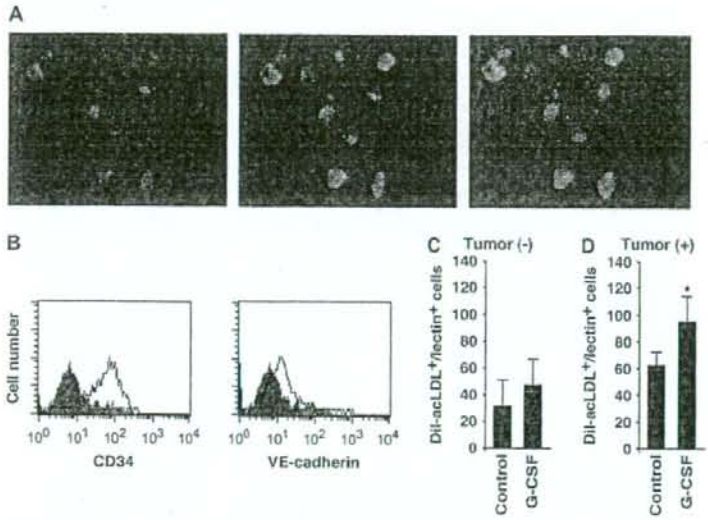

Fig. 4. G-CSF treatment increased circulating EPCs in cancer animal models. (A) Mononuclear cells were isolated from peripheral blood and cultured. DiI-acLDL uptake (left panel) and lectin binding (middle panel) of adherent cells were determined by fluorescence microscopy. Double-positive cells (merged) were considered as EPCs (right panel). (B) Expression of CD34 and VE-cadherin on EPCs. The blank histograms indicate staining with the indicated mAb, and the shaded histograms indicate background staining with control IgG. (C) G-CSF (8 μg kg^{-1}) was injected into mice for 3 days daily. The mice were then sacrificed. Mononuclear cells (4 × 10^6 cells per mouse) were isolated from peripheral blood and cultured. Adherent DiI-acLDL and lectin double-positive cells were counted. The number is the average of three high-power fields per mouse. Results are indicated as mean ± SD of eight mice in each group. (D) Mice were inoculated with LLCs on day 0. From day 25, G-CSF (8 μg kg^{-1}) was injected into mice for 3 days daily. Mice were sacrificed on day 28. Mononuclear cells (4 × 10^6 cells per mouse) were isolated from peripheral blood and cultured. Adherent DiI-acLDL and lectin double-positive cells were counted. The number is the average of three high-power fields per mouse. Results are indicated as mean ± SD of eight mice in each group. Compared with the control, the number of double-positive cells in G-CSF-treated mice was significantly increased (*$P < 0.01$).

G-CSF treatment increased Gr1+CD11b+ cells both in tumor-free and tumor-bearing mice

In tumor-bearing hosts, increase of Gr1+CD11b+ cells and immunosuppressive effect of these cells have been reported (25). Moreover, Gr1+CD11b+ cells in spleens of tumor-bearing mice were shown to directly differentiate into ECs in tumors and contribute to tumor angiogenesis and growth (15). They also indicated that Gr1+CD11b+ cells were different from EPCs. To investigate whether G-CSF increases Gr1+CD11b+ cells in spleens, mice were treated with G-CSF for 3 days. The spleens were isolated and the splenocytes were subjected to FACS analysis. The cell population of Gr1+CD11b+ cells in spleens of control mice was 3.61 ± 0.17%, and G-CSF treatment significantly increased Gr1+CD11b+ cells up to 7.52 ± 1.04% (Fig. 6A; mean ± SD). As the G-CSF treatment induced splenomegaly, the difference in the absolute number of Gr1+CD11b+ cells in spleens became much more evident between control and G-CSF-treated mice (Fig. 6C). Gr1+CD11b+ cells in spleens were indicated to increase 21–28 days after LLC inoculation (15). We treated mice with G-CSF daily for 3 days from 19 days after LLC inoculation, and isolated spleens 22 days after LLC inoculation. On day 22, there was no significant difference in tumor size between control and G-CSF-treated mice. The cell population of Gr1+CD11b+ cells in spleens of tumor-bearing mice was 21.6 ± 2.2% (Fig. 6B). G-CSF treatment in tumor-bearing mice significantly increased the cell population of Gr1+CD11b+ cells up to 41.1 ± 0.5% (Fig. 6B; mean ± SD). G-CSF treatment also significantly increased the absolute number of Gr1+CD11b+ cells in spleens of tumor-bearing mice (Fig. 6D).

Discussion

G-CSF has been reported to activate angiogenesis in malignancy (7), but its precise mechanism has not been fully clarified. Here, we demonstrated that accelerated tumor growth by G-CSF was accompanied with angiogenesis activation via increase of circulating EPCs and Gr1+CD11b+ cells (Figs 2–4 and 6).

G-CSF had no accelerating effects on cell proliferation in both cancer cells and ECs *in vitro* (Figs 2 and 3), suggesting that the effect on tumor progression and angiogenesis cannot be explained by their direct action on cells *in situ*. Recently, it was reported that G-CSF enhanced tumor neovascularization in which bone marrow cells participated (7). However, in the study, the involvement of EPCs was not shown. In our study, G-CSF significantly increased the ratio of circulating EPCs in the tumor-bearing mice (Fig. 4). Since EPCs are known to become a part of tumor vessels (9, 10), these studies might

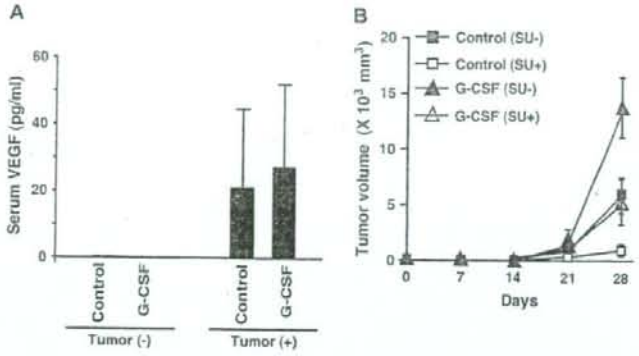

Fig. 5. LLCs inoculation elevated the serum VEGF level, and VEGFR-2 kinase inhibitor SU1498 inhibited the tumor growth. (A) Tumor (−) indicates mice without tumors, and tumor (+) indicates mice with tumors. For tumor (−) models, mice were treated with G-CSF (8 μg kg^{-1}) for 3 days, and the serum VEGF level was determined by ELISA. For tumor (+) models, mice were inoculated with LLCs at day 0, and treated with G-CSF (8 μg kg^{-1}) for 3 days from day 25. At day 28, the serum VEGF level was determined. Inoculation of LLCs induced the elevation of serum VEGF. Results are indicated as mean ± SD of eight mice in each group. (B) At day 0, mice were inoculated with LLCs. From day 9 and every following week, SU1498 was injected into mice three times a week (SU+). From day 10, G-CSF (8 μg kg^{-1}) was injected into mice daily for 3 days, and every following week, G-CSF was injected daily for 3 days. DMSO was injected into mice as a control (SU−). Results are indicated as mean ± SD of five mice in each group.

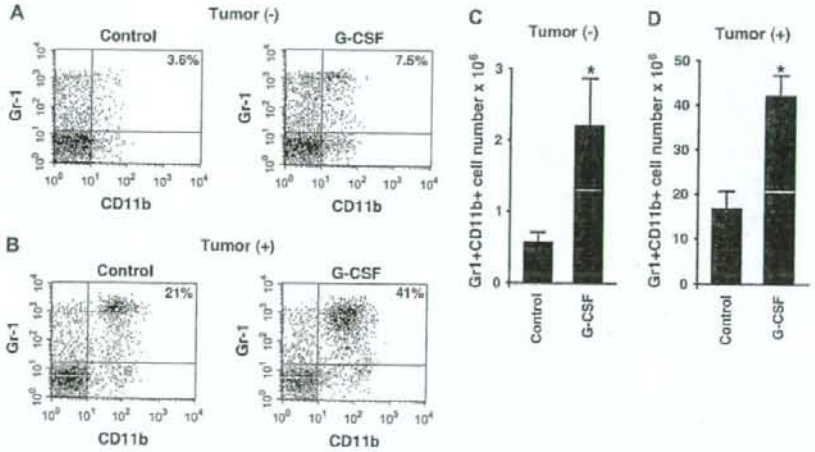

Fig. 6. G-CSF treatment increased Gr1+CD11b+ cells both in tumor-free and tumor-bearing mice. G-CSF indicates mice treated with G-CSF (8 μg kg^{-1}) daily for 3 days. Numbers in panels indicate the cell population of Gr1+CD11b+ cells in splenocytes. Tumor (−) indicates tumor-free mice, and tumor (+) indicates tumor-bearing mice. (A) G-CSF treatment significantly increased the cell population of Gr1+CD11b+ cells in spleens ($*P < 0.03$, $n = 8$ for each group). (B) Mice were inoculated with LLCs on day 0 and treated with G-CSF from day 19 for 3 days, and sacrificed on day 22. G-CSF treatment significantly increased the cell population of Gr1+CD11b+ cells in spleens ($*P < 0.01$, $n = 8$ for each group). (C) Absolute number of Gr1+CD11b+ cells in spleens of tumor-free mice. The number of splenocytes was determined and multiplied by the cell population of Gr1+CD11b+ cells for each mouse. G-CSF treatment significantly increased Gr1+CD11b+ cells ($*P < 0.01$). Results are indicated as mean ± SD of eight mice in each group. (D) Absolute number of Gr1+CD11b+ cells in spleens of tumor-bearing mice. Mice were treated with G-CSF as shown in (B), and this treatment significantly increased Gr1+CD11b+ cells in tumor-bearing mice ($*P < 0.01$). Results are indicated as mean ± SD of eight mice in each group.

suggest that the circulating EPCs mobilized after G-CSF treatment contributed to the vessel formation in the tumors. In contrast to the tumor-bearing mice, G-CSF did not increase the ratio of the EPCs in the mice without tumors (Fig. 4), and these might indicate that G-CSF requires some other factors to increase EPCs. VEGF has been reported to mobilize EPCs from bone marrow (12, 14). Previously, we have shown that LLCs continuously release VEGF (16), and the baseline serum VEGF level was elevated in mice inoculated with LLCs (Fig. 5A). VEGFR-2 tyrosine kinase inhibitor SU1498 partially inhibited

the tumor growth effect of G-CSF (Fig. 5B), and this might indicate a crucial role of a certain level of VEGF to increase EPCs in the periphery.

In addition to suggesting the involvement of circulating EPCs in tumor angiogenesis after G-CSF treatment, here we showed the possibility that the increase of Gr1+CD11b+ cells might contribute to G-CSF-induced activation of tumor angiogenesis (Fig. 6B and D). Previously, Gr1+CD11b+ cells were reported to increase in tumor-bearing hosts, and these cells were also reported to impair immune responses (25). Moreover, very recently, these cells were reported to directly differentiate into ECs and to contribute to tumor angiogenesis (15). EPCs in peripheral blood (or in circulation) are also called circulating endothelial precursor cells, and these cells are described as CD11b negative (9, 26). Therefore, Gr1+CD11b+ cells might represent another population of cells that participate in tumor angiogenesis in addition to EPCs. These results suggest that Gr1+CD11b+ cells contribute G-CSF-induced acceleration of tumor growth not only by immunosuppressive action but also by inducing angiogenesis.

A recent study showed that the majority of cultured EPCs expressed monocyte/macrophage markers and only a minority expressed specific endothelial markers and questioned the nomenclature EPC (27). Our results indicated that cultured EPC expressing CD34 and VE-cadherin is not a minority (Fig. 4). This discrepancy might be due to the difference in culture conditions (concentration of VEGF or FCS or cell density). Moreover, transplanted EPCs cultured in the essentially same conditions as in Fig. 4 were shown to functionally improve neovascularization after hind-limb ischemia in mice, and transplanted EPCs were shown to differentiate into ECs (28, 29). They also showed that macrophages or dendritic cells were significantly less effective in improving neovascularization than EPCs (28). These results indicate the importance of culture conditions for EPCs and difficulties of EPC characterization.

In a previous study, 20 $\mu g\ kg^{-1}$ human recombinant G-CSF was reported to promote tumor angiogenesis in mice (7). Here, we showed that relatively low-dose (similar to clinical dose) G-CSF has the capability to promote tumor angiogenesis. We used N-terminal-mutated human recombinant G-CSF, nartograstim. Nartograstim is known to have a three to five times higher potency than human recombinant G-CSF filgrastim (30). In order to confirm the biological effects of nartograstim at this dose, we showed that nartograstim increased the number of WBCs and neutrophils in peripheral blood (Fig. 1A), and reduced the SDF-1 level in bone marrow (Fig. 1C) as previously described (21). Moreover, for tumor growth models, we treated mice with 50 $\mu g\ kg^{-1}$ G-CSF as a positive control.

As previously shown, a 1-day G-CSF treatment did not change the serum SDF-1 level (Fig. 1) (21). They also showed that a 5-day G-CSF treatment reduced the serum SDF-1 level in mice but not in humans (21). Here, we showed that a 3-day G-CSF treatment to mice at our dose did not change the serum SDF-1 level (Fig. 1). The bone marrow SDF-1 level we showed here (Fig. 1) was lower than that of Petit *et al.* (21), but comparable to that of Hattori *et al.* (31). Petit *et al.* obtained bone marrow by flushing femurs, tibias, humeri and pelvis with 500 µl of PBS. In contrast, we obtained bone marrow by single flush of a right femur with 500 µl of PBS. Moreover, a large difference of SDF-1 levels in bone marrows among different strains of mice was indicated (32). The SDF-1 level in bone marrow has also been described as SDF-1 per femur, and various SDF-1 levels have been reported (ranging from 100 pg per femur to 2.7 ng per femur) (32–34). The bone marrow SDF-1 levels in Fig. 1 were shown as 110–220 pg per femur. Taken together, the different method in obtaining bone marrow and the difference in strains [BALB/c mice by Petit *et al.* (21) and C57BL/6 mice in this paper] might be the reason for the discrepancy of the SDF-1 level in the bone marrow.

In summary, we demonstrated that G-CSF increased circulating EPCs and Gr1+CD11b+ cells, which activated tumor angiogenesis that led to the acceleration of tumor growth in cancer animal models. Moreover, we demonstrated that relatively low-dose G-CSF (nartograstim) showed such effects. These results may suggest that clinicians should be careful with an excessive use of G-CSF in cancer patients with residual tumors.

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Abbreviations

APC	allophycocyanin
DiI-acLDL	acetylated low-density lipoprotein–DiI complex
DMSO	dimethyl sulfoxide
EC	endothelial cell
EPC	endothelial progenitor cell
G-CSF	granulocyte colony-stimulating factor
HUVEC	human umbilical vein endothelial cell
KDR	kinase insert domain containing receptor
LLC	Lewis lung carcinoma cell
MVD	microvessel density
SDF-1	stromal cell-derived factor-1
VE-cadherin	vascular endothelial cadherin
VEGF	vascular endothelial growth factor
WST	water-soluble terazolium

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Green tea consumption and cognitive function: a cross-sectional study from the Tsurugaya Project[1–3]

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ABSTRACT

Background: Although considerable experimental and animal evidence shows that green tea may possess potent activities of neuroprotection, neurorescue, and amyloid precursor protein processing that may lead to cognitive enhancement, no human data are available.

Objective: The objective was to examine the association between green tea consumption and cognitive function in humans.

Design: We analyzed cross-sectional data from a community-based Comprehensive Geriatric Assessment (CGA) conducted in 2002. The subjects were 1003 Japanese subjects aged ≥70 y. They completed a self-administered questionnaire that included questions about the frequency of green tea consumption. We evaluated cognitive function by using the Mini-Mental State Examination with cutoffs of <28, <26, and <24 and calculated multivariate-adjusted odds ratios (ORs) of cognitive impairment.

Results: Higher consumption of green tea was associated with a lower prevalence of cognitive impairment. At the <26 cutoff, after adjustment for potential confounders, the ORs for the cognitive impairment associated with different frequencies of green tea consumption were 1.00 (reference) for ≤3 cups/wk, 0.62 (95% CI: 0.33, 1.19) for 4–6 cups/wk or 1 cup/d, and 0.46 (95% CI: 0.30, 0.72) for ≥2 cups/d (P for trend = 0.0006). Corresponding ORs were 1.00 (reference), 0.60 (95% CI: 0.35, 1.02), and 0.87 (95% CI: 0.55, 1.38) (P for trend = 0.33) for black or oolong tea and 1.00 (reference), 1.16 (95% CI: 0.78, 1.73), and 1.03 (95% CI: 0.59, 1.80) (P for trend = 0.70) for coffee. The results were essentially the same at cutoffs of <28 and <24.

Conclusion: A higher consumption of green tea is associated with a lower prevalence of cognitive impairment in humans. *Am J Clin Nutr* 2006;83:355–61.

KEY WORDS Cognitive function, elderly, green tea, Japanese, Mini-Mental State Examination

INTRODUCTION

Dementia is a rapidly growing public health concern as a result of aging of the population (1, 2). In developed countries, dementia has a reported prevalence of ≈1.5% at age 65 y, doubling every 4 y to reach ≈30% at age 80 y (1). Environmental factors associated with the risk of Alzheimer disease (AD), a common cause of dementia, remain largely undefined, although several risk factors for vascular dementia have been identified (1, 3–6).

Experimental and animal studies have shown that tea and tea polyphenols (which include catechins and their derivatives), particularly those from green tea, may possess potent neuroprotective activity that can help to ameliorate neurodegenerative diseases such as AD and Parkinson disease (PD) (7). Green tea catechins, especially (−)-epigallocatechin-3-gallate (EGCG), formerly thought to be simple radical scavengers, are now considered to invoke a spectrum of cellular mechanisms related to neuroprotective as well as neurorescue activities (8–10). One of these mechanisms includes protective effects against β-amyloid (Aβ)–induced neurotoxicity by enhancing the release of the nonamyloidogenic soluble form of amyloid precursor protein (APP) (8). Aβ protein is formed by proteolytic cleavage of APP (11) and is the main constituent of the neuritic plaques that are the physiologic hallmark of AD (12). In addition, EGCG was shown to have neuroprotective activity in a mice model of PD (13), and an epidemiologic study indicated that the risk of PD was reduced if tea consumption was ≥2 cups/d (14). Despite this considerable evidence that tea, especially green tea, can protect against neurodegenerative diseases, to our knowledge, no data are available on any association between green tea intake and dementia or cognitive impairment in humans.

We therefore designed this cross-sectional analysis to investigate the association between consumption of green tea and cognitive function in elderly Japanese subjects, among whom green tea was widely consumed. We considered it important to search for modifiable factors underlying cognitive impairment

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because early detection and management of cognitive decline contribute to the prevention of dementia rather than to treatment (15, 16).

SUBJECTS AND METHODS

Study population

The Tsurugaya Project was a community-based Comprehensive Geriatric Assessment (CGA) conducted among elderly Japanese subjects living in Tsurugaya district, a suburban area of Sendai City in northern Japan, between July and October 2002 (17, 18). CGA is a structured approach to measuring the physical, mental, and social functioning of elderly people to assess early deterioration that may result in the need for long-term care and to promote healthy aging (19, 20).

At the time of the study, 2730 people aged ≥70 y were living in the Tsurugaya district. We sent letters to all of these people and invited them to participate in the health survey. Of those invited, 1198 participated in the survey and 1178 (43.2%) gave written informed consent to be included in the analysis. The study protocol was approved by the institutional review board of Tohoku University Graduate School of Medicine.

Data about consumption of green tea, black or oolong tea, and coffee and cognitive function were obtained from 1151 of the subjects who gave written informed consent. We excluded 148 subjects with missing data on body weight, height, blood glucose concentrations, blood pressure values, or depressive symptoms (described in Measurements). Thus, data from 1003 subjects contributed to the final analyses.

Measurements

The questionnaire in the CGA included items about the frequency of recent consumption of 5 beverages (green tea, black or oolong tea, coffee, cola or juice, 100% fresh vegetable juice) and 55 items about food intake during the previous month. The frequency of consumption of green tea was divided into 8 categories: never, <1 cup (0.1 L)/wk, 1 cup/wk, 2–3 cups/wk, 4–6 cups/wk, 1 cup/d, 2–3 cups/d, and ≥4 cups/d. In the study region, the volume of a typical cup of green tea is 100 mL. We grouped the subjects into 3 categories according to their beverage consumption: ≤3cups/wk, 4–6 cups/wk or 1 cup/d, and ≥2 cups/d.

The questionnaire in the CGA also included *1*) demographic characteristics (age, sex, and duration of education); *2*) social factors (visiting friends); *3*) lifestyle habits (smoking, alcohol use, and physical activity); and *4*) physical health [history of chronic medical conditions such as stroke or myocardial infarction, regular intake of supplements and medication, and self-rated health (excellent, good, normal, poor, or very poor)].

Cognitive function was tested by using the Japanese language version of the 30-point Mini-Mental State Examination (MMSE) (21). The test was administered by specially trained research assistants. The MMSE includes questions on orientation to time and place, registration, attention and calculation, recall, language, and visual construction. This screening test was originally created for a clinical setting (21) and is used extensively in epidemiologic studies (22). Higher MMSE scores indicate higher cognitive function, and the maximum score is 30 points. The analyses were conducted by using 3 cutoff points to define different levels of cognitive impairment. The initial cutoff point was <26, because a score of <26 points on the MMSE generally indicates cognitive impairment (23). The second was <28, which we regarded as slight cognitive impairment, and the third was <24, which we regarded as relatively severe cognitive impairment. In the initial analyses, the group with cutoff points of <26 included subjects with cutoff points of <24, and the group with cutoff points of <28 included subjects with cutoff points of <26 and <24. In further analyses, we reanalyzed the data by using cutoff points of <26 or <28 after excluding subjects with a MMSE score of <24.

Data were obtained about *1*) body mass index (BMI; in kg/m^2; as calculated from participants' measured weight and height); *2*) the presence or absence of diabetes mellitus, defined as a nonfasting blood glucose concentration ≥ 140 mg/dL or a history of diabetes mellitus; *3*) the presence or absence of hypertension, defined as a self-measured systolic blood pressure ≥ 135 mm Hg (measured at home) or a history of hypertension; *4*) the presence or absence of depressive symptoms, as assessed by using the Japanese version of the 30-item Geriatric Depression Scale (24); and *5*) physical functioning status, assessed by using the 6-item physical functioning status measure of the Medical Outcomes Study (MOS) Short-form General Health Survey (lower MOS scores indicate lower physical functioning status) (25).

Statistical analysis

The subjects' characteristics according to categories of green tea consumption were compared by using analysis of variance or chi-squared test, as appropriate. We used multivariate logistic regression analysis to calculate odds ratios (ORs) for cognitive impairment relative to the consumption frequencies of green tea or other beverages, with the lowest frequency category (≤3 cups/wk) treated as the reference group. Trend tests were performed by including the ordinal variable in a linear regression analysis. In these analyses, we regarded the following data as covariates: age (continuous variable); sex; consumption of green tea (≤3 cups/wk, 4–6 cups/wk or 1 cup/d, ≥2 cups/d; when calculating the ORs for consumption of black or oolong tea or coffee); consumption of black or oolong tea (≤3 cups/wk, 4–6 cups/wk or 1 cup/d, ≥2 cups/d; when calculating the ORs for consumption of green tea or for coffee); consumption of coffee (≤3 cups/wk, 4–6 cups/wk or 1 cup/d, ≥2 cups/d; when calculating the ORs for the consumption of green tea or black or oolong tea); BMI (<18.5, 18.5–24.9, 25.0–29.9, ≥30.0); diabetes mellitus (presence or absence); hypertension (presence or absence); history of stroke (presence or absence); history of myocardial infarction (presence or absence); depressive symptoms (Geriatric Depression Scale scores of <11 or ≥11); duration of education (≤12 y or >12 y); living with a spouse (yes or no); self-rated health (excellent or good or other); visiting friends (yes or no); physical functioning status (MOS scores of 0–1, 2–4, or 5–6); energy intake (continuous variable); intake of nondietary vitamin C or E including supplement vitamin C, supplement vitamin E, prescribed vitamin C, and prescribed vitamin E (yes or no); consumption of fish (<1 time/wk, 1–6 times/wk, or ≥1 time/d); consumption of green or yellow vegetables (<1 time/wk, 1–6 times/wk, or ≥1 time/d); mild leisure-time physical activity such as walking (yes or no); vigorous leisure-time physical activity such as tennis or jogging (yes or no); smoking (never, former, currently smoking <20 cigarettes/d, and currently smoking ≥20 cigarettes/d); and use of alcohol (never, former, and currently drinking).

Interactions between consumption of green tea and all confounders were tested through the addition of cross-product terms

TABLE 1
Characteristics of the study subjects according to categories of green tea consumption[1]

Characteristics	Green tea consumption			
	≤3 cups/wk (n = 170)	4–6 cups/wk or 1 cup/d (n = 108)	≥2 cups/d (n = 725)	P[2]
Women (%)	51.2	47.2	60.0	0.01
Age (y)[3]	74.2 ± 4.4	74.6 ± 4.3	74.8 ± 4.7	0.23
Mini-Mental State Examination score				
$\bar{x}$ ± SD	26.7 ± 3.3	27.3 ± 2.6	27.6 ± 2.5	0.0006
<28 (%)	48.8	44.4	39.2	0.06
<26 (%)	25.3	17.6	14.3	0.002
<24 (%)	11.2	8.3	6.3	0.09
Black or oolong tea consumption (%)				
≥2 cups/d	32.4	14.8	19.3	
4–6 cups/wk or 1 cup/d	11.8	31.5	17.4	<0.0001
Coffee consumption (%)				
≥2 cups/d	21.2	18.5	10.5	
4–6 cups/wk or 1 cup/d	27.1	37.0	31.3	0.0004
BMI (%)[4]				
<18.5 kg/m^2	6.5	3.7	4.7	
25.0–29.9 kg/m^2	29.4	32.4	30.5	
≥30.0 kg/m^2	4.1	3.7	4.1	0.96
Diabetes mellitus (%)[5]	22.4	26.9	22.1	0.54
Hypertension (%)[6]	69.4	67.6	68.1	0.94
History of stroke (%)	8.8	9.3	4.0	0.007
History of myocardial infarction (%)	12.4	17.6	10.1	0.06
Depressive symptoms (%)[7]	41.8	34.3	30.8	0.02
Duration of education ≤12 y (%)	30.0	31.5	30.5	0.97
Living with a spouse (%)	63.5	71.3	61.9	0.17
Self-rated health excellent or good (%)	57.6	63.8	67.3	0.06
Visiting friends (%)[8]	66.1	73.3	77.5	0.008
Physical functioning status (%)[9]				
Capable of moderate but not vigorous activity	27.1	20.4	25.7	
Only capable of low physical activity	15.3	12.0	8.7	0.06
Energy intake (kcal/d)[3]	1528.4 ± 417.8	1626.8 ± 389.4	1619.5 ± 391.8	0.02
Intake of nondietary antioxidants (%)[10]	11.8	11.1	16.0	0.20
Fish consumption (%)				
≥1 time/d	3.0	2.8	2.2	
1–6 times/wk	75.2	75.7	75.8	0.98
Green or yellow vegetable consumption (%)				
≥1 time/d	29.2	26.9	41.4	
1–6 times/wk	63.7	71.3	57.5	<0.0001
Mild leisure-time physical activity ≥1 time/wk (%)[11]	51.7	52.9	57.7	0.38
Vigorous leisure-time physical activity ≥1 time/wk (%)[12]	4.8	7.8	8.5	0.32
Smoking (%)				
Never	42.9	49.1	60.6	
1–19 cigarettes/d	11.9	11.3	8.6	
≥20 cigarettes/d	6.0	2.8	2.8	0.001
Alcohol use (%)				
Never	45.1	34.7	47.1	
Current	38.9	50.5	41.5	0.10

[1] 1 cup = 0.1 L.
[2] Determined by ANOVA or chi-square test.
[3] All values are $\bar{x}$ ± SD.
[4] Calculated from participants' measured weight and height.
[5] Defined as a nonfasting blood glucose concentration of ≥140 mg/dL or a history of diabetes mellitus.
[6] Defined as a self-measured systolic blood pressure of ≥135 mm Hg (measured at home) or a history of hypertension.
[7] Measured based on the Japanese version of the 30-item Geriatric Depression Scale, with a cutoff point of ≥11.
[8] Answer to the question, "Do you visit your friends?"
[9] Assessed by using the 6-item physical functioning status measure of the Medical Outcomes Study Short-form General Health Survey.
[10] Nondietary antioxidants included supplemental vitamin C, supplemental vitamin E, prescribed vitamin C, and prescribed vitamin E.
[11] For example, walking.
[12] For example, tennis and jogging.

TABLE 2
Odds ratios (ORs) and 95% CIs from logistic regression models for the association between consumption of green tea and cognitive impairment[1]

	Green tea consumption			
Logistic regression models	≤3 cups/wk	4–6 cups/wk or 1 cup/d	≥2 cups/d	*P* for trend[2]
Cognitive impairment, defined as MMSE score <28				
Model 1[3]	1.00 (reference)	0.84 (0.52, 1.36)	0.68 (0.48, 0.94)	0.02
Model 2[4]	1.00 (reference)	0.82 (0.50, 1.34)	0.61 (0.44, 0.87)	0.004
Model 3[5]	1.00 (reference)	0.83 (0.50, 1.38)	0.62 (0.43, 0.88)	0.005
Model 4[6]	1.00 (reference)	0.86 (0.52, 1.43)	0.69 (0.48, 0.98)	0.03
Model 5[7]	1.00 (reference)	0.85 (0.51, 1.40)	0.62 (0.43, 0.89)	0.005
Cognitive impairment, defined as MMSE score <26				
Model 1[3]	1.00 (reference)	0.63 (0.35, 1.15)	0.50 (0.33, 0.74)	0.0007
Model 2[4]	1.00 (reference)	0.61 (0.33, 1.13)	0.43 (0.29, 0.66)	< 0.0001
Model 3[5]	1.00 (reference)	0.64 (0.34, 1.21)	0.43 (0.28, 0.67)	0.0001
Model 4[6]	1.00 (reference)	0.63 (0.33, 1.19)	0.51 (0.33, 0.78)	0.003
Model 5[7]	1.00 (reference)	0.66 (0.35, 1.27)	0.47 (0.30, 0.74)	0.0008
Cognitive impairment, defined as MMSE score <24				
Model 1[3]	1.00 (reference)	0.72 (0.31, 1.66)	0.54 (0.31, 0.95)	0.03
Model 2[4]	1.00 (reference)	0.69 (0.30, 1.62)	0.47 (0.26, 0.83)	0.008
Model 3[5]	1.00 (reference)	0.82 (0.35, 1.96)	0.48 (0.27, 0.88)	0.01
Model 4[6]	1.00 (reference)	0.69 (0.29, 1.64)	0.55 (0.30, 1.00)	0.05
Model 5[7]	1.00 (reference)	0.77 (0.32, 1.89)	0.48 (0.25, 0.89)	0.02

[1] Multivariate logistic regression analysis was used to calculate ORs and 95% CIs for cognitive impairment relative to the consumption frequencies of green tea, with the lowest frequency category (≤3 cups/wk) treated as the reference group. Cognitive function was tested by using the Japanese language version of the 30-point Mini-Mental State Examination. 1 cup = 0.1 L.

[2] Trend tests were performed by including the ordinal variable in a linear regression analysis.

[3] Crude model.

[4] Adjusted for age and sex.

[5] Adjusted for model 2 + black or oolong tea consumption, coffee consumption, BMI, diabetes mellitus, hypertension, history of stroke, and history of myocardial infarction.

[6] Adjusted for model 2 + depressive symptoms, duration of education, living with a spouse, self-rated health, visiting friends, and physical functioning status.

[7] Adjusted for model 2 + energy intake, intake of nondietary vitamin C or E, fish consumption, green or yellow vegetable consumption, mild leisure-time physical activity, vigorous leisure-time physical activity, smoking, and alcohol use.

to the regression model. All statistical analyses were performed with the use of SAS software, version 9.1 (26). All the statistical tests that we report were two-sided. A P value of < 0.05 was accepted as statistically significant.

RESULTS

The subjects' characteristics according to categories of green tea consumption are shown in **Table 1**. Of the subjects, 16.9% consumed ≤3 cups green tea/wk, 10.8% consumed 4–6 cups/wk or 1 cup/d, and 72.3% consumed ≥2cups/d. The mean ± SD overall MMSE score was 27.4 ± 2.7. The prevalence of cognitive impairment decreased with increasing consumption of green tea for every cutoff point (P for the cutoff points of <28, <26, <24 = 0.06, 0.002, 0.09, respectively). Subjects who consumed ≥2 cups green tea/d were more likely to be women, have better self-rated health ($P = 0.06$), visit friends, have more total energy intake, consume green or yellow vegetables, never have smoked, and never have used alcohol ($P = 0.10$). They were less likely to consume black or oolong tea or coffee, have a history of stroke or myocardial infarction ($P = 0.06$), have depressive symptoms, and have limited physical functioning status ($P = 0.06$). No apparent associations were observed among mean age, BMI, presence or absence of diabetes mellitus or hypertension, duration of education, living with a spouse, intake of nondietary antioxidants, consumption of fish, or mild and vigorous leisure-time activities and frequency of green tea consumption.

Statistically significant inverse associations were observed between green tea consumption and cognitive impairment (**Table 2**). With the use of the <26 MMSE score cutoff point, the crude ORs of cognitive impairment associated with the different frequencies of green tea consumption were 1.00 (reference) for ≤3 cups/wk, 0.63 (95% CI: 0.35, 1.15) for 4–6 cups/wk or 1 cup/d, and 0.50 (95% CI: 0.33, 0.74) for ≥2 cups/d. We included a variety of potential confounders in our multivariate logistic models; however, the results did not change substantially even after adjustment for these variables. The results for MMSE score cutoff points of <28 and <24 were essentially the same as those for the <26 cutoff point.

In the final model used to investigate the association between different frequencies of green tea consumption and cognitive impairment, we chose the following data as covariates according to their relative contribution to the model outlined in Table 2 and their clinical importance: age, sex, consumption of green tea (when calculating ORs for consumption of black or oolong tea or coffee), consumption of black or oolong tea (when calculating ORs for consumption of green tea or coffee), consumption of coffee (when calculating ORs for consumption of green tea or black or oolong tea), presence or absence of diabetes mellitus,

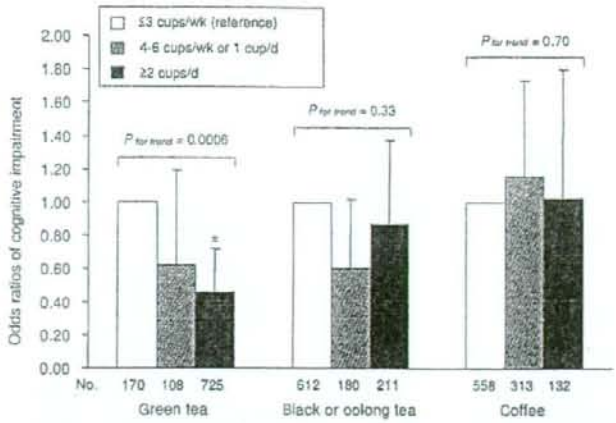

FIGURE 1. Odds ratios (ORs) for the association between different frequencies of beverage consumption and cognitive impairment. The bars indicate adjusted ORs for the association between different beverage consumption frequencies and cognitive impairment, respectively; error bars represent the corresponding 95% CIs. Multivariate logistic regression analysis was used to calculate ORs for cognitive impairment relative to the consumption frequencies of green tea or other beverages, with the lowest frequency category (≤3 cups/wk) treated as the reference group. Trend tests were performed by including the ordinal variable in a linear regression analysis. The ORs and 95% CIs for the ORs were adjusted for age, sex, green tea consumption (when calculating ORs for black or oolong tea or coffee consumption), black or oolong tea consumption (when calculating ORs for green tea or coffee consumption), coffee consumption (when calculating ORs for green tea or black or oolong tea consumption), presence or absence of diabetes mellitus, presence or absence of hypertension, history of stroke, depressive symptoms, duration of education, visiting friends, energy intake, intake of nondietary vitamin C or E, and fish consumption. Cognitive impairment was defined as a Mini-Mental State Examination score <26. *$P < 0.001$. 1 cup = 0.1 L.

presence or absence of hypertension, history of stroke, depressive symptoms, duration of education, visiting friends, energy intake, intake of nondietary vitamin C or E, and consumption of fish. The ORs (95% CIs) in the final model (using a cutoff point of <26) and corresponding ORs (95% CIs) for consumption of black or oolong tea or coffee are shown in **Figure 1**. The multivariate ORs according to frequencies of green tea consumption were 1.00 (reference) for ≤3 cups/wk, 0.62 (95% CI: 0.33, 1.19) for 4–6 cups/wk or 1 cup/d, and 0.46 (95% CI: 0.30, 0.72) for ≥2 cups/d. In contrast, a weak or null association was observed between intake of black or oolong tea or coffee and the prevalence of cognitive impairment. The ORs for black or oolong tea were 1.00 (reference), 0.60 (95% CI: 0.35, 1.02), and 0.87 (95% CI: 0.55, 1.38), whereas those for coffee were 1.00 (reference), 1.16 (95% CI: 0.78, 1.73), and 1.03 (95% CI: 0.59, 1.80). When cutoff points of <28 or <24 were used, the results for the final model were similar to those for the <26 cutoff point (data not shown). We were unable to examine the associations between cola or juice and 100% fresh vegetable juice and cognitive impairment because an insufficient number of subjects consumed these beverages. Tests for interaction between consumption of green tea and all confounders in the final models were not statistically significant.

We repeated the analysis after expanding the highest category of green tea consumption in the final model. With a cutoff point of <26, the ORs for the different frequencies of green tea consumption were 1.00 (reference) for ≤3 cups/wk, 0.62 (95% CI: 0.33, 1.19) for 4–6 cups/wk or 1 cup/d, 0.42 (95% CI: 0.25, 0.71) for 2–3 cups/d ($n = 258$), and 0.49 (95% CI: 0.30, 0.79) for ≥4 cups/d ($n = 467$) (P for trend = 0.004). With a cutoff point of <28, the corresponding ORs were 1.00 (reference), 0.80 (95% CI: 0.48, 1.34), 0.59 (95% CI: 0.39, 0.90), and 0.67 (95% CI: 0.45, 0.98) (P for trend = 0.04). With a cutoff point of <24, the corresponding ORs were 1.00 (reference), 0.77 (95% CI: 0.32, 1.86), 0.54 (95% CI: 0.26, 1.10), and 0.50 (95% CI: 0.26, 0.98) (P for trend = 0.04).

We also repeated the analysis for the final model after excluding subjects with relatively severe cognitive impairment (MMSE score < 24; $n = 74$). The results did not change substantially. With a cutoff point of <26, the ORs for the different frequencies of green tea consumption were 1.00 (reference) for ≤ 3cups/wk, 0.55 (95% CI: 0.24, 1.27) for 4–6 cups/wk or 1 cup/d, and 0.44 (95% CI: 0.25, 0.78) for ≥2 cups/d (P for trend = 0.006). With a cutoff point of <28, the corresponding ORs were 1.00 (reference), 0.82 (95% CI: 0.47, 1.41), and 0.68 (95% CI: 0.46, 1.00) (P for trend = 0.05).

DISCUSSION

Our study showed inverse dose-response relations between consumption of green tea and the prevalence of cognitive impairment. In contrast, a weak or null relation between consumption of black or oolong tea or coffee and cognitive impairment was observed. To our knowledge, this is the first study to examine the association between consumption of green tea and cognitive function in humans.

Our study had several methodologic strengths. We recruited subjects from the general population, and a substantial variation was observed in the consumption of green tea among our subjects. We conducted a CGA that allowed us to carefully consider cardiovascular risk factors, which were causes of vascular dementia. Our study had a reasonably large sample size, which gave us the opportunity to test the association between consumption of green tea and various grades of cognitive impairment (from slight to relatively severe).

Several methodologic limitations should be considered in the interpretation of our results. First, our study had a cross-sectional

design; therefore, no temporal relation between consumption of green tea and cognitive function can be inferred.

Second, our observational study design does not allow us to fully exclude the possibility of residual confounding by unmeasured factors. For example, healthier and more active individuals might have more opportunities to consume green tea. Among the Japanese, green tea is often consumed as a social activity, and this in itself may contribute to maintaining higher cognitive function (27). However, we controlled for many potential confounders, and the findings were robust to adjustments for these confounders.

Finally, because functional impairments of daily living were not fully assessed here, we cannot diagnose the presence or absence of dementia or the subtype of dementia syndromes, but we did evaluate cognitive impairment by using MMSE scores. However, cognitive decline is generally regarded as a core symptom of dementia. Furthermore, reduced cognition may be a key predictor of the development of dementia and may be considered a preclinical marker of the early stages of dementia (15, 16). Therefore, we believe that our data provide a useful clue to effective preventive interventions for dementia.

Green tea polyphenols, especially EGCG, might explain the observed association with improved cognitive function (7–10). Green tea is much richer in catechins than other beverages; Khokhar et al (28) reported that green tea contains 67.5 mg catechins/100 mL, whereas black tea contains only 15.5 mg/100 mL. The weak or null relations observed between consumption of black or oolong tea or coffee and cognitive impairment might reflect the important neuroprotective effects of catechins described in numerous experimental and animal studies (7–10). EGCG is brain permeable (29–31), and its neuroprotective and neurorescue effects were explained in terms of various mechanisms in addition to its well-established antioxidant and iron-chelating properties (7). These properties include modulation of cell survival and cell cycle genes (9) and promotion of neurite outgrowth activity (10). Furthermore, Levites et al (8) have shown that EGCG exerts neuroprotective and neurorescue effects against $A\beta$ toxicity by regulating the secretory processing of nonamyloidogenic APP through the protein kinase C pathway. In addition to the above-mentioned experimental and animal evidence, recent epidemiologic studies have suggested that red wine, which is also rich in polyphenols, may be associated with reduced risk of dementia (32, 33).

In addition to polyphenols, green tea contains vitamin C, caffeine, and other nutrients (34). Intake of vitamin C accompanied by high consumption of green tea might contribute to the observed association (3–6). Green tea contains 6 mg vitamin C/100 mL (10 g tea leaf/430 mL water, 90 °C, 1 min) (34) and is, in fact, the most common source of vitamin C (13.6%) among the population in our study region (35). Therefore, we cannot exclude a possible effect of vitamin C in the green tea on cognitive function. However, our results were not substantially changed even after adjustment for intake of nondietary vitamin C or E, indicating that the effects of vitamin C may be small. The contribution of caffeine to higher cognitive function also appears to be small because of the null relation observed between consumption of coffee and cognitive impairment. Green tea contains 0.02 g caffeine/100 mL (10 g tea leaf/430 mL water, 90 °C, 1 min), whereas coffee contains 0.06 g caffeine/100 mL (10 g coffee powder/150 mL water, 100 °C) (34). Nutrients in green tea other than polyphenols, vitamin C, and caffeine remain to be studied.

In conclusion, the present results suggest that higher consumption of green tea is associated with lower prevalence of cognitive impairment in humans. The results might partly explain the relatively lower prevalence of dementia, especially AD, in Japan than in Europe and North America (1). Given the high prevalence, worldwide rapid increase, and clinical significance of dementia (1, 2), any association between the intake of green tea, a drink with little toxicity and no calorific value, and cognitive function could have considerable clinical and public health relevance. The results of this cross-sectional study generate a new hypothesis and warrant further investigation.

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SK, AH, KO, and TS participated in the study design, data acquisition, data analysis, data interpretation, preparation of the written report, and final review of the report. TM, SE, SA, RN, HA, and IT participated in the study design, data acquisition, data interpretation, and final review of the report. None of the authors had any conflict of interest.

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LETTER TO THE EDITOR

Interleukin-1β gene polymorphisms associated with risk of lung cancer in Japanese

KEYWORDS
Interleukin-1β;
Polymorphisms;
SCLC;
NSCLC;
COPD;
Smoking

To the Editor,

We read with interest the article by Lind et al. [1]; however, there has been no evidence showing that IL-1β gene polymorphisms are associated with small cell lung cancer (SCLC). Therefore, IL-1β gene polymorphisms were studied for their association with NSCLC and SCLC in Japanese.

Our study involved 444 individuals of Japanese origin (Table 1). The subjects were newly diagnosed lung cancer patients at Tohoku University Hospital and the Miyagi Cancer Center Research Institute in Miyagi prefecture between 2001 and 2004. Both patients with lung cancer and controls were recruited because they met our enrollment criteria, as follows: (1) newly diagnosed as patients with lung cancer by pathohistological diagnosis between 2001 and 2004, (2) patients without IL-1 beta related disease including gastric cancer, hepatocellular carcinoma, breast cancer, collagen diseases, and gastroduodenal ulcer, (3) age more than 50, (4) Japanese, and (5) informed consent regarding attendance for this study could be obtained. Ninety-five of 220 patients had adenocarcinomas, 92 patients had squamous cell carcinoma, and 33 patients had small cell carcinoma. The 224 controls were recruited from Miyagi health screening 2001–2002. DNA was extracted from peripheral blood. IL-1β genotyping was carried out, as previously described [2,3]. The genotype distributions and frequencies of alleles were compared between lung cancer cases and control groups.

Table 1 shows that the C allele at −511 SNP was of particularly higher risk for lung cancer with a relative risk of 1.9 (95% confidence interval = 1.3–2.7). In this study, IL-1β −31 and −511 loci did not have complete linkage disequilibrium ($D' = 0.77$, $r^2 = 0.58$). There was no difference in the IL-1β −31 and genotyped frequency of IL-1β between lung cancer patients and controls.

Next, the genotypes of IL-1β gene polymorphisms at loci −511 C-T were further analyzed in NSCLC cases, SCLC cases and healthy controls. The distribution of IL-1β −511 genotypes with SCLC or NSCLC cases was similar to lung cancer cases. The C allele at −511 SNP was also at particularly higher risk for cancer with a relative risk of 3.0 (95% CI = 1.1–9.0) in SCLC cases and 1.9 (95% CI = 1.3–2.7) in NSCLC cases, but there were no differences in the homogeneous types of −31 SNP and the genotyped frequency of IL-1β between SCLC cases and controls.

Herein, the IL-1β −511 C allele was demonstrated to be associated with onset risks in not only NSCLC but SCLC in Japanese, but the positive relationship between the IL-1β −31 SNP and NSCLC onset risk in this study is not consistent with previous reports from Central and Eastern Europe [1,4,5]. The onset of chronic obstructive pulmonary disease (COPD) was reported to be associated with the IL-1β −511 C allele SNP in Japanese [6]. Furthermore, an increased risk of lung cancer onset in Japanese patients with COPD was reported [6,7]. This suggests that lung cancer risk may be increased in Japanese patients with chronic pulmonary inflammation regulated by IL-1β and may not be related with the IL-1β −31 T allele but with the IL-1β −511 C allele. Moreover, the effects of different ethnic and genetic backgrounds on the associations between malignancy onset and chronic inflammation cannot be ruled out.

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Table 1 Clinical features of study population in healthy controls and lung cancer cases

Characteristics	Controls ($n=224$)	Lung cancer ($n=220$)	Logistic regression analysis	
			Relative risk (95% CI)	P value
Age (years)[a]	66.9 ± 10.6	66.4 ± 9.8		NS
Pack-years[a]	12 ± 5	34 ± 3		$P<0.01$
Gender				
Male	106 (47.3)	160 (72.7)		
Female	118 (52.7)	60 (27.3)		NS
IL-1β −511 (T-C)				
Genotypes				
T/T	67 (29.9)	29 (13.2)	1.0	
T/C	122 (54.5)	127 (57.7)	2–4 (1.3–4.3)	$P<0.01$
C/C	35 (15.6)	64 (29.1)	3.8 (1.9–7.4)	$P<0.01$
Alleles				
1	67 (29.9)	29 (13.2)		
2	157 (70.1)	191 (86.8)	1.9 (1.3–2.7)	$P<0.01$
Subgroup analysis				
IL-1β −511 (T-C) in NSCLC cases; $n=187$				
Genotypes				
T/T		25 (13.4)	1.0	
T/C		110 (58.8)	2.4 (1.3–4.5)	$P=0.03$
C/C		53 (28.3)	3.8 (1.9–7.7)	$P<0.01$
Alleles				
1		25 (13.4)		
2		163 (87.2)	1.9 (1.3–2.7)	$P<0.01$
IL-1β −511 (T-C) in SCLC cases; $n=33$				
Genotypes				
T/T		4 (12.1)	1.0	
T/C		17 (51.5)	2.5 (0.9–7.9)	$P=0.09$
C/C		12 (36.3)	4.2 (1.2–14.2)	$P=0.02$
Alleles				
1		4 (12.1)	1.0	
2		29 (87.9)	3.0 (1.1–9.0)	$P=0.04$

Relative risk was obtained by performing logistic regression analysis after adjusted for age, sex and smoking status.
[a] Data are presented as mean ± S.E.M. or no. (%).

In summary, this is the first report demonstrating that the IL-1β −511C allele was associated with significantly increased onset risk in not only NSCLC but SCLC as well as COPD in Japanese.

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Heme oxygenase-1 gene promoter polymorphism and decline in lung function in Japanese men

Chronic obstructive pulmonary disease (COPD) is characterised by progressive airflow limitation in the lung airway. Cigarette smoking is the most important risk factor for COPD.[1] Heme oxygenase-1 (HO-1) is a key enzyme in heme metabolism and provides cytoprotection against oxidants in cigarette smoke.[2] A $(GT)_n$ dinucleotide repeat in the human *HO-1* promoter shows length polymorphism which is categorised into three groups: S-allele (<27 GT repeats), M-allele (27–32 GT repeats), and L-allele (≥33 GT repeats).[3] The L-allele was found to be associated with reduced *HO-1* inducibility and susceptibility to pulmonary emphysema in a case-control study of Japanese male smokers.[3] Conversely, He *et al*[4] reported that *HO-1* polymorphism was not related to a rapid decline in lung function in a prospective study of white smokers.

To evaluate the role of *HO-1* polymorphism in the decline in lung function in Japanese subjects, 101 Japanese male ex-smokers with mild to severe COPD (forced expiratory volume in 1 second (FEV_1) 40–90% predicted and FEV_1/FVC <70%) were enrolled from January 2000 to December 2001 and *HO-1* polymorphism was checked by PCR with peripheral blood DNA. Spirometric tests were performed at the beginning of the study and annually for 3 years. All participants sustained smoking cessation and were treated with bronchodilators including β_2 agonists and/or anticholinergic agents but not the long acting anticholinergic tiotropium. Rapid decliners are defined as subjects with a mean annual decrease in FEV_1 of ≥3.0% predicted,[1] whereas non-rapid decliners were subjects with a mean annual decline in FEV_1 of <3.0% predicted. Patients with active pneumonia, bronchial asthma, and malignant disease were excluded.

There were 28 individuals with the L-allele (L-allele carriers) and 73 without the L-allele (non-L-allele carriers). The baseline characteristics of L-allele carriers and non-carriers did not differ (table 1). At the end of the follow up period there were 25 subjects with a rapid decline in lung function and 76 non-rapid decliners. The mean annual decline in FEV_1 % predicted in L-allele carriers was significantly larger than in non-carriers (mean (SE) 2.74 (1.22)% per year *v* −0.57 (0.89)% per year, $p = 0.044$, unpaired *t* test, table 1). The proportion of rapid decliners was significantly higher among L-allele carriers than in non-L-allele carriers (12 (42.9%) *v* 13 (17.8%), $p = 0.009$, χ^2 test, table 1). Furthermore, the factors associated with a rapid decline in lung function were calculated by multivariate logistic regression analysis to adjust for potential risk factors including age, smoking status (pack-years), baseline FEV_1 predicted, and L-allele carrier status. As a result, the adjusted odds ratio of L-allele carrier status for rapid decliners was 3.9 (95% CI 1.4 to 10.6), $p = 0.009$ (12 (48.9%) in rapid decliners *v* 16 (21.1%) in non-rapid decliners). Other factors were not significantly associated with a rapid decline in lung function.

Table 1 Mean (SE), baseline characteristics and decline in lung function in L-allele carriers and non-L-allele carriers

Characteristics	L-allele carrier (n = 28)	Non-L-allele carrier (n = 73)	p value
Age (years)	70.3 (1.7)	70.6 (0.9)	0.84*
Sex (M/F)	28/0	73/0	>0.99**
Smoking status			
No of ex-smokers	28 (100%)	73 (100%)	>0.99**
Pack-years	44.7 (4.6)	49.3 (3.6)	0.47*
Pulmonary function			
FEV$_1$%FVC	59.6 (1.4)	61.0 (1.0)	0.83*
FEV$_1$ (l)	1.41 (0.1)	1.47 (0.1)	0.69*
FEV$_1$ (% predicted)	62.6 (4.8)	65.7 (3.3)	0.61*
Treatment			
Smoking cessation	28 (100%)	73 (100%)	>0.99**
Bronchodilator	28 (100%)	73 (100%)	>0.99**
Complications			
Hypertension	7 (25 %)	15 (20.5%)	0.60**
Diabetes mellitus	3 (10.7%)	8 (11.0%)	>0.99**
Hyperlipidaemia	3 (10.7%)	5 (6.8%)	0.68**
Cardiovascular disease	5 (17.9%)	9 (12.3%)	0.52**
Gastrointestinal disease	6 (21.4%)	13 (17.8%)	0.78**
Lung function decline			
Decrease in FEV$_1$ (% pred)	2.74 (1.22)	−0.57 (0.89)	0.044*
No of rapid decliners†	12 (42.9%)	13 (17.8%)	0.009***

All subjects had a smoking history of at least 10 pack-years and had quit smoking at least 6 months before the study. Lung function was assessed as post-bronchodilator values of spirometry.
*Unpaired *t* test; **Fisher's exact test; ***χ^2 test.
†Rapid decline is defined as a mean annual decrease in FEV$_1$ ≥ 3.0% predicted.

The results of this study suggest that polymorphism of the *HO-1* promoter gene may be associated with the rate of decline in lung function in Japanese male ex-smokers. A larger study is needed to confirm this result. Although the reason for the discrepancy between the results of our study and that of He *et al*[4] is not clear, it might result from the difference in ethnic background of the participants. Since the susceptibility to COPD and/or decline in lung function could be influenced by a number of genetic and environmental factors, different polymorphisms in different ethnic groups may cause the same COPD phenotype. It is therefore important to confirm the associations of polymorphisms in each population. The L-allele carrier of the *HO-1* promoter gene in Japanese men is significantly associated with risks of developing lung adenocarcinoma,[5] pulmonary emphysema,[3] and less longevity.[2] Modification of *HO-1* gene expression may offer a new target for therapeutic intervention in lung disease in the Japanese population.

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Randomized Phase II Trial Comparing Nitroglycerin Plus Vinorelbine and Cisplatin With Vinorelbine and Cisplatin Alone in Previously Untreated Stage IIIB/IV Non–Small-Cell Lung Cancer

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ABSTRACT

Purpose

To investigate the efficacy and safety of nitroglycerin plus vinorelbine and cisplatin in patients with previously untreated stage IIIB/IV non–small-cell lung cancer (NSCLC) as the experimental arm for the next phase III trial.

Patients and Methods

One hundred twenty patients with stage IIIB/IV NSCLC were randomly assigned to vinorelbine 25 mg/m^2 on days 1 and 8 and cisplatin 80 mg/m^2 on day 1, with transdermally applied nitroglycerin (25 mg/patient daily for 5 days; arm A) or with placebo patch (arm B) every 3 weeks for a maximum of four cycles in a double-blind and controlled trial. Primary efficacy end points were the best confirmed response rate and time to disease progression (TTP).

Results

The response rate in arm A (72%; 43 of 60 patients) was significantly higher than that for patients in arm B (42%; 25 of 60 patients; $P < .001$). Median TTP in arm A was longer than that in arm B (327 *v* 185 days). No severe adverse effect was recognized for either arm. The rate of grade 1 to 2 headache in arm A (30%; 18 of 60 patients) was significantly higher than that in arm B (2%; one of 60 patients; $P < .001$, χ^2 test).

Conclusion

Use of nitroglycerin combined with vinorelbine and cisplatin may improve overall response and TTP in patients with stage IIIB/IV NSCLC. The arm A regimen is being evaluated in a large phase III trial.

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INTRODUCTION

Low levels of oxygenation due to relative vascular insufficiency have been demonstrated to exist in solid cancers but not in normal tissues,[1-4] and hypoxic conditions in solid cancers are associated with resistance to cancer therapy.[5-7] Hypoxia-inducible factor-1 (HIF-1) activates the transcription of many genes that code for proteins involved in angiogenesis, cell growth, metastasis, and resistance to chemotherapy.[8-12] Hypoxia in solid cancers promotes stabilization of HIF-1,[13] and anticancer therapy to inhibit HIF-1 has been reported recently.[12,14,15] The administration of nitric oxide (NO)–donating drugs decreased hypoxia-induced resistance to anticancer drugs in cancer cell lines.[16] However, the effects of NO and NO-donating drugs on inhibition of HIF-1 activation during hypoxia remains controversial.[17-20]

Isosorbide dinitrate and inducible NO synthase gene transfer have various effects on tumor tissue and cells, including augmentation of oxygen pressure in tumor tissue through an increase in blood flow[21]; cytotoxicity in tumor cells[22,23]; programmed cell death that is dependent on position in the cell cycle[24]; and p53 protein activation, apoptosis, and growth inhibition in cancer cells.[20,25] In contrast, NO promotes tumor angiogenesis and tumor progression.[26,27]

A variety of anticancer drugs have been developed for treatment of lung cancer and have contributed to prolonged survival.[28,29] However, even

third-generation regimens such as vinorelbine plus cisplatin (VC) result in survival rates of only 26% to 36% at 1 year and in median overall survival of 8 to 9 months among patients with advanced non–small-cell lung cancer (NSCLC) and good performance status (PS).[30-32]

In our preliminary survey, the response rate to chemotherapy using VC was significantly higher in patients with lung cancer and angina pectoris treated with nitroglycerin than in patients with lung cancer who did not have angina pectoris and did not use nitroglycerin treatment (unpublished data). However, the beneficial effects of NO-donating drugs on response to chemotherapy and on time to progression (TTP) in patients with lung cancer have not been reported to date.

PATIENTS AND METHODS

Patient Characteristics

A total of 193 patients with inoperable advanced NSCLC were recruited onto this study, and 120 of 193 patients fit the 15 inclusion criteria (Table 1). Grounds for exclusion at enrollment for 73 of 193 recruited patients were as follows: use of vasodilators including antihypertensive drugs in 41 patients; Eastern Cooperative Oncology Group[33] PS ≥ 2 in 17 patients; brain metastasis in 12 patients; renal, hematologic, or cardiac dysfunction in three patients.

The 120 eligible patients were randomly assigned to receive VC with or without nitroglycerin during chemotherapy in a double-blind phase II trial at the Department of Geriatric and Respiratory Medicine, Tohoku University School of Medicine (Sendai, Japan), and at the Division of Internal Medicine, Furukawa City Hospital (Furukawa, Miyagi Prefecture, Japan). Enrollment took place between April 2001 and February 2003. The random allocation sequence was generated by a random-number table at the coordinating center at the Department of Geriatric and Respiratory Medicine, Tohoku University School of Medicine.

PS was rated using the Eastern Cooperative Oncology Group scale.[33] Staging of NSCLC was determined using computed tomography (CT) scans of the brain, chest, and abdomen, positron emission tomography, gallium-67 citrate scintigraphy, and technetium-99m scintigraphy of the bone. Stage was defined using the revised lung cancer staging system of the American Joint Committee on Cancer.[34] Participant characteristics are listed in Table 2.

Table 1. Enrollment Criteria

The diagnosis of lung cancer was confirmed with histologic or cytologic examination
Age ≥ 40 years old
No treatment with a vasodilator such as calcium channel blockers
Stage IIIB or stage IV
No prior chemotherapy or radiotherapy
A measurable or evaluable tumor lesion according to WHO criteria
Good performance status: a performance status of 0-1 according to the ECOG scale
Without brain metastasis
Adequate renal function (calculated creatinine clearance of > 50 mL/min)
Adequate hepatic function (serum bilirubin, ALT, and AST < 2× ULN)
Adequate hematologic function (neutrophil count > 2,000/mL, hemoglobin > 10 g/dL, platelet count > 100,000/mL)
Adequate cardiac function (cardiothoracic ratio < 55%)
Informed consent to receive chemotherapy and attend this study was obtained
Scheduled treatment with chemotherapy and without radiotherapy
No ischemic heart diseases

Abbreviations: ECOG, Eastern Cooperative Oncology Group; ULN, upper limit of normal.

Table 2. Characteristics of the Patients With Non–Small-Cell Lung Cancer

Characteristic	Arm A, With Nitroglycerin (n = 60) Value	Arm A, With Nitroglycerin (n = 60) No. of Patients	Arm B, Without Nitroglycerin (n = 60) Value	Arm B, Without Nitroglycerin (n = 60) No. of Patients	P
Age, years					
Median	64		64		.63
Range	40-75		41-75		
Sex					
Male		53		52	.78
Female		7		8	
Performance status					
0		44		42	.69
1		16		18	
Smoking history, pack-year					
Median	46		47		.81
Range	0-135		0-125		
Nonsmoker		12		15	.64
Ex-smoker		17		13	
Current smoker		31		32	
Cell type					
Squamous cell		29		23	.15
Adenocarcinoma		27		36	
Large cell		4		1	
Staging					
IIIB		26		22	.46
IV		34		38	

Chemotherapy Treatment

Of the 120 patients, 60 were treated with VC (vinorelbine 25 mg/m^2 on days 1 and 8; cisplatin 80 mg/m^2 on day 1) every 3 weeks for a maximum of four cycles with transdermally applied nitroglycerin (25 mg/patient daily for 5 days between 3 days before the start of each cycle of chemotherapy and cycle day 2; arm A). Nitroglycerin transdermal patches (5 to 25 mg/patient daily) are widely and safely used in treatment of coronary artery disease and heart failure.[35] Therefore, we used 25 mg/patient nitroglycerin transdermal patches daily as the NO donor. The other 60 patients were treated with VC every 3 weeks for a maximum of four cycles with placebo patches (arm B). Nitroglycerin was used only with first-line chemotherapy.

Change in Chemotherapy Timing and Dose Adjustments

Drug administration was postponed for a maximum of 2 weeks if there was incomplete hematologic recovery on day 22 (leukocytes < 2,000/mL and/or platelets < 100,000/mL) or there was persistent grade 2 or more nonhematologic toxicity according to the National Cancer Institute Common Toxicity Criteria for Adverse Events, version 2.0.[36] Dosage of anticancer drugs for the subsequent course was reduced to 80% in the event of grade 3 to 4 nonhematologic toxicity (except nausea, vomiting, and headache). Treatment was stopped if the same toxicity occurred with chemotherapy at a reduced dose level. Nonsteroidal anti-inflammatory drugs were used if nitroglycerin-induced headache occurred.

Estimation of Response to Treatment and Follow-Up Assessments

To assess nitroglycerin effects on response to chemotherapy, we compared identifiable tumor sizes with a chest CT scan after the finish of the second and fourth cycles of chemotherapy. Response rate was evaluated by two independent radiologists and an independent oncologist according to WHO criteria.[37] Patients were categorized as responders when they experienced either a partial response or a complete response. Patients with no change or progressive disease were categorized as nonresponders.

Once patients came off protocol treatment, they were evaluated by physical examination every 4 weeks and by CBC, biochemical tests, and chest

radiograph every 3 months. If necessary, CT scans of the brain, chest, or abdomen were appropriately performed to assess disease progression. CT scans were reviewed by two independent radiologists and an independent oncologist to confirm disease progression.

Treatment Toxicity

Toxic effects of anticancer drugs were graded according to National Cancer Institute Common Toxicity Criteria for Adverse Events, version 2.0.[36]

Study Design and Sample Size

The primary efficacy end point was comparison of response rate and TTP between arms A and B. A secondary efficacy end point was overall survival. Efficacy analyses were based on an intent-to-treat analysis.

We estimated that we needed to enroll 54 patients per arm on the basis of an experimental-treatment group to confer a power of 80% for a two-sided .05-level test to detect an increase in 1-year progression-free probability of 26% (from 26% to 52%) in the pooled nitroglycerin-treated arm.[37,38] Actual accrual was 60 eligible patients and 56 assessable patients for both arms A and B (Fig 1). This is the report of an interim analysis; the final analysis is planned for 2 years from the end of accrual. This study was approved by the Tohoku University Ethics Committee and informed consent was obtained from each subject.

Measurements of Plasma Vascular Endothelial Growth Factor Levels

To study nitroglycerin effects on the HIF-1 pathway, we measured plasma levels of vascular endothelial growth factor (VEGF), which is regulated by HIF-1.[39] Plasma levels of VEGF were measured as previously described[40] before and after 3 days of treatment with transdermally applied nitroglycerin patches (arm A) or placebo patches (arm B).

Statistical Methods

For statistical analysis, age, sex, performance status, smoking history, cancer cell type, cancer staging, treatment delivery, anticancer drugs dose-intensity, adverse effects due to chemotherapy, and response rate were compared using Pearson's χ^2 contingency table analysis (or Fisher's exact probability test whenever appropriate) between arms A and B. Age and smoking history (pack-year) between arms A and B, and plasma VEGF levels before and after use of nitroglycerin in arm A and placebo patches in arm B were compared using the Student's t test. Factors associated with response rate such as age, sex, performance status, cancer cell type, cancer staging, and use of nitroglycerin during chemotherapy were calculated with logistic regression analysis. Relative risks (RRs) and 95% CIs were calculated to assess response rate.

TTP was defined as the time from date of random assignment to date of disease progression. The probability of remaining free of progression or of surviving was estimated using the Kaplan-Meier product-limit method. P values indicated the significance of differences between arms A and B by log-rank test. Overall survival was calculated from the date of random assignment to the date of death or a cutoff date for patients alive at the time of closure of the data set.

Multivariate analysis by Cox regression analysis was performed to assess the prognostic significance of several variables, including age, sex, performance status, cancer cell type, cancer staging, and use of nitroglycerin combined with anticancer drugs.

All statistical analyses in this study were carried out using the Stat View program (SAS Institute Inc, Cary, NC). Results of interim significance tests were not considered significant unless the P values were less than .001.

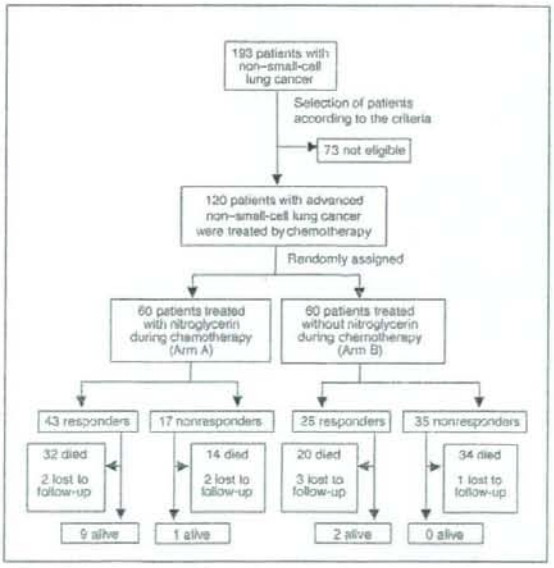

Fig 1. Trial profile. A total of 120 patients with advanced non-small-cell cancer were randomly assigned to chemotherapy with or without nitroglycerin and were observed to evaluate the effects of nitroglycerin combined with vinorelbine and cisplatin on response to chemotherapy and time to progression.

RESULTS

Patient Characteristics

There were no statistically significant differences in baseline characteristics between arms A and B (Table 2).

Chemotherapy Treatment

In arm A, 44 (73%) of 60 patients received all four courses of chemotherapy. Of the 44 patients, 17 received chemotherapy at the full prescribed dose, and 10 of 44 received chemotherapy without any modification of dose. Conversely, in arm B, 35 (58%) of 60 patients received all four courses of chemotherapy. Of those 35 patients, 16 received chemotherapy at the full prescribed dose and 12 of 35 received chemotherapy without any modification of dose. The mean number of chemotherapy courses was 3.52 for arm A and 3.27 for arm B. There were no significant differences between arms in dose of anticancer drugs or the number of chemotherapy courses (Table 3).

Treatment Toxicity

In first-line chemotherapy, the frequency of adverse effects grade ≥ 3 in arm A did not differ from that in arm B (Table 4).[36] The rate of grade 1 (15 of 60 patients) and grade 2 (3 of 60) headache in arm A (30%; 18 of 60) was significantly higher than that in arm B (2%; one of 60; $P < .001$, χ^2 test). However, there were no severe headaches of grade ≥ 3 in arm A. Conversely, grade 1 hypotension was observed in arm A (5%; three of 60). There was no severe hypotension of grade ≥ 3 in arm A during treatment with nitroglycerin.

There was a high rate of severe neutropenia in arm A (58%; 35 of 60) and arm B (57%; 34 of 60; Table 4). Furthermore, higher frequencies of persistent neutropenia on day 8 were observed in the fourth course in arm A (64%; 28 of 44) and in arm B (63%; 22 of 35). Therefore, the start timing of the fourth course of chemotherapy was postponed for some of the patients in arm A (48%; 21 of 44) and arm B (40%; 14 of 35).

Response Rate

Table 5 shows the response rate for arms A and B: the response rate in arm A (72%; 43 of 60 patients) was significantly higher than

Table 3. Treatment Delivery

Treatment	Arm A, With Nitroglycerin (n = 60) No.	%	Arm B, Without Nitroglycerin (n = 60) No.	%	P
No. of chemotherapy courses delivered					
1	4	7	6	10	.38
2	5	8	7	23	
3	7	12	12	20	
4	44	73	35	47	
Total No. of courses	211		196		
Mean		3.52		3.27	
Median		4		3	
4 cycles without dose reduction	17	28	16	27	.84
4 cycles without delay in chemotherapy timing	16	27	20	33	.43
4 cycles without treatment modification	10	17	12	20	.64
Dose-intensity (% prescribed doses)					
Vinorelbine		79		78	.86
Cisplatin		76		74	.74

that in arm B (42%; 25 of 60; odds ratio = 3.5; 95% CI, 1.7 to 7.6; $P < .001$). Conversely, the rate of no change in arm A (13%; eight of 60) was lower than that in arm B (35%; 21 of 60; odds ratio = 0.29; 95% CI, 0.1 to 0.7; $P = .006$). The rate of progressive disease in arm A did not differ from that in arm B (Table 5).

The use of nitroglycerin (RR = 4.3; 95% CI, 1.8 to 10.5; $P = .001$) and squamous cell carcinoma cell type (RR = 2.6; 95% CI, 1.0 to 6.5; $P = .049$) were associated positively with response rate in logistic regression analysis (Table 6).

TTP

The median follow-up period was 326 days (range, 32 to 1,380 days). Median TTP in arm A was 327 days (range, 32 to 1,151 days) compared with 185 days (range, 32 to 998 days) in arm B; use of nitroglycerin during chemotherapy (hazard ratio [HR] = 2.1; 95% CI, 1.3 to 3.2; $P = .002$) was associated with prolongation of TTP even after adjustment for age, sex, cancer cell type, and cancer staging in the Cox regression method. High performance status (PS 0; HR = 1.9; 95% CI, 1.4 to 2.7; $P < .001$) was also associated with prolongation of TTP. Kaplan-Meier analysis showed that progression-free probability in arm A was higher than that in arm B ($P = .006$; Fig 2).

Survival

We confirmed 100 deaths within the total of 120 patients by February 2005. In arm A, we confirmed that 46 of 60 patients had died and that 10 of 60 patients were alive at the end of the follow-up period, with four of 60 patients lost to follow-up. In arm B, we confirmed that 54 of 60 patients had died and that two of 60 patients were alive at the end of the follow-up period, with four of 60 patients lost to follow-up (Fig 1). Median survival time was 413 days (range, 32 to 1,380 days) in arm A, and 289 days (range, 56 to 1,117) in arm B. Treatment with nitroglycerin in arm A (HR = 2.5; 95% CI, 1.6 to 3.9; $P < .001$) was a significantly good prognostic factor compared with treatment without nitroglycerin even after adjustment for age, sex, cancer cell type, and cancer staging (Table 7). Kaplan-Meier analysis showed

Table 4. Toxic Effects

Toxicity	Grade	Arm A, With Nitroglycerin (n = 60)	Arm B, Without Nitroglycerin (n = 60)	P
Leukopenia	2	21	22	.89
	3	15	17	
	4	22	20	
Neutropenia	2	23	16	.59
	3	17	14	
	4	18	20	
Anemia	2	32	28	.63
	3	5	7	
	4	1	2	
Thrombocytopenia	2	29	25	.55
	3	3	4	
	4	1	0	
Nausea or vomiting	2	21	19	.62*
	3	13	15	
	4	0	0	
Diarrhea	2	14	18	.92
	3	1	2	
	4	1	1	
Anorexia	2	33	31	.72
	3	15	19	
	4	2	3	
Cardiac toxic effects	2	2	2	> .99*
	3	1	1	
	4	0	0	
Renal dysfunction	2	5	3	> .99*
	3	0	0	
	4	0	0	
Neuropathy	2	2	3	.81*
	3	1	1	
	4	0	0	
Headache	2	3	1	> .99*
	3	0	0	
	4	0	0	
Hypotension	2	0	0	> .99*
	3	0	0	
	4	0	0	

*Comparison of grade 2 to 3 by Fisher's exact probability test.

that survival probability in arm A was significantly higher than in arm B ($P < .001$; Fig 3).

Plasma VEGF Levels

In arm A patients, plasma VEGF levels after 3 days of treatment with nitroglycerin patches were significantly lower than levels before

Table 5. Response to Chemotherapy

Outcome	Arm A, With Nitroglycerin (n = 60)	Arm B, Without Nitroglycerin (n = 60)	P
Complete response	1	1	< .001
Partial response	42	24	
No change	8	21	
Progressive disease	9	14	

Table 6. Analysis of Risk Factors for Chemosensitivity Assessed by Multivariate Analysis

Characteristics	PR + CR (n = 68)		NC + PD (n = 52)		Logistic Regression Analysis		
	No. of Patients	%	No. of Patients	%	Relative Risk	95% CI	P
Age, years							
≥ 60	46	61	30	39	1.28		.59
< 60	22	50	22	50		0.51 to 3.23	
Sex							
Male	59	51	46	49	0.47		.30
Female	9	60	6	40		0.11 to 1.96	
Performance status							
0	49	57	37	43	0.98		.96
1	19	56	15	44		0.49 to 1.96	
Cell type							
Squamous cell	39	75	13	25	2.56	1.00 to 6.54	.05
Adenocarcinoma	26	41	37	59	—		—
Large cell	3	60	2	40	0.63	0.07 to 5.28	.67
Staging							
IIIB	35	73	13	27	2.22		.10
IV	33	46	39	54		0.86 to 5.56	
Use of nitroglycerin							
Yes	43	72	17	28	4.31		.001
No	25	42	35	58		1.77 to 10.53	

Abbreviations: CR, complete response; PR, partial response; NC, no change; PD, progressive disease.

treatment (mean ± SE, 293 ± 50 *v* 205 ± 28 pg/mL; n = 6; $P = .03$). In arm B patients, plasma VEGF levels after 3 days of use of placebo patches did not differ from levels before use (286 ± 47 *v* 290 ± 48 pg/mL; n = 6; $P = .40$).

DISCUSSION

This randomized phase II trial was designed to evaluate the safety and efficacy of nitroglycerin combined with VC regimen in patients with stage IIIB/IV NSCLC. We demonstrated that treatment with nitroglycerin improved response rate, TTP, and survival time in patients with advanced NSCLC without the appearance of major adverse effects. The response rate in arm B (42%) is consistent with rates given in previous reports.[41-43] Furthermore, the response rate in arm A of our study (72%) was more than two times higher than that achieved in patients treated with VC alone in previous reports.[41,42] Median TTP and overall survival in arm A were longer than those in arm B (1.8 and 1.4 times, respectively). These findings suggest that use of nitroglycerin during chemotherapy may have beneficial effects on chemosensitivity in patients with NSCLC.

Although VC is a well-tolerated regimen,[41-43] we observed a high rate of severe neutropenia, especially on day 8 in the fourth course in both arm A (58%) and arm B (57%) in the present study. Therefore, we partially postponed the start timing of the fourth course of chemotherapy in both arms. A larger randomized trial is needed to study the toxicity profile in arm A.

Additional clinical benefit beyond four courses of VC therapy for patients with advanced NSCLC had not been reported at the start of our study. Smith et al[44] reported no evidence for additional clinical benefit by continuing mitomycin plus vinblastine and cisplatin beyond three courses in patients with NSCLC. Therefore, in our study, treatment for each arm was to be administered for a maximum of

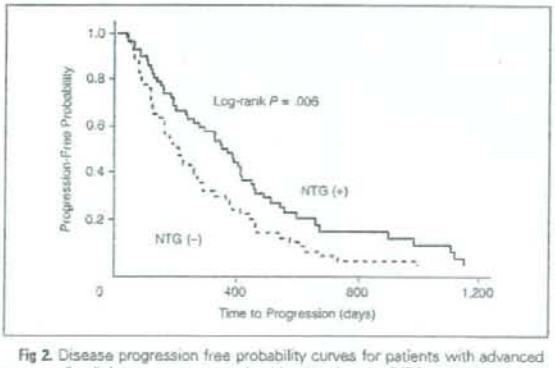

Fig 2. Disease progression free probability curves for patients with advanced non-small-cell lung cancer treated with nitroglycerin [NTG (+), [——]] and without nitroglycerin [NTG (−), [· · ·]] during chemotherapy. The *P* value was calculated by the log-rank test.

Table 7. Multivariate Analysis of Risk Factors Related to Survival

Variable	P	Risk Ratio	95% CI
Use of nitroglycerin combined with anticancer drugs, Yes* *v* No	< .001	2.5	1.6 to 3.9
PS 0* *v* PS 1	< .001	1.9	1.3 to 2.6

NOTE. All data were adjusted by age, sex, cancer staging, and smoking history (pack-year).
Abbreviation: PS, performance status.
*The group with better outcome.