (15.4%)	1.234 (0.537-2.837)	22 (27.2%)	**2.619 (1.242-5.524)**
TT	0 (0%)	0 (0%)	---	2 (2.4%)	---
GT+TT	14 (12.8%)	12 (15.4%)	1.234 (0.537-2.837)	24 (29.6%)	**2.857 (1.368-5.967)**

Note: There were 9 patients not included in the analysis, due to unknown or inconclusive real-time mutation results.

Note: Bold text indicated a significant association with p-value <0.05.

Abbreviations: OR, odds ratio; CI, confidence interval.

Table 4. Clinicopathologic characteristics of lung adenocarcinoma patients with EGFR L858R mutation, stratified by polymorphic genotypes of *eNOS* 894 G/T (rs1799983)

Variables	GG (N=66)	GT + TT (N=12)	p-value
Tumor AJCC stages, n (%)			
I+II	22 (33.3%)	1 (8.3%)	p=0.081
III+IV	44 (66.7%)	11 (91.7%)	
Tumor "T" classification, n (%)			
T1+T2	50 (75.8%)	7 (58.3%)	p=0.211
T3+T4	16 (24.2%)	5 (41.7%)	
Lymph node status, n (%)			
Negative	26 (39.4%)	0 (0%)	**p=0.008***
Positive	40 (60.6%)	12 (100%)	
Distant Metastasis, n (%)			
Negative	37 (56.1%)	6 (50.0%)	p=0.698
Positive	29 (43.9%)	6 (50.0%)	
Tumor differentiation, n (%)			
Good	9 (13.6%)	2 (16.7%)	p=0.912
Moderate	53 (80.3%)	9 (75.0%)	
Poor	4 (6.1%)	1 (8.3%)	

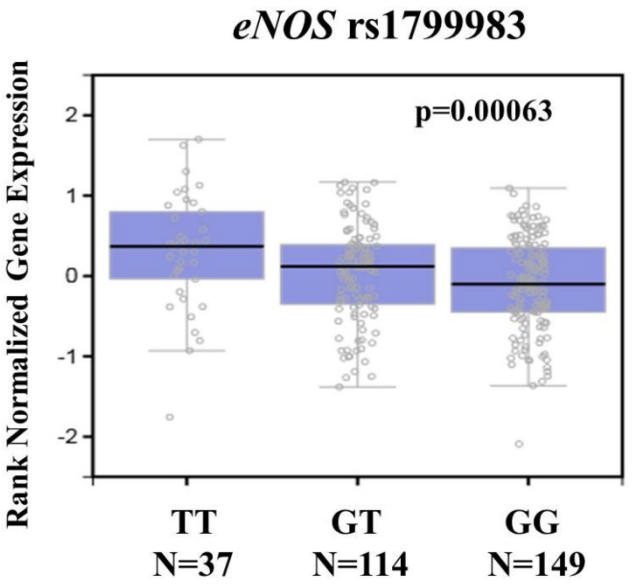

Figure 1. eNOS displays a significant expression quantitative trait locus (eQTL) association with rs1799983 genotypes in the transformed fibroblasts cell of the Genotype-Tissue Expression (GTEx) database.

To further support our findings, we assessed eNOS expression in the transformed fibroblasts cell using the Genotype-Tissue Expression database (GTEx) database to conduct the putative functional relevance of *eNOS* SNP rs1799983. As shown in figure 1, the GTEx database revealed a statistically significant upregulation of eNOS mRNA expression in the transformed fibroblasts cell of rs1799983-variant genotypes (GT or TT) compared with that of the WT homozygous GG genotype (p=0.00063) (Figure 1). The data suggested that changes in eNOS expression due to genetic polymorphisms may affect the development of cervical cancer.

Discussion

The purpose of this study was to examine the associations between *eNOS* gene polymorphism and *EGFR* mutation of lung adenocarcinoma, which refers to a predictor of responsiveness to targeted therapy. Among the 277 patients, variant types (GT + TT) of *eNOS* 894 G/T polymorphism (rs1799983) were significantly positively correlated with *EGFR* mutation type, specifically exon 19 in-frame deletion. With the subgroup of *EGFR* L858R mutation, variant genotypes (GT + TT) of *eNOS* 894 G/T were significantly associated with lymph node invasion.

EGFR mutation of NSCLCs was predominantly observed among Asian population, as well as females and non-smokers [3]. Hsu CH et al. [21] also performed a study in 2016, utilizing National Taiwan Lung Cancer Registry, and noticed *EGFR* mutation prevalence higher than 50%. Our study revealed a consistently high prevalence of *EGFR* mutation. Within this subgroup, females and non-smokers were predominant, as well as better differentiation of lung tumors.

In recent two decades, polymorphisms of *eNOS* gene have gained attention for susceptibility of tumorigenesis or carcinogenesis, as well as cancer invasion and metastasis. Several studies focused on the associations of its three major polymorphisms, namely G894T, T-786C and VNTR 4a/b, and different cancers, such as bladder [13-15, 22], colorectum [14, 15, 23], breast [11, 13, 14, 24-26], prostate [14, 17, 27-33], stomach [14, 34], lung [35-41], liver [10, 14, 42], gallbladder [43], pancreas [44], and others [14, 45-47]. There were also different researches investigating correlation of *eNOS* polymorphisms and responsiveness of different therapies toward malignancies, such as chemotherapy, targeted therapy and radiotherapy [5, 10, 38, 48-53]. There were three significant meta-analyses performed in 2014-2015 regarding the associations between three *eNOS* polymorphisms and cancer risks. Wu X et al. [15] concluded: 1) *eNOS* -786 variants were associated with increased cancer risk; 2) *eNOS* G894T was associated with risk for females and for breast cancer; and 3) *eNOS* intron 4a/b polymorphisms increased susceptibility was revealed for prostate cancer. Conversely, Haque S et al. [14] collected 29 research articles and revealed that *eNOS* 4a/b and G894T polymorphisms were not associated with cancer risk. Still, no consistent conclusion was obtained from these meta-analyses.

In the field of lung cancer, unlike other malignancies, there were very few studies examining the role of *eNOS* gene polymorphism in carcinogenesis and invasiveness. And yet, the answer was conflicting so far. One study showed that VNTR polymorphism was the culprit [35], while another believed high expression of *NOS* was a favorable prognostic sign [40]. Moreover, Donnini et al. also reports that NO and PGE2 exist cross-talk effects in

EGFR-driven epithelial tumor cells and may as the better therapeutic strategies for cancer treatment [54]. In our study, we performed analysis of lung adenocarcinoma patients based on their *EGFR* mutation types and *eNOS* gene polymorphisms (-786 T/C and 894 G/T). There was significantly positive correlation between 894 G/T variant types (GT + TT) and *EGFR* mutation type, but no significant relationship was found between *EGFR* mutation type and -786 T/C (T/G, C/T, and others). To further exploring this association, we noticed that 894 G/T genotypes (GT and GT + TT) was highly correlated with *EGFR* mutation type of exon 19 in-frame deletion, instead of L858R. To determine if there was any correlation between *EGFR* L858R mutation and *eNOS* 894 G/T genotypes, we performed a subgroup analysis of L858R mutation based on clinicopathological characteristics of lung cancer. We noticed that GT + TT genotypes were associated with more invasive disease as compared with the wild type of GG, represented by higher AJCC staging (stage III + IV) percentage, higher AJCC "T" classification (T3 + T4) percentage, positive lymph node invasion, more distant metastasis, and poorer tumor differentiation. But these findings did not reach statistically significant difference, except lymph node invasion. This is mostly due to limited study sample. The limitation of our study is lacking of healthy control group. The possible association of *eNOS* gene polymorphisms with the risk of developing lung cancer is worth for further investigation, which will be included in our future work. To our best knowledge, our study is the first one investigating association between *eNOS* polymorphisms and *EGFR* mutation of lung adenocarcinoma. Further exploration of this correlation with a large-scale study population was mandatory for better understanding of this gene prediction.

To apply the knowledge of our findings in the clinical practice, we may utilize genetic analysis of *eNOS* polymorphism to predict future cancer risk, especially among NSCLC. Also, with the significant association between *eNOS* 894 G/T variant types and *EGFR* mutation of lung cancer, which was considered as a predictor of therapy responsiveness, we may provide with EGFR-TKI therapy in the early phase of disease once diagnosed and predict a better response.

In conclusion, our study showed that *eNOS* 894 G/T variant were significantly associated with *EGFR* mutation types of lung adenocarcinoma, specifically exon 19 in-frame deletion. This may be utilized as a prediction of tumor invasiveness and therapy responsiveness.

Competing Interests

The authors have declared that no competing interest exists.

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