

RESEARCH ARTICLE

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Genetic polymorphisms in the endothelial nitric oxide synthase gene correlate with overall survival in advanced non-small-cell lung cancer patients treated with platinum-based doublet chemotherapy

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Abstract

Background: Nitric oxide (NO) is a free radical that is involved in carcinogenesis. Endothelial NO, synthesized from L-arginine by endothelial NO synthase (eNOS), inhibits apoptosis and promotes angiogenesis, tumor cell proliferation and metastasis. The aim of this study was to evaluate the influence of polymorphisms in the eNOS gene on prognosis of patients with advanced stage non-small-cell lung cancer (NSCLC).

Methods: Unresectable, chemotherapy naïve stage III or IV NSCLC patients who were treated with standard platinum-containing doublet regimens were analyzed. All individuals were genotyped for the single-nucleotide polymorphism G894T in exon 7 of the eNOS gene and for a variable number of tandem repeats (VNTR) polymorphism in intron 4 that results in a rare smaller allele (a) and a common larger allele (b), to investigate the association between these polymorphisms and clinical outcomes. The primary endpoint was correlation with overall survival.

Results: From October 2004 to December 2007, 108 patients (male/female, 66/42; Stage IIIA/IIIB/IV, 6/30/72) aged 29-77 years (median 63) with good performance status were consecutively enrolled in this study. Using Kaplan-Meier estimates, we showed that 5-year overall survival was significantly increased in patients carrying the VNTR a-allele compared with VNTR b/b patients ($P = 0.015$). In multivariate Cox proportional hazard analysis, the VNTR polymorphism was an independent prognostic factor for survival.

Conclusions: The results support the role of the VNTR polymorphism in intron 4 as a marker for survival in patients with advanced stage NSCLC who are candidates for standard chemotherapy.

Background

Lung cancer is the leading cause of cancer-related death in most developed countries [1]. Non-small-cell lung cancer (NSCLC) accounts for 80% of all lung cancers, and platinum-based chemotherapy is considered the standard of care worldwide for patients with advanced stage disease [2]. The treatment response is related to

various factors, most of which are defined by tumor and clinical characteristics, such as disease stage and performance status. However, recent studies have shown that genetic factors may also influence the effectiveness of treatment [3,4].

Nitric oxide (NO) is a small free radical and is involved in numerous physiologic and pathophysiologic processes, including vasodilation, neurotransmission, immunity and carcinogenesis. NO is synthesized from L-arginine and oxygen by four major isoforms of NO synthase (NOS): neuronal NOS, endothelial NOS

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(eNOS), inducible NOS, and mitochondrial NOS. Although eNOS produces low levels of NO, many clinical observational studies have shown a dysregulation of eNOS expression in human solid tumors [5].

The number of reported polymorphisms in the gene encoding eNOS (*NOS3*) exceeds 160. The G894T polymorphism (rs1799983), which is located in exon 7 of the gene and leads to the amino acid change from Glu298Asp, is associated with reduced basal NO production [6]. In addition, a 27-bp variable number of tandem repeat (VNTR) polymorphism in intron 4 has been associated with variations in plasma levels of NO and its metabolites [7,8]. This polymorphism has three alleles: a short 4 repeat allele (a-allele), a more common long 5 repeat allele (b-allele), and a rare allele with 6 repeats. Recent studies have indicated that this repeat polymorphism in intron 4 is the source of a 27 nt-long RNA derived from pre-mRNA splicing. This RNA species inhibits eNOS expression and may represent a new class of small RNA [9,10]. Endothelial cells containing the a-allele produce a lower level of 27-nt small RNA and a higher level of eNOS mRNA compared with cells with the more common b-allele [10]. The association between eNOS mRNA levels and the a-allele appeared to be dose-dependent [11].

To date, very few studies have investigated the association of these polymorphisms with the prognosis of various neoplastic disorders, and no studies have examined them in relation to survival of advanced stage NSCLC patients who are candidates for standard platinum-based chemotherapy. In this study, we evaluated the potential association between these polymorphisms and survival of NSCLC patients.

Methods

One hundred and eight patients who were judged to have inoperable stage III or IV NSCLC at the time of diagnosis were consecutively enrolled in our study between October 2004 and December 2007. All patients were native-Japanese of Asian ethnicity. Staging was based upon the 6th tumor node metastasis (TNM) staging system [12]. Patients with symptomatic brain metastasis, malignancies other than NSCLC within the last 5 years, presence of acute or chronic infections rendering them unsuitable for chemotherapy, or an Eastern Cooperative Oncology Group (ECOG) performance status (PS) of between 2 and 4 were excluded from the study. Patients with operable disease were also excluded, because surgery was considered to be a substantial confounding factor that masks the genetic effect on survival. Tumor response was evaluated according to the Response Evaluation Criteria in Solid Tumors (RECIST) [13], and all responses were evaluated at least 4 weeks after initial assessment. The local ethical committee

(Kyoto University Graduate School and Faculty of Medicine, Ethics Committee) approved the study, and written informed consent was obtained from each patient before enrollment. The study was conducted in accordance with the Helsinki declaration.

The patients received one of the following three chemotherapy treatments: carboplatin/paclitaxel, carboplatin/gemcitabine or cisplatin/gemcitabine. With regard to the carboplatin/paclitaxel regimen, paclitaxel was administered either at a lower dose (70 mg/m²) on a weekly basis for 3 out of 4 weeks, or at 180 mg/m² on day 1 (carboplatin dose was area under the curve (AUC) = 5 or 6 mg/mL • min on day 1). The combination of carboplatin and gemcitabine consisted of carboplatin AUC 5 mg/mL • min on day 1 plus gemcitabine 1000 mg/m² on days 1 and 8 every 3 weeks. Carboplatin was replaced with cisplatin (80 mg/m² on day 1) in the cisplatin/gemcitabine regimen. Each treatment was repeated for three to six cycles, unless unacceptable toxicity or disease progression was apparent. Patients who are candidate of definite thoracic irradiation therapy received carboplatin AUC 5 mg/mL • min on day 1 plus paclitaxel 40 mg/m² on days 1, 8 and 15 every 4 weeks in a concurrent fashion. Two to four cycles of carboplatin/paclitaxel were administered subsequently for consolidation.

Blood samples were collected and DNA was isolated from whole blood cell fractions using the QIAamp DNA Blood Mini Kit (Qiagen, Tokyo, Japan). The single nucleotide polymorphism (SNP) of G894T (a G-to-T transversion at nucleotide 894 in exon 7) was analyzed with TaqMan genotyping assays, using the ABI 7500 real-time PCR system (Applied Biosystems, Inc., Tokyo, Japan). Probes, primers and TaqMan universal PCR master mix were those from ABI and TaqMan assays were performed according to the manufacturer's instructions. The intronic 27 bp insertion/deletion VNTR was genotyped using PCR amplification and agarose gel electrophoresis with ethidium bromide staining [14].

The distribution of genotypes was tested for Hardy-Weinberg equilibrium with the goodness-of-fit χ^2 test. The linkage disequilibrium of the two polymorphisms was analyzed. The association between overall survival times was estimated using the Kaplan-Meier method and assessed using the log-rank test. The effects of different genotypes on overall survival were estimated using hazard ratios (HRs), and 95% confidence intervals (95% CIs) were estimated using the multivariate Cox proportional hazards regression model, with adjustment for gender, age (<70 vs. older), ECOG PS (0 vs. 1), smoking status (never-smoker vs. former/current smoker), clinical stage (III vs. IV), histology (adenocarcinoma vs. others), thoracic radiotherapy (yes vs. no) and the two genotypes examined. We used Akaike's

information criterion for entering and removing a variable in a stepwise Cox regression [15]. Survival time was calculated from diagnosis to death or last follow-up. A *P* value of <0.05 was considered statistically significant.

We estimated the false-positive report probability for the observed statistically significant associations using the methods described by Wacholder et al [16]. HR values from 2.0 to 4.0 were considered likely threshold values. The prior probability used was 0.1 for these polymorphisms and the false-positive report probability value for noteworthiness was set at 0.5.

Statistical analyses were performed using JMP version 6 (SAS Institute, Cary, NC, USA.), R software (R Foundation for Statistical Computing., Vienna, Austria.), and Haploview version 4.0 [17].

Results

Patients' characteristics are summarized in Table 1. Eighty percent of patients had a cytological or histological diagnosis of adenocarcinoma. Treatment characteristics are shown in Table 2. By July 31, 2009, 73 (68%) of the 108 patients had died. The median follow-up period was 21.5 months.

Median survival for all 108 patients was 26.8 months (95%CI, range 21.9 - 30.7 months). Median survival was 23.3 months for male patients and 32.3 months for

Table 1 Characteristics of the 108 patients with non-small-cell lung cancer enrolled in the study

	Patients	
	N	%
Age, Years		
Median	63	
Range	29 - 77	
Gender		
Male	66	61.1
Female	42	38.9
Stage		
IIIA	6	5.5
IIIB	30	27.8
IV	72	66.7
PS		
0	23	21.3
1	85	78.7
Chemotherapy regimen		
CBDCA+PAC	89	82.4
CBDCA+GEM	17	15.7
CDDP+GEM	2	1.9
Definitive thoracic radiotherapy		
Yes	20	18.5
No	88	81.5

Abbreviations: CBDCA, carboplatin; PAC, paclitaxel; CDDP, cisplatin; GEM, gemcitabine

Table 2 Treatment characteristics of the enrolled patients

	No. of patients		
	Stage IIIA	Stage IIIB	Stage IV
CBDCA + PAC + TRT	6	14	0
CBDCA + PAC	0	13	56
CBDCA + GEM	0	3	14
CDDP + GEM	0	0	2

Abbreviations: TRT, thoracic radiotherapy

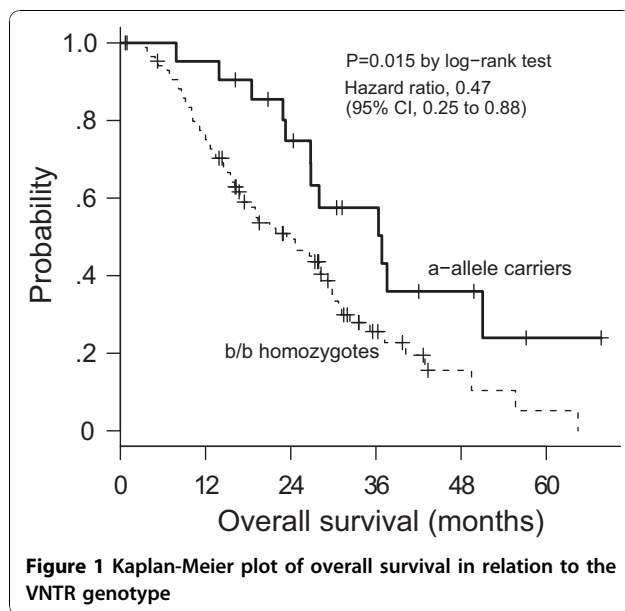
female patients (HR, 1.72; 95%CI, 1.04-2.85; *P* = 0.034). Median survival was 24.6 months for smokers and 35.2 months for never-smokers (HR, 1.69; 95%CI, 1.00-2.85; *P* = 0.048). There was substantial agreement between gender and smoking status (Cohen's kappa coefficient, 0.64). Age, ECOG PS, disease stage, histology, and thoracic radiotherapy were not significantly associated with overall survival in the univariate analysis. Regarding epidermal growth factor receptor (EGFR) gene mutations, tumor samples of 34 patients (31.5%) were analyzed and 21 were positive for mutations. Eleven tumors had an L858R mutation in exon 21 and nine contained in-frame nucleotide deletions (including E746-A750) in exon 19. A G719S mutation was found in one tumor. EGFR mutation status (positive vs. negative/unknown) was not prognostic in the univariate analysis.

The distribution of all genotypes and allelic frequencies is shown in Table 3. These frequencies were in agreement with previous reports, and with those expected according to the Hardy-Weinberg equilibrium model. Linkage disequilibrium between these polymorphisms was not observed. Since there was no TT homozygote carrier with respect to G894T, and analyses were performed between the GG homozygote group and the GT heterozygote group. In addition, because the frequency of intron 4 short repeat (a-allele) homozygotes was low (0.9%; *n* = 1), b/a heterozygotes and a/a homozygotes were combined into an a-allele carrier group for analyses.

Median survival was 36.8 months for a-allele carriers and 23.4 months for b/b homozygotes (Figure 1). The univariate Cox regression model showed that presence of the a-allele significantly correlated with prolonged survival (HR, 0.47; 95% CI, 0.25-0.88; *P* = 0.015). Correspondingly, carriers of the common b/b genotype had a

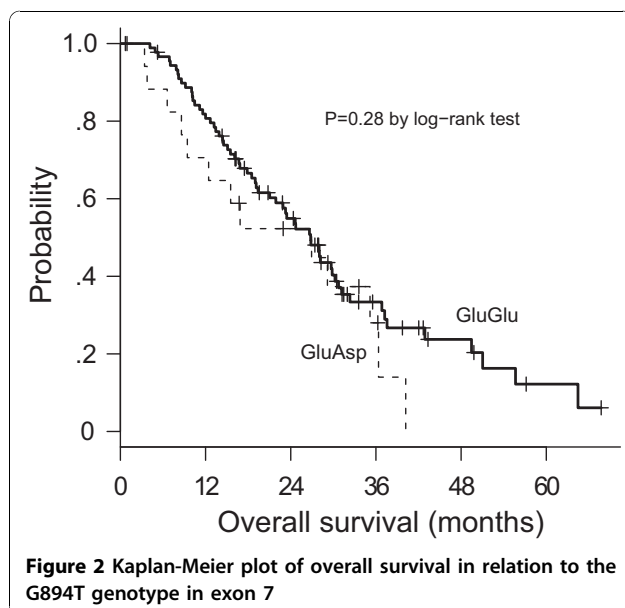
Table 3 Genotypic and allelic frequencies of the G894T SNP in exon 7 and the VNTR in intron 4

	Genotypic frequencies (%)			Allelic frequencies (%)	
	GluGlu	GluAsp	AspAsp	Glu	Asp
G894T	84.3	15.7	0	92.1	7.9
VNTR in intron 4	bb	ba	aa	b	a
	80.6	18.5	0.9	89.8	10.2



higher risk of death than carriers of the a-allele (HR, 2.14). The false-positive report probability for the VNTR was 0.16, indicating noteworthiness. No significant difference in survival was observed according to the G894T SNP ($P = 0.28$) (Figure 2).

EGFR mutation status was not included in a Cox proportional hazards model because the number of analyzed patients was small and statistically significant results were not observed in the univariate analysis. Including gender, age, ECOG PS, smoking status, clinical stage, histology, thoracic radiotherapy and two genotypes in a model, the common b/b genotype (HR, 2.33; 95% CI, 1.24 - 4.36; $P = 0.0085$), male gender (HR, 2.02;



95% CI, 1.20-3.43; $P = 0.0084$) and stage IV disease (HR, 1.76; 95% CI, 1.04-2.99; $P = 0.036$) were selected as the predictors for overall survival. Correspondingly, HRs of female gender and stage III disease were 0.50 and 0.57, respectively.

With regard to clinical and pathological parameters, there were no discrepancies between carriers of the VNTR common b/b genotype and the VNTR a-allele carriers (Table 4). To investigate whether the eNOS intron 4 VNTR was associated with clinical benefit of chemotherapy, we analyzed the treatment response among patients without definitive thoracic radiotherapy. The overall response rate in 82 evaluable patients was 27%, with 22 (27%) partial responders (PR), 50 patients (61%) with stable disease (SD), and 10 patients (12%) with disease progression (PD). There was no significant distribution of a-allele carriers when patients were separated into two groups (PR+SD group vs. PD group; Table 5), indicating that the effect of the polymorphism on survival was not associated with the clinical benefit of first-line platinum-containing chemotherapy. We restricted the population to patients who received carboplatin plus paclitaxel without definitive thoracic radiotherapy and re-analyzed. The PR+SD rate was 81% in a-allele carriers and 86% in b/b genotype patients, respectively. Again there was no significant difference between the groups ($P = 0.69$).

Discussion

Our results show that polymorphism of the 27-bp VNTR in intron 4 of the eNOS gene is strongly associated with survival in advanced stage NSCLC patients treated with standard platinum-based chemotherapy. Median survival was significantly prolonged in patients carrying the shorter a-allele (i.e., those with a higher level of eNOS mRNA production).

Published studies indicate a procarcinogenic role of eNOS. In solid tumors, eNOS plays an essential role in VEGF-induced angiogenesis and vascular permeability [18]. A recent study showed that blocking phosphorylation of eNOS inhibited tumor initiation and maintenance through inactivation of the PI3K-AKT-eNOS-RAS pathway [19]. Moreover, a higher eNOS level was shown to correlate with cisplatin resistance in ovarian cancer cell lines [20]. Our findings were unexpected and in contrast to these observations.

However, our results can be explained by considering the following factors. NO derived from eNOS or eNOS itself can be cytotoxic to cancer cells through direct DNA damage and can be antitumorigenic [21]. The cytotoxic effects of NO can also be mediated through the generation of peroxynitrite (ONOO⁻) in the presence of superoxide anion (O₂⁻) [22]. Moreover, clearance of metastatic cells from organ microvasculature is

Table 4 Comparative analysis of demographic and pathological information

	No. of bb genotype pts.	No. of a-allele carriers	P
Gender			
Female	35	7	P = 0.63
Male	52	14	
Age			
<70	64	15	P > 0.9
70 or older	23	6	
ECOG PS			
0	17	6	P = 0.38
1	70	15	
Smoking Status			
Never or Light	29	7	P > 0.9
Current or Former	58	14	
Clinical Stage			
III	31	5	P = 0.44
IV	56	16	
Histology			
Adenocarcinoma	68	18	P = 0.56
Non-adenocarcinoma	19	3	
EGFR mutation			
Activating mutation	19	2	P = 0.30
Wild type	9	4	
Unknown	59	15	
Thoracic radiotherapy			
Yes	17	3	P = 0.76
No	70	18	

regulated in part by NO. NO has been reported to reduce the attachment of tumor cells to the endothelium and NO-mediated vasodilation may also contribute to the clearance of tumor cells [23,24]. Using an eNOS knockout mice model and the B16F1 melanoma cell line, Wang and colleagues have shown that arrest of tumor cell metastasis in the portal and pulmonary circulation can trigger the immediate release of NO in an eNOS-dependent manner, and subsequently induce apoptosis in these tumor cells [25,26]. Our observations were consistent with these reports. Furthermore, Mortensen et al. showed that expression of immunoreactive eNOS in the peritumoral microvasculature is a favorable prognostic indicator in premenopausal breast cancer patients, providing clinical evidence for a defensive role of eNOS against cancer cells [27]. With respect to

treatment modality, NO was shown to increase the efficacy of chemotherapy and improve survival of patients with NSCLC in one phase II trial. Yasuda et al. randomized 120 patients with advanced stage NSCLC to receive standard chemotherapy (cisplatin and vinorelbine) alone, or the same regimen with nitroglycerin, a NO-donating drug [28]. The median time to progression was longer (10.9 vs. 6.2 months) in the nitroglycerin treatment group. Thus, in certain situations NO appears to act as a defense against the progression and/or metastasis of malignant tumors.

The median survival time of 26.8 months observed in the present study was favorable compared with that described in other reports [29,30]. This is probably due to the inclusion of a heterogeneous treatment group (including patients who received definitive chemoradiation) and the relatively young population (median 63 years of age).

Our study has several limitations. The single-institutional study design contains the potential to introduce a selection bias. Although we estimated the false-positive report probability to confirm the statistical significance obtained by established methods, analysis based upon a small number of enrolled patients could potentially lead to false-positive results. Two recent Japanese trials

Table 5 Frequency and distribution of eNOS intron 4 VNTR alleles in 72 patients with partial response or stable disease and 10 patients with progressive disease

	PR+SD		PD		P
	No. of Patients	%	No. of Patients	%	
a-allele carriers	15	83	3	17	0.68
bb genotype	57	89	7	11	

confirmed that EGFR mutation status is a predictive factor for response to EGFR-TKI treatment with a progression free survival of 10 months [31,32] and this finding paves the way for the personalized therapy for NSCLC. EGFR mutation status was evaluated in only a small proportion of the enrolled patients (31.5%) and the favorable effect of the mutations on prognosis was not observed in our study. Finally, because we restricted the study subjects to those of East-Asian ethnicity, it is uncertain whether these results can be generalized to other populations.

Conclusions

Our results indicate that the VNTR polymorphism in intron 4 of the eNOS gene may be associated with the progression of NSCLC and shed light on the association between polymorphisms in the eNOS gene and survival of patients with NSCLC.

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Authors' contributions

SF participated in the design of the study, carried out the molecular genetic analysis and performed the statistical analysis. KM carried out the molecular genetic analysis, contributed to acquisition of data and helped to draft the manuscript. YH, AF, YHK and TM have made substantial contribution to acquisition of data. AH and RK performed the statistical analysis and helped to draft the manuscript. MM participated in the design of the study and helped to draft the manuscript. NK participated in the design of the study and conceived of the study. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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