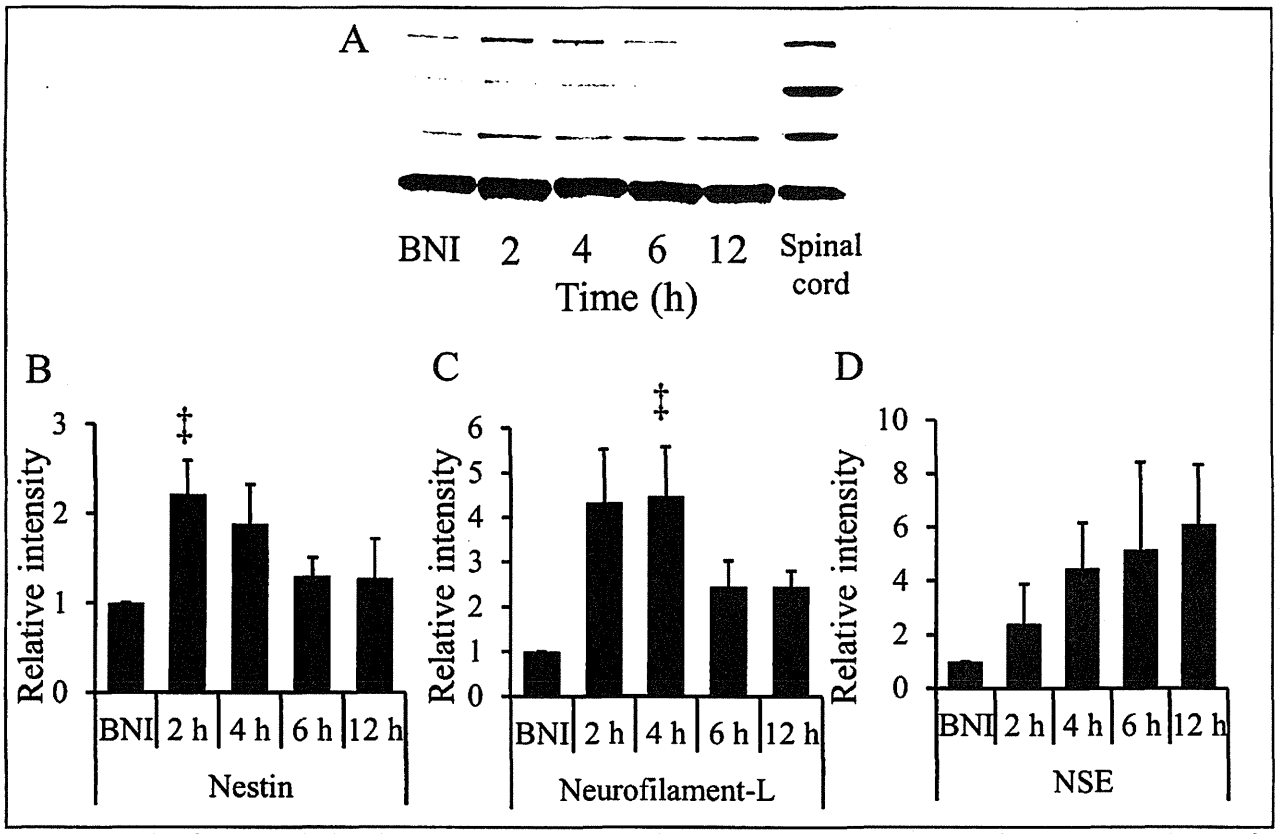

Figure 5—Results of western blotting (A) to illustrate protein expression levels of nestin (first row), neurofilament-L (second row), NSE (third row), and β-actin (fourth row) and relative protein expression rates (chemiluminescent intensity) of nestin (B), neurofilament-L (C), and NSE (D) in BMSCs (obtained from 6 dogs) BNI and at 2, 4, 6, and 12 hours after commencement of neuronal induction. Western blotting included samples of spinal cord obtained from 3 adult Beagles that were euthanized for other studies. The expression of these proteins generally increased after neuronal induction. *See* Figure 3 for remainder of key.

ens[28] have revealed decreases in mRNA expression levels for *SOX2* and *NES* in association with neuron maturation. The findings of the present study have suggested that neural stem or progenitor cell–specific mRNA expression is inhibited during the neuronal induction of canine BMSCs, as in previous studies.[13,28] Furthermore, mRNA expression levels for all evaluated neuron-specific markers increased during the neuronal induction of canine BMSCs. Specifically, significant increases were observed in the mRNA expression level of *MAP2* (as determined in a previous study[13]), *NEFL*, *NEFH*, *SLC1A1*, and *SLC2A3*. To our knowledge, the present study is the first in which such changes in canine BMSCs have been identified.

To confirm whether the observed increases in the mRNA expression levels of neuron markers were associated with concomitant changes in protein expression, we performed western blotting for determining protein expression levels of neuron markers. Results of another study[29] indicated that the protein expression level of nestin temporarily increases during the neuronal differentiation of human BMSCs. A similar pattern of change was evident during the neuronal induction of canine BMSCs in the present study. The expression levels of neurofilament-L and NSE proteins were found to increase in human BMSCs that differentiated into neurons.[26] Similarly, in the present study, the protein expression of neurofilament-L increased significantly and the protein expression of NSE appeared to increase (albeit not significantly) after the neuronal induction of canine BMSCs.

Furthermore, the mRNA expression levels of sodium ion channel markers *SCN1A* and *SCN2A* and a calcium ion channel marker *CACNA1G* significantly increased during the neuronal induction of canine BMSCs in the present study. Together, these results suggested that chemical induction with β-mercaptoethanol and BHA could enhance the mRNA expression of both neuron and ion channel markers and could induce canine BMSCs into a neuronal lineage.

In addition, we also investigated the electrophysiological function of the neuron-like cells derived from canine BMSCs to determine whether canine BMSCs can differentiate into functional, mature neurons. If canine BMSCs are able to differentiate into functional and mature neurons, these cells may be able to integrate into preexisting neural circuits and be used in regenerative therapies for neurologic disorders in dogs. However, in the present study, exposure of the neuron-like cells derived from canine BMSCs to 50mM KCl did not result in an increase in intracellular calcium concentration, indicating the absence of a typical neuronal response.

The protocol used in the present study was probably insufficient to induce development of canine BMSCs into functional neurons.[20] The study data indicated that the mRNA expression levels of some neuron and ion channel markers did not reach levels compara-

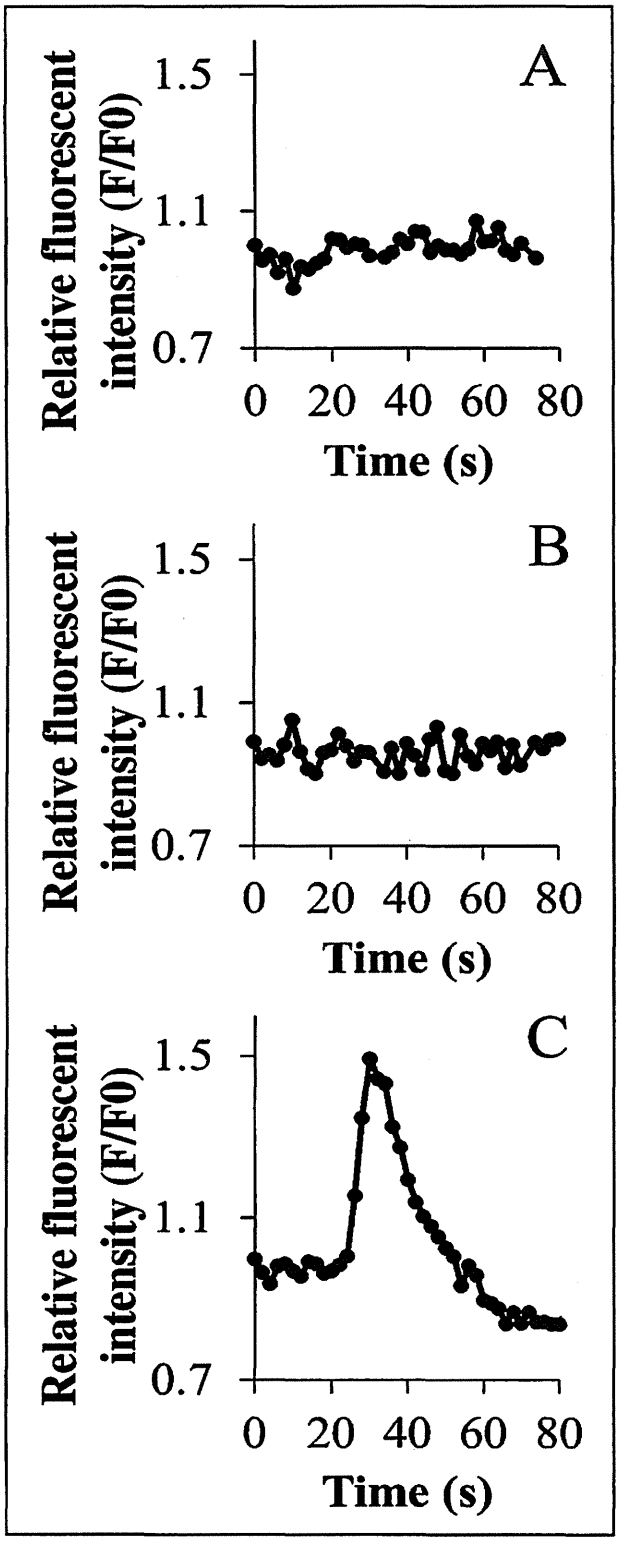

Figure 6—Intracellular Ca^{2+} concentrations determined with confocal laser scanning microscopy and a 75-W xenon arc lamp with appropriate filter sets (excitation, 488 nm; emission, 527 nm) in BMSCs (obtained from 6 dogs) before (A) and after (B) neuronal induction and in cultured canine neurons (C; positive control) obtained from 3 adult Beagles that were euthanized for other studies. After baseline data were acquired, the cells were stimulated (0 seconds) with KCl (50mM). The relative changes in intracellular Ca^{2+} concentrations over time were calculated as fluorescence at a given time point divided by baseline fluorescence (F/F0). Canine BMSCs did not respond to stimulation with 50mM KCl before or after neuronal induction.

ble to those found in canine spinal cord samples, which may have hindered the normal ion channel kinetics required for generating action potentials. Unfortunately, a large number of canine BMSCs detached from the culture flask within 6 to 12 hours after the start of neuronal induction in the present study, an occurrence that has been observed in previous studies.[19,20] Tondreau et al[15] reported that neuronal differentiation of human BMSCs requires cascades of transcriptional events that do not occur within a few hours. A protocol of longer duration might be more successful in inducing canine BMSCs into functional neurons. Lu et al[22] reported that the combination of β-mercaptoethanol with BHA may have cytotoxic effects on BMSCs and may induce apoptosis. The results of the present study suggested the possibility that the cellular damage caused by these chemical compounds may interfere in electrophysiological function of the canine BMSCs. In addition, other factors or supplements may be needed for canine BMSCs to differentiate into functional neurons. Other researchers have succeeded in differentiating human and mouse BMSCs into functional, mature neurons with basic fibroblast growth factor or brain-derived neurotrophic factor.[16,30] For canine BMSCs, it seems probable that protocols that involve the use of such growth factors and that can maintain cell viability for longer durations are needed. Longer durations of cell viability will be necessary to confirm whether canine BMSCs can differentiate into fully functional, mature neurons.

The results of the present study indicated that canine BMSCs develop neuron-like morphology and are positive for neuron markers following chemical induction with β-mercaptoethanol and BHA. In addition, the mRNA and protein expression levels of some neuron and ion channel markers increased after the neuronal induction of canine BMSCs. However, the mRNA expression levels of almost all the neuron and ion channel markers in canine BMSCs after neuronal induction in the present study were lower than levels in canine spinal cord samples, and the neuron-like cells induced from canine BMSCs had no electrophysiological function. Further investigations with improved neuronal induction methods are necessary to confirm whether canine BMSCs have the ability to differentiate into fully functional, mature neurons.

a. Midazolam hydrochloride, Astellas Pharma Inc, Tokyo, Japan.
b. Butorphanol tartrate, Meiji Seika Pharma Co Ltd, Tokyo, Japan.
c. Propofol, Schering-Plough Co, Osaka, Japan.
d. Isoflurane, Intervet KK, Osaka, Japan.
e. Histopaque-1077, Sigma-Aldrich Inc, St Louis, Mo.
f. 25-cm^2 plastic culture flask, Corning Life Sciences Inc, Lowell, Mass.
g. α-Modified Eagle minimum essential medium, Invitrogen Co, Carlsbad, Calif.
h. Fetal bovine serum, Invitrogen Co, Carlsbad, Calif.
i. Trypsin-EDTA, Invitrogen Co, Carlsbad, Calif.
j. DMEM-LG, Invitrogen Co, Carlsbad, Calif.
k. BME, Sigma-Aldrich Inc, St Louis, Mo.
l. Dimethylsulfoxide, Sigma-Aldrich Inc, St Louis, Mo.
m. BHA, Sigma-Aldrich Inc, St Louis, Mo.
n. 35-mm Glass base dish, Iwaki Co Ltd, Tokyo, Japan.
o. Paraformaldehyde, Nacalai Tesque Inc, Kyoto, Japan.
p. Triton X-100, Sigma-Aldrich Inc, St Louis, Mo.
q. Serum-free blocking solution, DAKO North America Inc, Carpinteria, Calif.

r. Anti-rat nestin mouse monoclonal IgG_1 antibody, Santa Cruz Biotechnology Inc, Santa Cruz, Calif.
s. Anti-human NSE mouse monoclonal antibody, DAKO North America Inc, Carpinteria, Calif.
t. Anti-human NF protein mouse monoclonal antibody, DAKO North America Inc, Carpinteria, Calif.
u. Alexa fluor 594 F $(ab')_2$ fragments of goat anti-mouse IgG (H+L), Invitrogen Co, Carlsbad, Calif.
v. ProLong Gold Antifade Reagent, Invitrogen Co, Carlsbad, Calif.
w. FV1000D IX81, Olympus Co, Tokyo, Japan.
x. TRIzol, Invitrogen Co, Carlsbad, Calif.
y. PrimeScript RT Master Mix, TaKaRa Bio Inc, Shiga, Japan.
z. SYBR Premix Ex Taq II, TaKaRa Bio Inc, Shiga, Japan.
aa. Thermal Cycler Dice Real Time System II, TaKaRa Bio Inc, Shiga, Japan.
bb. TP900 DiceRealTime, version 4.02B, TaKaRa Bio Inc, Shiga, Japan.
cc. Complete Mini EDTA-free, Roche Pharma AG, Mannheim, Germany.
dd. Mini-PROTEAN TGX gel, Bio-Rad Laboratories Inc, Hercules, Calif.
ee. Immobilon-P Transfer Membranes, Merck Millipore, Billerica, Mass.
ff. Block Ace, DS Pharma Biomedical Co Ltd, Osaka, Japan.
gg. Anti-human NF protein mouse monoclonal antibody, Thermo Fisher Scientific Inc, Rockford, Ill.
hh. Anti-β-actin mouse monoclonal antibody, Sigma-Aldrich Inc, St Louis, Mo.
ii. Horseradish peroxidase–conjugated secondary anti mouse IgG, GE Healthcare, Piscataway, NJ.
jj. ECL plus Western blotting Analysis System, GE Healthcare, Piscataway, NJ.
kk. ImageQuant LAS 4000 mini, GE Healthcare, Piscataway, NJ.
ll. Fluo3-AM, Dojindo Laboratories, Kumamoto, Japan.
mm. LSM-510, Carl Zeiss AG, Oberkochen, Germany.
nn. StatMate IV, ATMS, Tokyo, Japan.

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Appendix

Primers specific for canine neural stem or progenitor cell, neuron, and ion channel markers.

Gene symbol	Protein	Sequence	Marker
GUSB	Glucuronidase β	F: ACATCGACGACATCACCGTCA R: GGAAGTGTTCACTGCCCTGGA	Housekeeping gene
SOX2	Transcription factor SOX2	F: ATGCACCGCTACGACGTGA R: TGCTGCGAGTAGGACATGCTG	Neural stem or progenitor cell
NES	Nestin	F: GGACGGGCTTGGTGTCAATAG R: AGACTGCTGCAGCCCATTCA	Neural stem or progenitor cell
TUBB3	β III tubulin	F: TACAACGCCACGCTGTCCA R: CTTGAGAGTGCGGAAGCAGATG	Neural stem or progenitor cell
MAP2	Microtubule-associated protein 2	F:AAGCATCAACCTGCTCGAATCC R: GCTTAGCGAGTGCAGCAGTGAC	Neuron
NEFH	Neurofilament heavy chain	F: GGAGGTTCCTGCCAAGGTGA R: CTCTGCTGCTTTGCTGGGTTC	Neuron
NEFL	Neurofilament light chain	F: TGAATATCATGGGCAGAAGTGGAA R: GGTCAGGATTGCAGGCAACA	Neuron
NEFM	Neurofilament medium chain	F: CTTAAGCCCAGCCGATGAA R: TGAGTGACGGTTACAGATTTAGTGA	Neuron
ENO2	Neuron specific enolase	F: GCATCCAGGCAGAGCAATCA R: AATGGGTGGATGCAGCACAA	Neuron
SLC1A1	Glutamate transporter	F: CATGTTGTCTGATTGTTCCACGTC R: AATGGTGATGCCACCTTGGAG	Neuron
SLC2A3	Glucose transporter 3	F: CTTCAGATCGCGCAGCTACC R: TGCATCTTTGAAGATTCCTGTTGAG	Neuron
SLC2A12	Glucose transporter 12	F: TTGTCAAGGTCATCAGCACCATC R: AATGAAGCCGCCATCACAGAG	Neuron
SCN1A	Nav 1.1	F: CTGAGCGAGGATGACTTTGAGATG R: TCGGTTGTGGCAAATTGAGTG	Sodium ion channel α subunit
SCN2A	Nav 1.2	F: TGGTCATGTTCATCTACGCCATC R: TCATGCTGTTGCCAAAGGTCTC	Sodium ion channel α subunit
SCN8A	Nav 1.6	F: GTCTGATCAAAGGCGCCAAAG R: GCGAAATTGGACATCCCAAAG	Sodium ion channel α subunit
CACNA1C	Cav 1.2	F: GCTGTGTCTGCTGCCTCTGAA R: GTCGCTTTGGTAGTAGCTGACGTG	Calcium ion channel α subunit
KCNC1	Kv 1.2	F: ACACCACAGTACTCAGAGTGACACA R: AGGAGTGAGGGCCTGGTCTA	Potassium ion channel α subunit
KCNA2	Kv 3.1	F: CCTGATCTCGATTGTGAGCTTCTG R: TGGTACCCGATTGTGCTGTTG	Potassium ion channel α subunit

F = Forward. R = Reverse.

Research article

Increased oxidative stress and coenzyme Q10 deficiency in juvenile fibromyalgia: amelioration of hypercholesterolemia and fatigue by ubiquinol-10 supplementation

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Fibromyalgia (FM) is characterized by generalized pain and chronic fatigue of unknown etiology. To evaluate the role of oxidative stress in this disorder, we measured plasma levels of ubiquinone-10, ubiquinol-10, free cholesterol (FC), cholesterol esters (CE), and free fatty acids (FFA) in patients with juvenile FM ($n = 10$) and in healthy control subjects ($n = 67$). Levels of FC and CE were significantly increased in juvenile FM as compared with controls, suggesting the presence of hypercholesterolemia in this disease. However, plasma level of ubiquinol-10 was significantly decreased and the ratio of ubiquinone-10 to total coenzyme Q10 (%CoQ10) was significantly increased in juvenile FM relative to healthy controls, suggesting that FM is associated with coenzyme Q10 deficiency and increased oxidative stress. Moreover, plasma level of FFA was significantly higher and the content of polyunsaturated fatty acids (PUFA) in total FFA was significantly lower in FM than in controls, suggesting increased tissue oxidative damage in juvenile FM. Interestingly, the content of monoenoic acids, such as oleic and palmitoleic acids, was significantly increased in FM relative to controls, probably to compensate for the loss of PUFA. Next, we examined the effect of ubiquinol-10 supplementation (100 mg/day for 12 weeks) in FM patients. This resulted in an increase in coenzyme Q10 levels and a decrease in %CoQ10. No changes were observed in FFA levels or their composition. However, plasma levels of FC and CE significantly decreased and the ratio of FC to CE also significantly decreased, suggesting that ubiquinol-10 supplementation improved cholesterol metabolism. Ubiquinol-10 supplementation also improved chronic fatigue scores as measured by the Chalder Fatigue Scale.

Keywords: Juvenile fibromyalgia, Oxidative stress, Coenzyme Q10 deficiency, Fatigue, Hypercholesterolemia

Introduction

Fibromyalgia (FM) is a chronic disorder characterized by idiopathic chronic widespread non-specific musculoskeletal pain with generalized tender points and allodynia, as well as a heightened and painful response to pressure.[1] The syndrome is associated with a constellation of symptoms, including debilitating fatigue, non-refreshing sleep, irritable bowel, and joint stiffness. Children with chronic musculoskeletal pain disorders, including FM, account for up to 25% of new referrals to pediatric rheumatologists in the United States.[2] Several factors, such as substance P, serotonin, reactive oxygen species, and genetic factors, are associated with the pathophysiology of FM,[3,4] but the etiology of this disorder remains unclear.

Oxidative stress may play a role in the pathophysiology of FM.[4,5] In fact, blood mononuclear cells derived from patients with FM have reduced levels of coenzyme Q10, increased formation of mitochondrial

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superoxide, increased levels of lipid peroxidation, and decreased mitochondrial membrane potential.[6,7] This mitochondrial dysfunction was also associated with increased expression of autophagic genes and the elimination of dysfunctional mitochondria with mitophagy.[7] These findings suggest that mitochondrial dysfunction may be the origin of oxidative stress in patients with FM.

Coenzyme Q10 is a lipid-soluble substance that functions as an essential cofactor in the mitochondrial respiratory chain. Its reduced form, ubiquinol-10, is an important antioxidant, and the redox balance of coenzyme Q10 is a potential biomarker of systemic oxidative stress.[8] Ubiquinol-10 acts as an antioxidant in the mitochondria and in lipid membranes by either directly scavenging free radicals or in conjunction with α-tocopherol.[8,9] Coenzyme Q10 is a popular dietary supplement among healthy individuals and those with various ailments, including neurodegenerative and cardiovascular diseases.[10,11]

The present study was conducted to characterize oxidative stress in juvenile patients with FM and to evaluate the effect of coenzyme Q10 supplementation in a double-blind, placebo-controlled trial.

Subjects

Patients were eligible for inclusion in this study if they met the American College of Rheumatology classification criteria for FM.[1] Oral supplementation with coenzyme Q10 or any other medications was not allowed for 3 months prior to the first administration of ubiquinol-10 in this the study. Ten children (two boys and eight girls; age, 14.7 ± 2.9 years) were enrolled. The mean age at onset of FM was 12.0 ± 2.0 years, and the mean duration of disease was 31.4 ± 29.3 months (Table 1). The Ethics Committee of Yokohama City University Hospital approved all study protocols (Approval No. B090702013). The parent or legal guardian of each child provided written informed consent, and child assent was obtained when appropriate.

As a healthy control, blood samples from 67 schoolchildren in Shinagawa, Tokyo were obtained (37 boys and 30 girls; age, 11.5 ± 2.0 years). All samples were included since all schoolchildren were diagnosed to be healthy.

Methods

Study design

This study consisted of three sequential double-blind phases: (1) treatment with ubiquinol-10 for 12 weeks, (2) treatment with placebo for 8 weeks, and (3) treatment with ubiquinol-10 for 8 weeks. All patients received either daily oral supplementation of ubiquinol-10 or a placebo (Softgel capsules, 100 mg/day; Kaneka, Osaka, Japan). Plasma levels of ubiquinol-10 (reduced form of coenzyme Q10), ubiquinone-10 (oxidized form of coenzyme Q10), vitamin E (VE), free cholesterol (FC), cholesterol esters (CE), and the percent contents of palmitoleic acid (16:1), oleic acid (18:1), and polyunsaturated fatty acids (PUFA) in total free fatty acids (FFA) (%16:1, %18:1, and %PUFA, respectively) were measured at 0, 2, 4, 8, 12, 16, 20, 24, and 28 weeks after initiation of the study. All data (week 0) were compared with those in age-matched healthy individuals.

Changes in the clinical manifestations of FM were evaluated by assessing subjective pain intensity, quality of life (QOL), and general fatigue using the pain Visual Analog Scale (VAS),[12] Pediatric Quality of Life Inventory (PedsQL),[13] and the Chalder Fatigue Scale,[14,15] respectively. All assessments were completed in the presence of clinical research coordinators. The Chalder Fatigue Scale consists of 14 items that assess symptoms of physical and mental fatigue, such as tiredness, sleepiness, lack of energy, lack of muscle strength, and difficulties with concentration and memory. Each item asks participants to rate the frequency of a symptom by choosing from among 'less than usual', 'no more than usual', 'more than usual', and 'much more than usual'. Scores ranging from 0 to 3 were given using the Likert scoring system. The total score of the scale was calculated by adding the rating for each item, resulting in a score that ranged from 0 to 42.

Analytical procedures

Plasma levels of VE, ubiquinol-10, ubiquinone-10, FC, and CE were determined as described[16] with modifications. In brief, plasma extracted with a 19-fold volume of 2-propanol was analyzed by HPLC using an analytical column (Type Supelcosil LC-8, 5 μm, 25 cm × 4.6 mm i.d.; Supelco Japan, Tokyo, Japan), a reduction column (Type RC-10-1; Irica, Kyoto, Japan), and an amperometric electrochemical detector (Model Σ985; Irica) with an oxidation potential of +600 mV (vs. Ag/AgCl) on a glassy carbon electrode. The mobile phase consisted of 50 mM sodium perchlorate in methanol/2-propanol (9/1, v/v) delivered at a flow rate of 0.8 ml/minute.

Plasma FFA were derivatized with monodansylcadaverine and then analyzed by HPLC.[17] Briefly, plasma samples (50 μl) were mixed with 200 μl of methanol containing 12.5 μM margaric acid (internal standard) and then separated by centrifugation at 13 000 × *g* for 3 minutes. Samples (50 μl) of supernatants were dried under a stream of nitrogen gas, and then each residue was mixed with diethyl phosphorocyanidate (1 μl) and *N,N*-dimethylformamide (50 μl) containing monodansylcadaverine (2 mg/ml) and then placed at room temperature in the dark for 20 minutes. A 5-μl sample was injected onto an

Table 1 Patient characteristics

Patient No	Age (years)	Gender	Disease duration (months)	Number of positive tender point*	Pain VAS (mm/100 mm)	Chalder's Fatigue Scale**
1	15.0	F	11.8	18	83.8	26
2	16.2	F	34.5	11	22.2	35
3	13.1	F	9.9	18	55.6	25
4	17.4	F	92.7	18	71.7	20
5	14.6	F	18.8	18	24.2	25
6	16.6	F	23.6	15	44.4	35
7	11.6	M	9.9	12	62.6	23
8	18.7	F	82.8	18	50.5	30
9	15.4	F	23.2	18	62.6	24
10	8.3	M	6.9	11	33.0	4
Average	14.7		31.4			25

*Number of positive tender point out of 18 tender points on the body highly sensitive to pressure in people with fibromyalgia as specified by the American College of Rheumatology criteria.
**Total score of 14-item instrument with a four-choice format which ranges from 0 to 3. It was calculated by adding the rating for each item and ranges from 0 to 42.

octadecylsilyl column (3 μm, 3.3 cm × 4.6 mm i.d.; Supelco Japan) and a pKb-100 column (5 μm, 25 cm × 4.6 mm i.d.; Supelco Japan) connected in series. The FFA components were measured by fluorescence detection (Model 821-FP; Japan Spectroscopic, Tokyo, Japan) with excitation at 320 nm and emission at 520 nm. The mobile phase consisted of acetonitrile/methanol/water (17.5/65.0/17.5, v/v/v) delivered at a flow rate of 1.5 ml/minute with the analytical columns maintained at 40°C.

Statistical analysis

Data were statistically analyzed by Student's *t*-test. To assess the time course efficacy of ubiquinol-10 supplementation, post-treatment data were assessed using a repeated-measure analysis of variance (ANOVA). $P < 0.05$ was considered statistically significant.

Results and discussion

Hypercholesterolemia and coenzyme Q10 deficiency in juvenile FM

Fig. 1 shows baseline plasma levels of FC and CE in juvenile FM before the ubiquinol-10 supplementation. Levels of FC and CE were two-fold greater in patients with juvenile FM than in age-matched healthy controls, suggesting that juvenile FM is associated with hypercholesterolemia. In adult patients with FM, one previous study showed the slight increase in total cholesterol as compared with healthy control subjects[18] while another study showed no change in cholesterol when comparing with controls.[19] The ratio of FC to CE is a good indicator of lecithin-cholesterol acyltransferase (LCAT) activity in plasma. There was a small but a significant difference in the FC/CE ratio when comparing juvenile FM patients and healthy control subjects (Fig. 1). This will be discussed later.

Total cholesterol (FC + CE) level in patients with juvenile FM was two-fold greater than that in healthy control subjects (Fig. 2). Levels of the lipid-soluble antioxidant, VE, were also two-fold higher in patients with juvenile FM than in healthy control subjects (Fig. 2). Therefore, the ratio of VE to TC was nearly identical in the two groups (Fig. 2).

On the other hand, plasma level of ubiquinol-10, another lipid-soluble antioxidant, was significantly lower in patients with juvenile FM when compared

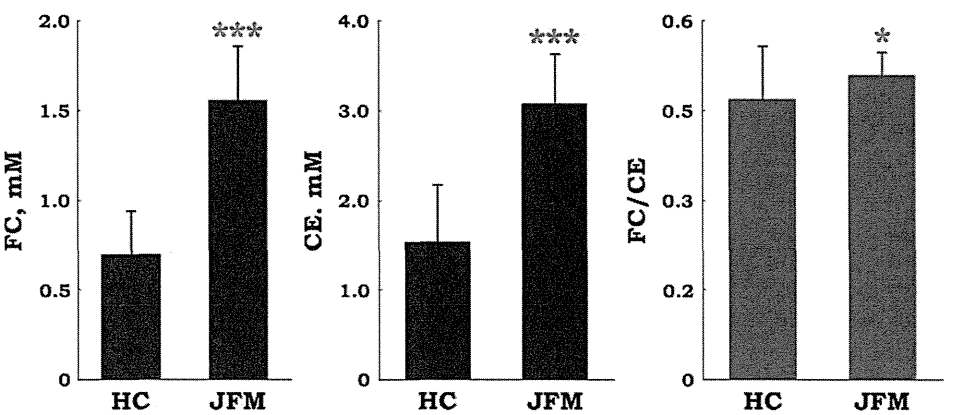

Figure 1 Plasma levels of free cholesterol (FC) and cholesterol esters (CE), and the ratio of FC to CE in patients with juvenile fibromyalgia (JFM, $n = 10$) and in healthy control (HC, $n = 67$) subjects. Data are means ± SD, *$P < 0.05$, ***$P < 0.001$ vs. HC.

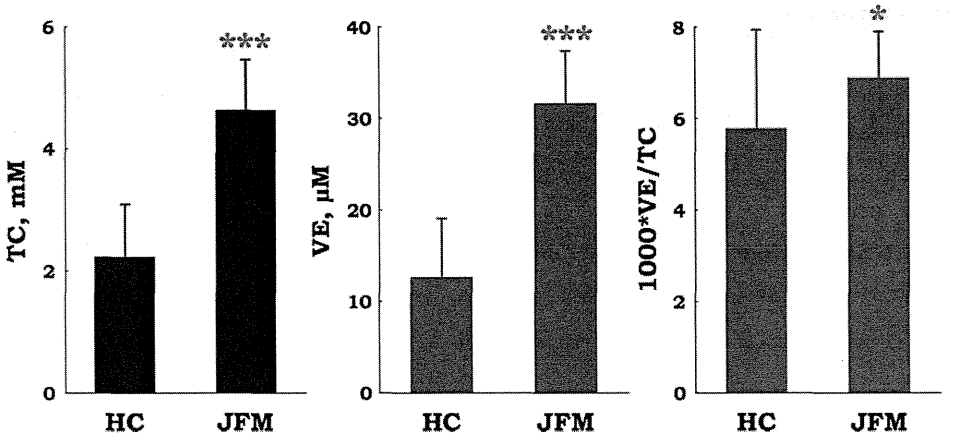

Figure 2 Plasma levels of total cholesterol (TC) and vitamin E (VE), and the ratio of VE to TC in patients with juvenile fibromyalgia (JFM, $n = 10$) and healthy control (HC, $n = 67$) subjects. Data are means ± SD, $^{*}P < 0.05$, $^{***}P < 0.001$ vs. HC.

with healthy control subjects (Fig. 3). Level of the oxidized form of coenzyme Q10, ubiquinone-10, was similar in the two groups (Fig. 3). The ratio of total coenzyme Q10 (ubiquinol-10 + ubiquinone-10) to total cholesterol was 13-fold higher in healthy control subjects than in juvenile FM patients (Fig. 3). This finding is consistent with coenzyme Q10 deficiency in patients with juvenile FM.

Oxidative stress in juvenile FM

Oxidative stress is defined as a disturbance in the prooxidant–antioxidant balance in favor of the former. When human plasma was incubated under aerobic conditions at 37°C, ubiquinol-10 levels decreased after the depletion of ascorbate, α-tocopherol level remained stable, and concomitant formation of ubiquinone-10 was observed.[7] Ubiquinol-10 is very reactive to oxygen radicals. Therefore, the ratio of ubiquinone-10 to total coenzyme Q10 (%CoQ10) is a useful biomarker of systemic oxidative stress.[7] The baseline value of %CoQ10 in juvenile FM patients was significantly higher than that in healthy control subjects (Fig. 3), suggesting that an increased formation of reactive oxygen species in circulating blood in patients with juvenile FM (i.e. increased oxidative stress state).

As discussed above, Cordero *et al.* reported that FM was associated with coenzyme Q10 deficiency, increased mitochondrial superoxide formation, and increased level of lipid peroxidation as measured by thiobarbituric acid reactive substances formation in blood mononuclear cells.[6] On the other hand, another report concluded that there was no increase in oxidative stress in FM, as there was no change in urinary levels of F_2 isoprostane (a free radical oxidation product of arachidonic acid (20:4) and one of

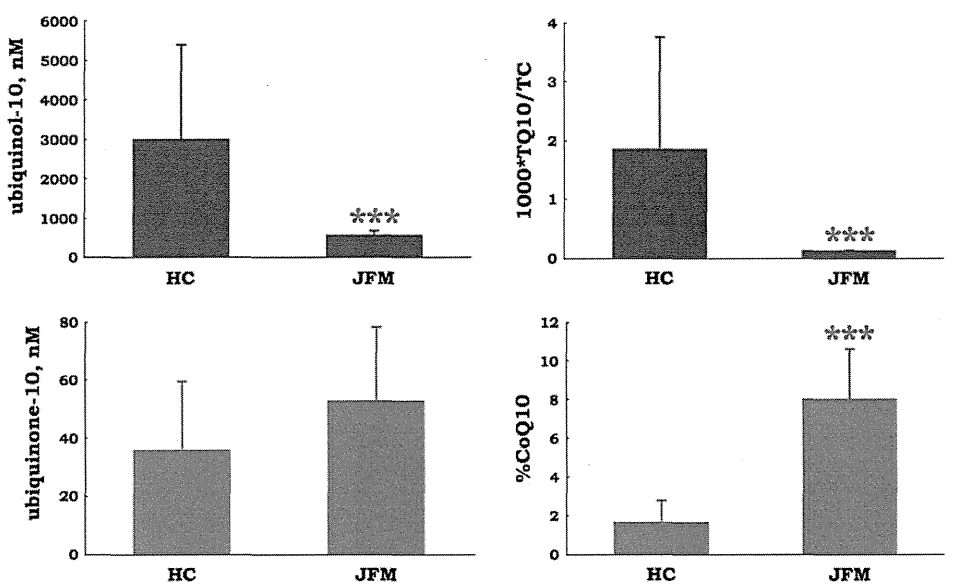

Figure 3 Plasma levels of ubiquinol-10 and ubiquinone-10, and the ratio of total coenzyme Q10 (TQ10) to total cholesterol (TC) and ubiquinone-10 to TQ10 (%CoQ10) in patients with juvenile fibromyalgia (JFM, $n = 10$) and healthy control (HC, $n = 67$) subjects. Data are means ± SD, $^{***}P < 0.001$ vs. HC.

the most reliable oxidative stress markers) when comparing patients with FM and controls.[20] These contradictory results prompted us to further investigate the role of oxidative stress in juvenile FM.

Tissue oxidative damage can be measured by studying plasma FFA and its composition. When tissues are under oxidative stress, plasma FFA are increased; this is because the activity of phospholipases A_2 and A_1 increases under oxidative stress[21–23] and because the FFA produced by this process may enter the bloodstream through leakage or lysis of oxidatively damaged brain cells. If this were indeed the case, we would expect a lower concentration of PUFA, such as linoleic acid (18:2), linolenic acid (18:3), 20:4, and docosahexaenoic acid (22:6) in the plasma, since those substances are highly susceptible to oxidation. A previous study reported that plasma concentrations of PUFA decreased while those of monoenoic acids, such as oleic acid (18:1) and palmitoleic acid (16:1), increased to compensate for the oxidative loss of PUFA under various conditions of oxidative stress.[24] Such changes were observed in patients with adult respiratory distress syndrome,[25] multiple sclerosis,[26] Papillon–Lefevre syndrome,[27] and in newborn babies.[28] We recently observed that an elevation of plasma FFA levels and the contents of 16:1 and 18:1 in total FFA (%16:1 and %18:1, respectively) was induced by 2-hour occlusion–reperfusion of the middle cerebral artery in rats and that this change was attenuated by the administration of a free radical scavenger drug, edaravone.[29]

Fig. 4 shows that plasma FFA level was four-fold higher in patients with juvenile FM when compared with healthy control subjects, while %PUFA was significantly lower in patients with juvenile FM when compared with healthy control subjects. Moreover, %16:1 and %18:1 were significantly higher in juvenile FM patients than in healthy control subjects (Fig. 4). These data indicate the presence of oxidative tissue damage in patients with juvenile FM. These results may correlate with the presence of lipid droplets and abnormal mitochondria in muscle[30] and an increase in the levels of the inflammatory cytokine, interleukin-8, in the serum and cerebrospinal fluid[31] from patients with FM.

Effect of ubiquinol-10 supplementation

The fact that patients with juvenile FM are coenzyme Q10-deficient led us to conduct a study of ubiquinol-10 supplementation in juvenile FM patients. Although plasma levels of VE remained unchanged, total levels of coenzyme Q10 significantly increased after ubiquinol-10 supplementation. Then, after switching to placebo, total levels of coenzyme Q10 returned to baseline. Subsequent reinitiation of ubiquinol-10 supplementation resulted in an increase in total levels of coenzyme Q10 (Fig. 5). Similarly, values of %CoQ10 significantly decreased after ubiquinol-10 supplementation, returned to baseline during the placebo period, and then decreased again with the second ubiquinol-10 supplementation period (Fig. 5). This suggests that oxidative stress in patients with juvenile FM can be ameliorated by ubiquinol-10 supplementation.

While ubiquinol-10 supplementation did not change the level of tissue oxidative damage, as indicated by the lack of change in FFA level, %16:1, and %18:1 (Fig. 6), hypercholesterolemia was attenuated (Fig. 7). In particular, the FC/CE ratio was significantly reduced after ubiquinol-10 supplementation (Fig. 7),

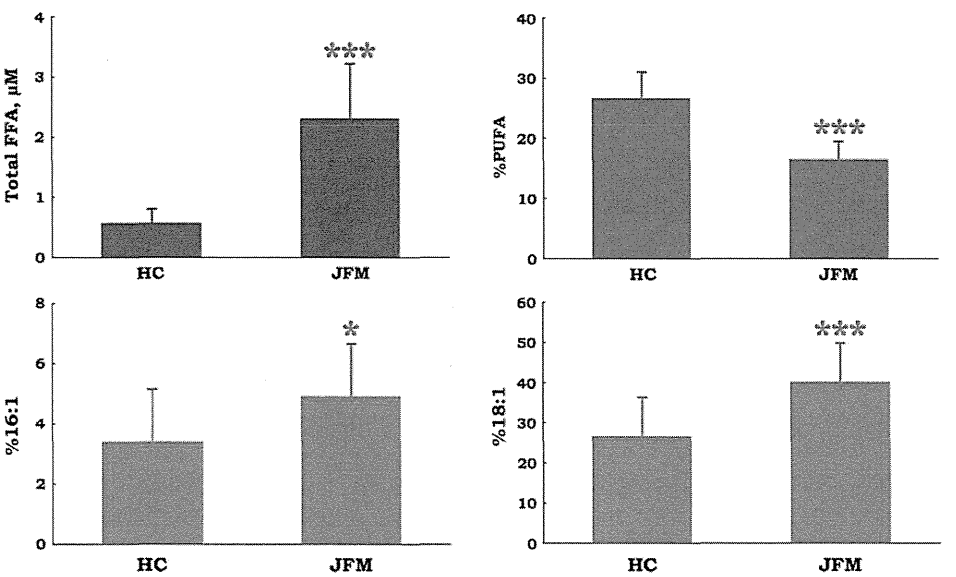

Figure 4 Plasma level of total free fatty acids (FFA), the ratios of polyunsaturated fatty acids (PUFA) to total FFA (%PUFA), oleic acid to total FFA ratio (%18:1), and palmitoleic acid to total FFA ratio (%16:1) in patients with juvenile fibromyalgia (JFM, $n = 10$) and healthy control (HC, $n = 67$) subjects. Data are means ± SD, *$P < 0.05$, ***$P < 0.001$ vs. HC.

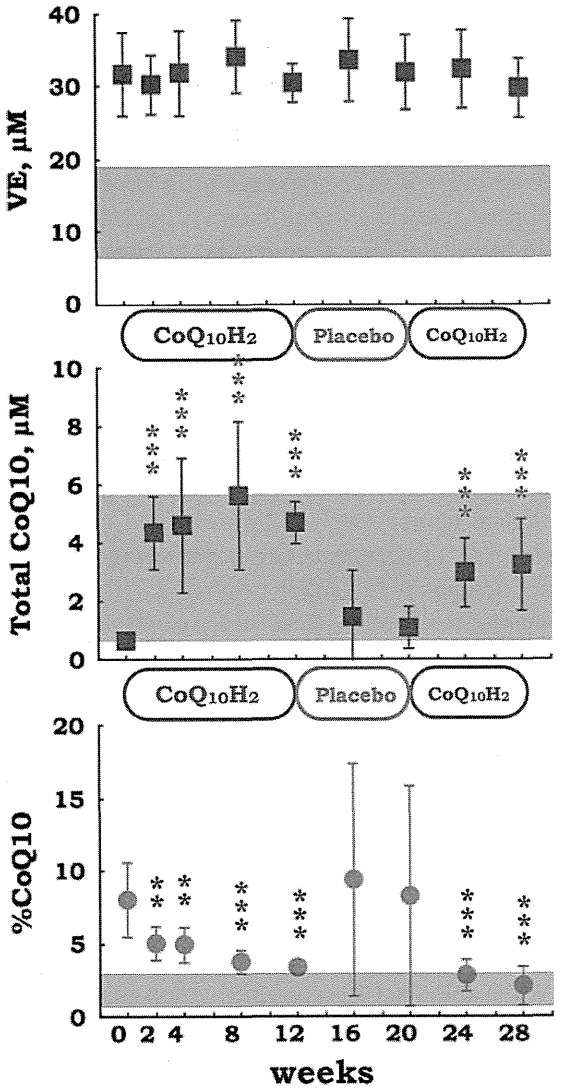

Figure 5 Plasma level of vitamin E (VE), total coenzyme Q10 (ubiquinol-10 + ubiquinone-10), and the ratio of ubiquinone-10 to total coenzyme Q10 (%CoQ10) in patients with juvenile FM ($n = 10$) during supplementation with reduced coenzyme Q10 (100 mg/day for 12 weeks, 0 mg/day for 8 weeks, and 100 mg/day for 8 weeks). Data are means ± SD, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. baseline value at week 0. Mesh shows the means ± SD values in healthy control ($n = 67$) subjects.

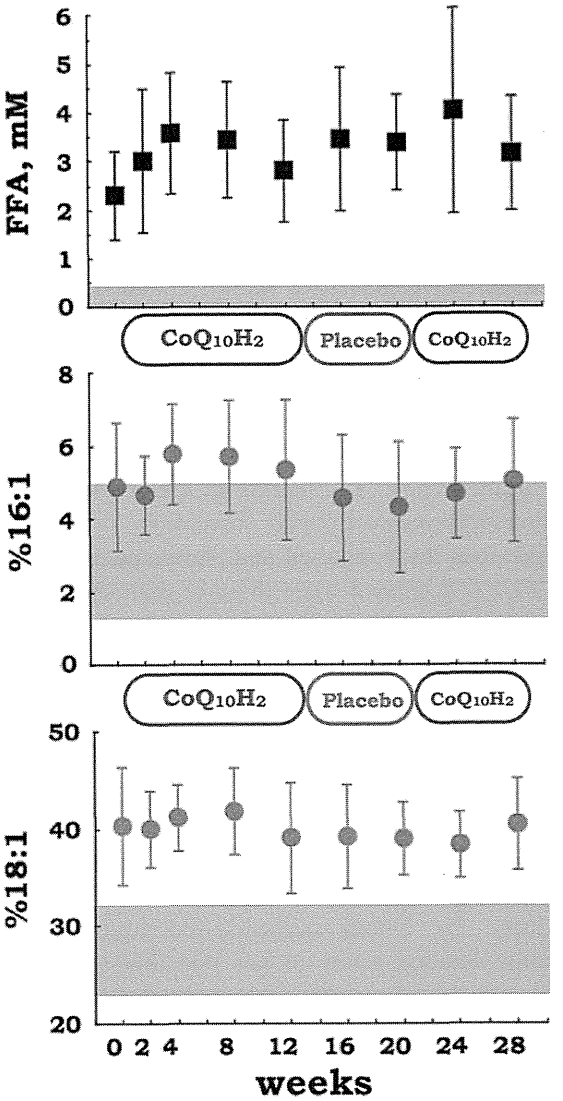

Figure 6 Plasma levels of total free fatty acids (FFA), and the ratios of palmitoleic acid and oleic acid to total FFA (%16:1 and %18:1, respectively) in patients with juvenile FM ($n = 10$) during supplementation with reduced coenzyme Q10 (100 mg/day for 12 weeks, 0 mg/day for 8 weeks, and 100 mg/day for 8 weeks). Data are means ± SD. Mesh shows the mean ± SD values in healthy control ($n = 67$) subjects.

suggesting that plasma LCAT activity was enhanced. LCAT catalyzes the transesterification of fatty acid at the 2 position of phosphatidylcholine (PC) to FC to yield CE and lyso-PC. LCAT is secreted from the liver, and its activity is often reduced in patients with liver disease, such as hepatitis, cirrhosis, and hepatoma.[32,33] It is noteworthy that ubiquinol-10 supplementation improved the FC/CE ratio in patients with juvenile FM, which suggests that ubiquinol-10 supplementation may help improve liver function.

Effect of ubiquinol-10 supplementation on QOL

The effect of ubiquinol-10 supplementation on QOL was assessed. Both subjective pain intensity (assessed using the 100-mm VAS) and health-related QOL (evaluated by self- and proxy-rating) did not change throughout the three study phases (data not shown). On the other hand, symptoms of chronic fatigue (as measured by the Chalder Fatigue Scale) significantly decreased in response to ubiquinol-10 supplementation (Fig. 8). This was also confirmed by repeated-measure ANOVA ($P = 0.041$).

It is noteworthy that in five adult patients with FM, ubiquinone-10 supplementation (300 mg/day for 9 months) results in clinical improvement such as tender points, VAS, FM impact questionnaire score, and headache impact test score.[34] This results were confirmed by a further study consisting of 20 adult patients with FM who took 300 mg ubiquinone-10/day for 3 months.[35] The reason as to why the effect

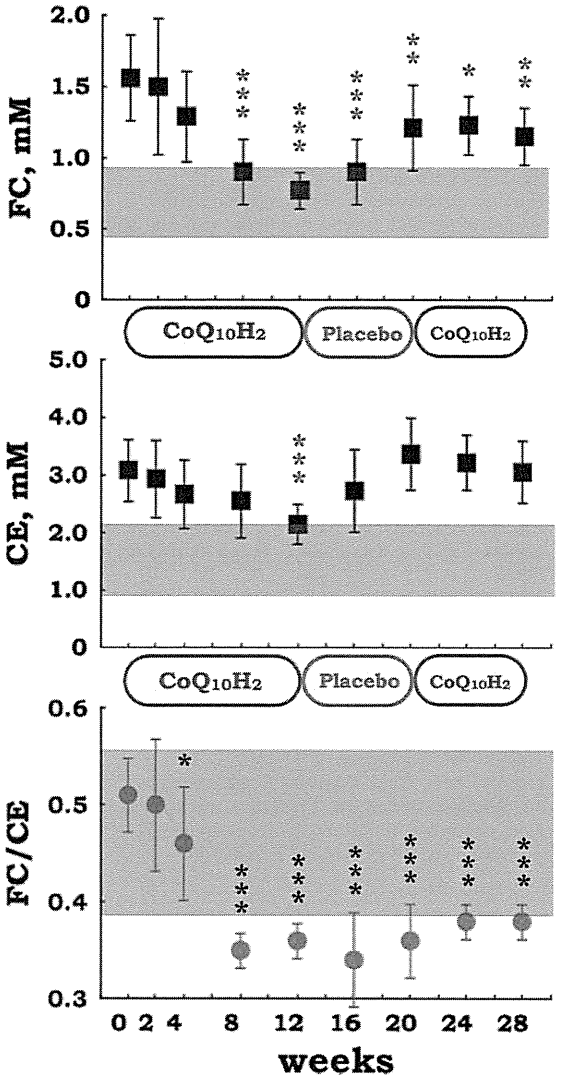

Figure 7 Plasma levels of free cholesterol (FC) and cholesterol esters (CE), and the ratio of FC to CE in patients with juvenile FM ($n = 10$) during supplementation with reduced coenzyme Q10 (100 mg/day for 12 weeks, 0 mg/day for 8 weeks, and 100 mg/day for 8 weeks). Data are means ± SD, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. baseline value at week 0. Mesh shows the mean ± SD values in healthy control ($n = 67$) subjects.

of ubiquinol-10 supplementation was limited in this juvenile FM study is not clear. Age difference may be critical but dose difference should be examined. We, therefore, planned to investigate the effect of higher doses of ubiquinol-10 in future studies.

In conclusion, we found that patients with juvenile FM are hypercholesterolemic and coenzyme Q10-deficient. Increased oxidative stress in the patients was suggested by a significant increase in %CoQ10, FFA level, %16:1, and %18:1, and a significant decrease in %PUFA when compared with healthy control subjects. Pain and tissue oxidative damage, indicated by FFA, %18:1, and %16:1, did not change, but general fatigue and hypercholesterolemia was attenuated in response to ubiquinol-10 supplementation.

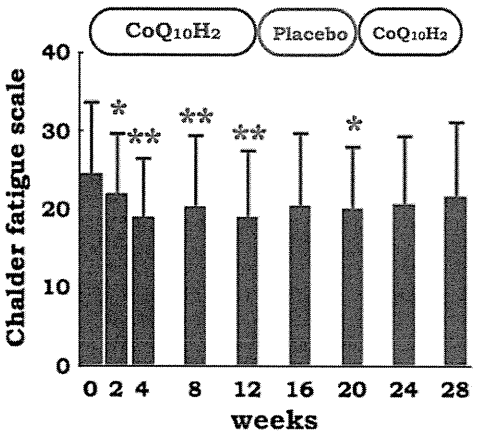

Figure 8 Chalder Fatigue Scale in patients with juvenile FM ($n = 10$) during supplementation with reduced coenzyme Q10 (100 mg/day for 12 weeks, 0 mg/day for 8 weeks, and 100 mg/day for 8 weeks). Data are means ± SD, *$P < 0.05$, **$P < 0.01$ vs. baseline value at week 0. Repeated-measure ANOVA indicates a significant time course effect of 12 weeks supplementation of reduced coenzyme Q10 ($P = 0.041$).

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PLOS ONE

SNP (–617C>A) in ARE-Like Loci of the NRF2 Gene: A New Biomarker for Prognosis of Lung Adenocarcinoma in Japanese Non-Smoking Women

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Abstract

Purpose: The transcription factor NRF2 plays a pivotal role in protecting normal cells from external toxic challenges and oxidative stress, whereas it can also endow cancer cells resistance to anticancer drugs. At present little information is available about the genetic polymorphisms of the *NRF2* gene and their clinical relevance. We aimed to investigate the single nucleotide polymorphisms in the *NRF2* gene as a prognostic biomarker in lung cancer.

Experimental Design: We prepared genomic DNA samples from 387 Japanese patients with primary lung cancer and detected SNP (c.–617C>A; rs6721961) in the ARE-like loci of the human *NRF2* gene by the rapid genetic testing method we developed in this study. We then analyzed the association between the SNP in the *NRF2* gene and patients' overall survival.

Results: Patients harboring wild-type (WT) homozygous (c.–617C/C), SNP heterozygous (c.–617C/A), and SNP homozygous (c.–617A/A) alleles numbered 216 (55.8%), 147 (38.0%), and 24 (6.2%), respectively. Multivariate logistic regression models revealed that SNP homozygote (c.–617A/A) was significantly related to gender. Its frequency was four-fold higher in female patients than in males (10.8% female vs 2.7% male) and was associated with female non-smokers with adenocarcinoma. Interestingly, lung cancer patients carrying *NRF2* SNP homozygous alleles (c.–617A/A) and the 309T (WT) allele in the *MDM2* gene exhibited remarkable survival over 1,700 days after surgical operation (log-rank $p = 0.021$).

Conclusion: SNP homozygous (c.–617A/A) alleles in the *NRF2* gene are associated with female non-smokers with adenocarcinoma and regarded as a prognostic biomarker for assessing overall survival of patients with lung adenocarcinoma.

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Introduction

Lung cancer is the leading cause of cancer-related death in many industrial countries. It is classified into two major types, namely, small-cell lung carcinoma (SCLC) and non-small-cell lung carcinoma (NSCLC). While long-term exposure to cigarette smoke is the most common cause of lung cancer (80–90% of lung cancers), non-smokers account for 10–15% of lung cancer cases, which are often attributed to a combination of genetic and environmental factors [1–3]. The transcription factor NF-E2-related factor 2 (NRF2) is known to control cellular adaptation/protection to reactive oxygen species and electrophiles by inducing antioxidation and detoxification genes [4–6] as well as mediate cancer cell proliferation and drug resistance [7–12]. We have undertaken the present study to examine the clinical impact of the

NRF2 gene and its genetic polymorphisms on the risk and prognosis of lung cancer.

NRF2 is a "cap'n'collar" basic region-leucine zipper (CNC-bZip) transcription factor and plays a pivotal role in the induction of antioxidant response element (ARE)-regulated genes [4–13]. Under non-stressed conditions, NRF2 protein is associated with Kelch-like ECH associating protein 1 (*KEAP1*) [14]. KEAP1 is known to be a negative regulator of NRF2 by retrieving it in the cytoplasm. Oxidative stress and/or electrophilic attack lead to the dissociation of NRF2 from KEAP1 and thereby the NRF2 protein is translocated into the nucleus. NRF2 together with small multiple alignment format (MAF) sequences binds to ARE sequences [15]. Many genes encoding detoxifying and antioxidant enzymes have been found to be regulated by NRF2 [4–6,15–18]. It has recently been reported that NRF2 contributes to malignant phenotypes of cancer cells *in vitro*, including aggressive cell proliferation, drug resistance, and metabolic re-programming [7–11,19,20]. In this context, the *NRF2* gene is considered to play split roles, for example, in the protection of normal cells and progression of cancer malignancy.

In 2004, Yamamoto and colleagues first reported the structure of the *NRF2* gene and found three SNPs (−653A>G, −651G>A, and −617C>A) and one triplet repeat polymorphism in its regulatory region [21]. Three years later, Marzec *et al.* examined the impact of those SNPs on the regulation of *NRF2* gene expression [22]. In transient transfection assays, they found that the −617C>A SNP significantly affects basal NRF2 protein levels and its function *in vitro* [22]. SNP −617C>A was found to be associated with a higher risk of oxidant-induced acute lung injury in humans [22]. It has been reported that a SNP (c.−617C>A) in the ARE-like loci of the human *NRF2* gene is important for self induction of the *NRF2* gene. *NRF2* regulates the transcription of numerous phase II drug-metabolizing enzymes and phase III drug-transporters (*e.g.*, ABCC1, ABCC2, ABCC4, and ABCG2) in response to oxidative stress *via* direct binding to the ARE sequences in those target genes [23–26]. At present, however, little information is available as to the clinical impact of genetic polymorphisms of the *NRF2* gene and the prognosis of lung cancer.

To gain insight into the genetic polymorphisms of the *NRF2* gene, we have developed rapid genotyping primer sets by utilizing the SmartAmp method, an isothermal DNA amplification process [27,28]. Among a total of 387 lung cancer patients, we found that SNP (c.−617C>A) in the *NRF2* gene is a prognostic biomarker for assessing the gender (female)-related risk of lung adenocarcinomas in the Japanese non-smoking sub-population of lung cancer patients. The epidermal growth factor receptor (*EGFR*) gene was frequently mutated in those female patients harboring the SNP homozygous SNP allele (−617A/A), suggesting a potential link between the SNP homozygote (−617A/A) and *EGFR* gene mutations. Furthermore, NRF2 reportedly regulates expression of the *MDM2* gene that encodes a negative regulator of p53, and this study shows that lung cancer patients with homozygous SNP alleles (−617A/A) in the *NRF2* gene and the 309T (WT) allele in the *MDM2* gene had markedly better overall survival. This is the first report providing clinical evidence that homozygous SNP (−617A/A), as one of the intrinsic genetic polymorphisms in the *NRF2* gene, is associated with the overall survival of lung cancer patients. Our clinical research data strongly suggest that the SNP homozygous allele (−617A/A) is a useful biomarker for clinical diagnosis.

Results

Clinicopathological Characterization

The clinicopathological characterization data for the 387 primary lung cancer patients are summarized in Table 1. The patient population comprised 221 men and 166 women, with an overall mean age of 66 years (range 35 to 87 years). The histological type of lung cancer was determined according to the protocol of the third World Health Organization/International Association for the Study of Lung Cancer Classifications [29]. Among the lung cancer patients, 298 were classified as having adenocarcinomas and 89 non-adenocarcinomas. The p-stage was determined by pathological examination of surgical specimens for 376 patients, and their tumours were staged according to the tumor nodes metastasis (TNM) classification of malignant tumours: 292, 46, 35, and 3 patients were respectively classified into stages I, II, III, and IV. For the remaining 11 patients, the p-stage could not be determined. The smoking history was obtained from each lung cancer patient at Kanagawa Cancer Center Research Institute: 154 patients had no smoking history, whereas 233 patients were smokers.

Preparation of Genotyping Primers for Detection SNP (c.−617 C>A) in *NRF2* Gene

The *NRF2* gene is located on the negative strand of genomic DNA at q31.2 of chromosome 2. To create the template for preparation of genotyping primers, we synthesized double stranded DNA encoding a 424-bp region of nt.178129735 to nt.178130158, including the SNP (c. −617 C>A) in the *NRF2* gene by means of PCR with human genomic DNA. PCR primers used for the DNA synthesis were GACCACTCTCCGACCTAAAGG (forward) and CGAGATAAAGAGTTGTTTGCGAA (reverse).

Table 1. Clinicopathological characterization of primary lung cancer patients.

Variable	No. of patients	(%)
Gender		
Male	221	(57.1)
Female	166	(42.9)
Age (years old)		
≤50	25	(6.4)
>50	362	(93.5)
Histopathology		
Adeno	298	(77.0)
Non-adeno	89	(23.0)
Smoking		
Non-smoker	154	(39.8)
Smoker	233	(60.2)
p-Stage		
I	292	(75.4)
II	46	(11.9)
III	35	(9.0)
IV	3	(0.8)
Undetermined	11	(2.8)

Ages of all patients, 66.4±9.9 (mean±S.D.).
Abbreviation: Adeno, adenocarcinoma.
doi:10.1371/journal.pone.0073794.t001

The resulting PCR product was then inserted into the TA-cloning site of the pGEM®-T Easy Vector (Promega, Madison, WI, USA). The vector was amplified in JM109 High Efficiency Competent Cells (Promega), and vector DNA was purified by using the GeneGET™ Plasmid Miniprep Kit (Fermentas, Thermo Fisher Scientific Inc., Waltham, MA, USA). The sequence of the inserted DNA was analyzed with a laser-based automated DNA sequencer (ABI PRISM 3100 DNA Analyzer, Applied Biosystems Ltd., Tokyo, Japan). We designed a number of SNP-typing primer candidates and repeatedly tested them by using the vector-inserted DNA as a template until we obtained the best primer set.

The schematic illustration in Figure 1A shows the annealing sites of the best primer set, which comprises four different primers, *i.e.*, TP, OP, FP, and BP. The TP primer was designed such that its turn-back region can discriminate the nucleotide of C or A at c.–617 in the negative DNA strand extended from the 3′-end of the primer (see Figure 1A). Furthermore, two SNPs, c.−651G>A and c.653A>G, were not included in the annealing sites of the four primers. Figure 1B depicts the sequences of primers in the WT (–617C)-typing and SNP (–617)-typing sets. Exciton dye was linked to thymine in the FP primer as symbolized by "Z" in the lower panel of Figure 1B.

Figure 1C shows the time courses of genotyping reactions as functions of fluorescence intensity. By using the genotyping primer sets, we could detect the WT homozygote (–617C/C), WT/SNP heterozygote (–617C/A), and SNP homozygote (–617A/A) in genomic DNA samples. These results were verified by DNA sequence analysis. Namely, we performed sequencing analysis for determination of the WT homozygote (–617C/C), WT/SNP heterozygote (–617C/A), and SNP homozygote (–617A/A) in those genomic DNA samples. We tested 24 samples for each group (i.e., C/C, C/A, and A/A) and confirmed the 100% accordance between the SmartAmp method and the DNA sequencing analysis method.

Detection of SNP (c.–617 C>A) in the *NRF2* Gene in Lung Cancer Patients

Using the genomic DNA samples prepared from a total of 387 lung cancer patients, we have detected WT and SNP alleles in the *NRF2* gene by the rapid genotyping method described above. Table 2 summarizes these results, showing that 216, 147, and 24 patients could be typed as WT homozygote (–617C/C), WT/SNP heterozygote (–617C/A), and SNP homozygote (–617A/A), respectively. Accordingly, the allele frequency was calculated to be 74.8% and 25.2% for WT (c.–617C) and SNP (c.–617A), respectively, when both male and female patients were grouped together. It is of interest to note, however, that the allele frequency of SNP (c.–617A) was 28.6% for female patients, compared with 22.6% for male patients. Indeed, 18 female patients carried the SNP homozygote (–617A/A), a number three-fold higher than that of male patients. The ratio of homozygous SNP (–617A/A) was 10.8% for female patients, about four-fold higher than the 2.7% found in males (Table 2; $P=0.004$). In contrast, the ratios of WT homozygote (–617C/C) and heterozygote (–617 C/A) were moderately higher in male patients than in female patients (Table 2). On the other hand, with respect to smoking experience, the ratio of homozygous SNP (–617A/A) was 10.4% in the non-smoker sub-population, being about three times ($P=0.021$) higher than the ratio (4.5%) observed in the smokers (Table 2).

By multivariate analysis, the SNP homozygote (–617A/A) in the *NRF2* gene was found to be independently associated with gender (Table 3). Among a total of 20 adenocarcinoma patients (females+males) carrying the SNP homozygote (–617A/A), 16 patients were females who had no cigarette-smoking experience (Table 4). In contrast, there were no male adenocarcinoma patients in the sub-group of non-smokers carrying the SNP homozygote (–617A/A) (Table 4). These results demonstrate a marked gender difference in terms of non-smoking patients carrying the SNP homozygote (–617A/A).

To gain more insight into the gender difference among 24 patients homozygous for the SNP (c.–617A/A), we have analyzed the genetic polymorphisms of human *CYP2A6*4* (whole gene deletion) and the numbers of $(GT)_n$ repeats in the *HO-1* gene 5′-flanking region. These data are summarized in Table 5. *CYP2A6*4/*4* (whole gene deletion) was found in only one female patient in this subgroup (Table 5), whereas among 387 lung cancer patients, *CYP2A6*4/*4* was detected in seven patients (1.8%) with lung adenocarcinoma. The number of $(GT)_n$ repeats in the *HO-1* gene 5′-flanking region varied greatly (14 to 34 repeats) among these 24 patients.

Interestingly, as shown in Table 5, either exon 19 or exon 21 of the *EGFR* gene was frequently mutated in female patients who were non-smokers and had homozygous SNP alleles (–617A/A). Two of those patients were diagnosed as p-stage IIA (cases 1 and 16). They were treated first surgically and then with gefitinib. These patients had no relapse over 1879 days (case 1) and 939 days (case 16).

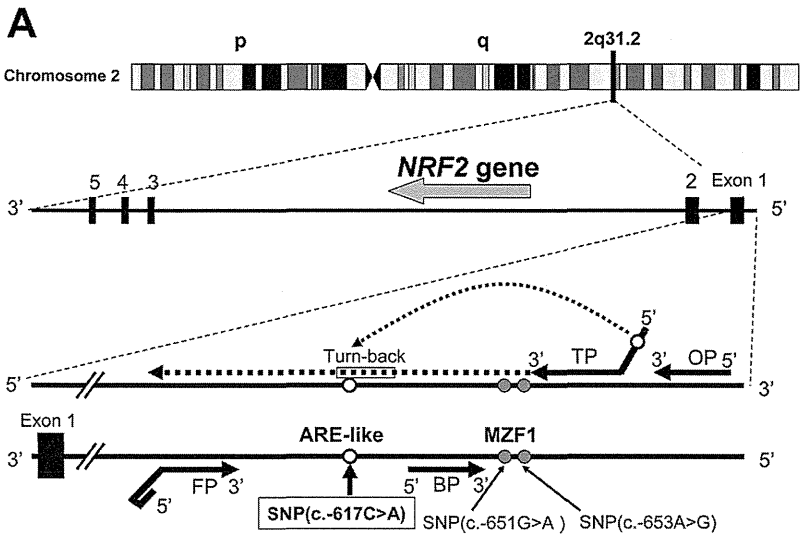
Association of SNP (c.–617 C>A) in the *NRF2* Gene with Overall Survival of Lung Cancer Patients

We investigated a potential association between SNP (c.–617 C>A) in the *NRF2* gene and the overall survival of lung cancer patients, since we could obtain follow-up information on 369 patients among the total of 387 lung cancer patients over 1,700 days after surgical operation at Kanagawa Cancer Center. A survival Kaplan-Mayer plot is shown in Figure 2A. Our univariate analysis has revealed that lung cancer patients (p-stages I to IV) carrying homozygous SNP alleles (–617A/A) in the *NRF2* gene experienced significantly better overall survival, as compared with patients with heterozygous alleles (c.–617C/A) (log-rank $P=0.021$). In contrast, no association was found between patients with homozygous WT alleles (–617C/C) and those with heterozygous alleles (c.–617C/A) (Figure 2A). It is important to mention that one female patient with the homozygous SNP (–617A/A) (case 5 in Table 5) died due to primary pancreatic cancer during the follow-up study.

We further analyzed the associations between the *NRF2* genotypes and patients' overall survival in the p-stage I of non-small cell lung cancer (NSCLC) including adenocarcinoma, squamous cell cancer, and large cell cancer. In the case of stage I, patients (n = 285) were treated by surgical excision of the tumor, with no follow-up treatment by adjuvant therapy or chemotherapy. Nonetheless, patients harboring homozygous alleles (–617A/A) in the *NRF2* gene exhibited the best record of overall survival among the members of these three different allele groups (Figure 2B).

Potential Link between *NRF2* and *MDM2* Genotypes

NRF2 reportedly regulates expression of the *MDM2* gene that encodes a crucial negative regulator of p53. To gain insight into a potential link between *MDM2* and *NRF2* genotypes, we have analyzed the SNP (c.309T>G) in the *MDM2* gene as well as the SNP (–617C>A) in the *NRF2* gene using the genomic DNA samples from lung cancer patients. Table 6 summarizes the corresponding results, where the number of patients harboring T/T, T/G, or G/G genotype in the *MDM2* gene has been given for each genotype of the *NRF2* gene (*i.e.*, –617C/C, C/A. or A/A). In the genotype groups of –617C/C and –617C/A, the 309G (SNP)



B

WT (c.-617C)-typing primer set

FP TP/Bc BP
5'-GTGGGCCCTGCCTAGGGGAGATGTGGACAGCGCCGGCAGCTCGTGTTCGCAGTCACCCTGAAG
Exciton

TP OP
CCCTCCTCTGAACTCCCACGTGTCTCCATTCTCCTAAGCTCAGGTCGTCA-3'

SNP (c.-617A)-typing primer set

FP TP/Bc BP
5'-GTGGGCCCTGCCTAGGGGAGATGTGGACAGCTCCGGCAGCTCGTGTTCGCAGTCACCCTGAAG
Exciton

TP OP
CCCTCCTCTGAACTCCCACGTGTCTCCATTCTCCTAAGCTCAGGTCGTCA-3'

TP (-617C)	5'-CGCCGGCATGGAGACACGTGGGAGTTCAGAGG-3'
TP (-617A)	5'-GCTCCGGCTGGAGACACGTGGGAGTTCAGAGG-3'
FP (-617C)	5'- GCATTCGCTAGGGGAGATGTGGAC-3'
FP (-617A)	5'-TTTATATATATATAAACCCTGCCTAGGGGAGAT-3'
Exciton	5'-AGGACGCTGAGATGCGTCCTCTAGGGGAGAZGTGGA-3'
BP	5'-CGTGTTCGCAGTCACC-3'
OP	5'-GACGACCTGAGCTTAGGA-3'

C

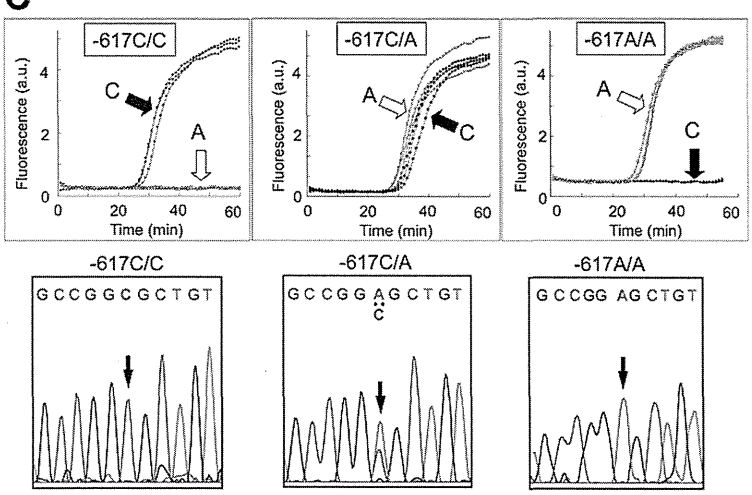


Figure 1. SmartAmp-based detection of SNP (c.–617C>A) in the *NRF2* gene. SNP (c.–617C>A) resides in the promoter region of the *NRF2* gene on chromosome 2q31.2. Panel **A** presents a schematic illustration of annealing sites of the TP, FP, and OP primers. Panel **B** shows cDNA encoding a partial sequence of the *NRF2* gene and primer annealing sites. Panel **C** depicts the results of SNP detection. a.u. = arbitrary unit.
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allele frequency was 0.606 and 0.541, respectively. In contrast, the SNP allele frequency was found to be markedly lower (0.333) in the genotype group of *NRF2* –617A/A. In the case of adenocarcinoma, female patients harboring 309T/T, T/G, and G/G genotype in the *MDM2* gene were 7, 8, and 1, respectively (Table S1); most patients were harboring the 309T (WT) allele in the *MDM2* gene.

Discussion

SNP (c.–617C>A) in the *NRF2* Gene and Female Non-smokers with Adenocarcinoma

Recent genome-wide association studies (GWAS) have identified several loci associated with lung cancer susceptibility in never-smoking women in Asia; they were, 5p15.33 (rs2736100) [30], 6p21.3 (rs3817963) [30], 3q28 (rs10937405 and rs4488809) [31], 10q25.2 (rs7086803) [32], 6q22.2 (rs9387478) [32], and 6p21.32 (rs2395185) [32].

In the present study, differing from those reports, we found that SNP (c.–617C>A; rs6721961) in the *NRF2* gene located on chromosome 2q31.2 is associated with Japanese non-smoking female patients with adenocarcinoma and their overall survival. While the allele frequency of SNP c.–617C>A in the *NRF2* gene was estimated to be 25.2%, non-smoking females harboring homozygous alleles (–617A/A) had a markedly higher incidence of adenocarcinoma (Table 4, Table 5), as compared with non-smoking males harboring the same genotype. In other words, the –617 C>A SNP in the ARE-like loci of the human *NRF2* gene seems to be associated with female non-smokers with adenocarcinoma. Furthermore, it is noteworthy that the *EGFR* gene was frequently mutated in female patients who were non-smokers and had homozygous SNP alleles (–617A/A) in the *NRF2* gene (Table 5), suggesting a potential link between the SNP homozygote (–617A/A) and *EGFR* gene mutations.

Recent studies have demonstrated that mutations in the tyrosine kinase domain of the EGFR are frequently found among non-smoker patients with NSCLC [33]. Approximately 90% of these mutations are exon 19 deletions or exon 21 L858R point mutations in the tyrosine kinase domain [34]. In the vast majority of cases, *EGFR* mutations are non-overlapping with other oncogenic mutations (e.g., *KRAS* mutations, *ALK* rearrangements) found in NSCLC [34]. A large randomized clinical study named the "IRESSA Pan-Asian Study (IPASS)" has reported that high rates of mutations in the *EGFR* gene were observed in female NSCLC patients without smoking experience [35]. A high incidence of *EGFR* gene mutations was reported in female non-smokers with adenocarcinoma of lung: 30–40% in East Asians, as compared with 15% in Caucasians [36–38]. Both *EGFR* gene mutations and homozygous SNP alleles (–617A/A) in the *NRF2* gene were frequently observed in Japanese female adenocarcinoma patients without smoking experience (Table 4). As shown in Table 7, ethnic group-dependent difference was observed in the *NRF2* genotype, where the frequency of the –617A allele is high in Japanese, Taiwanese, and Chinese populations. Thus, it is of great interest to investigate the link between the SNP homozygote (–617A/A) and *EGFR* gene mutations and to gain insight into the underlying molecular mechanism.

SNP (c.–617C>A) in the *NRF2* Gene as a Biomarker for Prognosis of Lung Cancer

The *NRF2* gene is regarded as a double-edged sword. It plays an important role in protecting normal cells from external toxic challenges and oxidative stress, whereas it can also endow cancer cells resistance to anticancer drugs. Recently it has been reported that NRF2 contributes to the malignant phenotypes of cancer cells *in vitro*, including aggressive cell proliferation, drug resistance, and metabolic re-programming [8,20]. Indeed, NRF2 activation is involved in the emergence of cancer resistance to various anticancer drugs by transcriptionally activating a battery of self-defense genes, such as those encoding antioxidant enzymes, phase

Table 2. Classification of primary lung cancer patients with respect to *NRF2* genotypes, gender, and histopathology.

	NRF2 gene SNP (–617)			
	C/C	C/A	A/A	*P*-value*
Patients	216 (55.8)	147 (38.0)	24 (6.2)	
Gender*				
Male	127 (57.5)	88 (39.8)	6 (2.7)	
Female	89 (53.6)	59 (35.5)	18 (10.8)	0.004
Histopathology				
Adeno	164 (55.0)	114 (38.3)	20 (6.7)	
Non-adeno	52 (58.4)	33 (37.0)	4 (4.5)	0.687
Smoking behavior*				
Smoker	133 (58.4)	92 (37.0)	8 (4.5)	
Non-smoker	83 (53.9)	55 (35.7)	16 (10.4)	0.021
p-Stage				
I	156 (53.4)	114 (39.0)	22 (7.5)	
II	28 (60.9)	16 (34.8)	2 (4.3)	
III	23 (65.7)	12 (34.3)	0 (0)	
IV	1 (33.3)	2 (66.7)	0 (0)	0.459

The number of patients (%).
*P- values were calculated by Fisher's exact test.
Abbreviation: Adeno, adenocarcinoma.
doi:10.1371/journal.pone.0073794.t002

Table 3. Logistic regression analysis for evaluation of the association among homozygous SNP alleles (–617A/A) in the *NRF2* gene and gender/smoking experience of lung cancer patients.

Variable	*P*	Odds Ratio	95% CI
Gender	0.041	3.48	1.05 to 11.51
Smoking	0.463	0.66	0.22 to 2.00

Abbreviation: CI, confidence interval.
Gender code: 1 = female; 0 = male.
Smoking experience code: 1 = smoker; 0 = non-smoker.
The multivariate logistic regression analysis was performed under two categories, *i.e.*, the gender (female and male) and the smoking experience (smoker and non-smoker).
doi:10.1371/journal.pone.0073794.t003

Table 4. Classification of primary lung cancer patients with respect to *NRF2* genotypes, smoking behavior, adenocarcinoma, and gender.

	NRF2 gene SNP (–617)			
	C/C	C/A	A/A	*P*-value*
Patients (M+F)	216	147	24	
Smoking behavior				
Smoker (M)	114	75	6	
Smoker (F)	19	17	2	
Non-smoker (M)	13	13	0	
Non-smoker (F)	70	42	16	0.014
Adenocarcinoma				
Smoker (M)	75	48	4	
Smoker (F)	15	16	0	
Non-smoker (M)	11	13	0	
Non-smoker (F)	63	37	16	0.003

*P-values were calculated by Fisher's exact test.
Abbreviation: M, male; F, female.
doi:10.1371/journal.pone.0073794.t004

II detoxifying enzymes, and ABC transporters [23–25]. ABCG2 is known to mediate the efflux of gefitinib (Iressa®) from cancer cells [39], and its expression is regulated by NRF2 [26] and the EGFR-tyrosine kinase cascade [40,41].

As revealed in the Kaplan-Meier plot (Figure 2), lung cancer patients (both females and males) with homozygous SNP alleles (–617A/A) in the *NRF2* gene had markedly high overall survival. Univariate analysis showed a significant difference between the –617 A/A and –617 C/A groups in terms of overall survival (log-rank $P = 0.021$). It is important to note that, except for one patient (case 5 in Table 5) who died because of primary pancreatic cancer, all of the adenocarcinoma patients with homozygous SNP alleles (–617A/A) in the *NRF2* gene survived over 1,000 days after surgical excision of the tumor that was followed up with neither adjuvant therapy nor chemotherapy, even when p-stage I patients were considered alone (Figure 2B). To our knowledge, this is the first report providing clinical evidence that homozygous SNP (–617A/A), as one of the intrinsic genetic polymorphisms in the *NRF2* gene, is associated with overall survival of lung cancer patients.

SNP –617C>A is considered to play a pivotal role in the positive feedback loop of transcriptional activation of the *NRF2* gene to regulate the NRF2 protein level (Figure 3). Since the SNP (c.–617A) in the ARE-like loci of the human *NRF2* gene decreases the binding affinity to the transcription factors of NRF2/small

Table 5. Clinicopathological profiling of 24 patients harboring homozygous SNP alleles (–617A/A) in the *NRF2* gene.

Case	Histology	p stage	Age	Gender	smoker	(GT)n repeats	CYP2A6	EGFR mutation	Gefitinib therapy
1	Ad	IIA	74	F	non-smoker	19,30	Wt	Exon 21	Yes
2	Ad	IA	53	F	non-smoker	23	*4/*4	Exon 19	–
3	Ad	IB	70	F	non-smoker	24	Wt	Exon 21	–
4	Mix	IB	63	F	non-smoker	34	Wt	Exon 21	–
†5	Ad	IB	61	F	non-smoker	23	Wt	Exon 19	–
6	Ad	IB	72	F	non-smoker	22	Wt	Exon 21	–
7	Ad	IA	40	F	non-smoker	19	Wt	Exon 19	–
8	Ad	IA	71	F	non-smoker	17	Wt	Exon 21	–
9	Ad	IA	45	F	non-smoker	30	Wt	Exon 21	–
10	Ad	IA	74	F	non-smoker	30	Wt	Exon 21	–
11	Ad	IA	73	F	non-smoker	22	Wt	Exon 19	–
12	Ad	IA	69	F	non-smoker	22	Wt	Exon 21	–
13	Ad	IA	62	F	non-smoker	28	Wt	None	–
14	Ad	IA	73	F	non-smoker	14	Wt	Exon 19	–
15	Ad	IA	63	F	non-smoker	15	Wt	Exon 19	–
16	Ad	IIA	73	F	non-smoker	30	Wt	None	Yes
17	Sq	IA	72	F	smoker	20	Wt	None	–
18	Ple	IA	75	F	smoker	29	Wt	None	–
19	Ad	IA	74	M	smoker	23,27	Wt	None	–
20	Sq	IA	78	M	smoker	23	Wt	None	–
21	Ad	IA	65	M	smoker	30	Wt	Exon 21	–
22	Sq	IB	75	M	smoker	20	Wt	None	–
23	Ad	IA	77	M	smoker	28,31	Wt	Exon 21	–
24	Ad	IA	80	M	smoker	29,30	Wt	Exon 19	–

Abbreviation: Ad, adenocarcinoma; Mix, adenocarcinoma and squamous cell carcinoma; Ple, pleomorphic carcinoma; Sq, squamous cell carcinoma; F, female; M, male; Wt, wild type.
†Patient (case 5) died because of primary pancreatic cancer.
doi:10.1371/journal.pone.0073794.t005

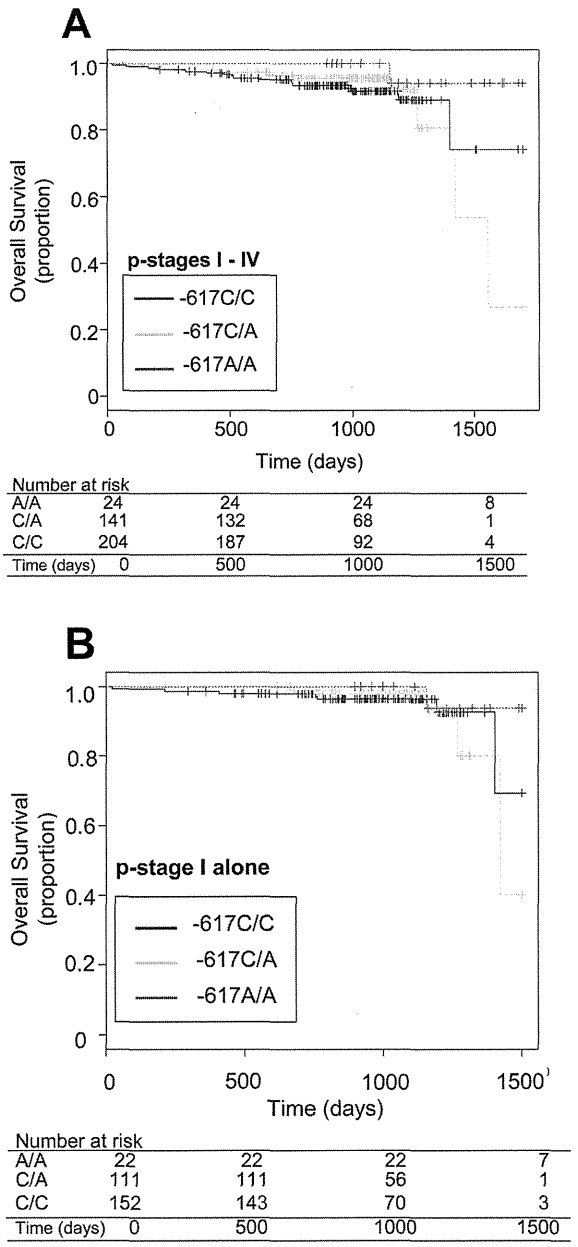

Figure 2. Kaplan-Meier plots showing the overall survival of patients harboring the WT homozygote (–617C/C), WT/SNP heterozygote (–617C/A), or SNP homozygote (–617A/A) in the *NRF2* gene. Patients with p-stages I to IV (**A**) and p-stage I only NSCLC (**B**). The number of patients at times 0, 500, 1000, or 1500 days after surgical operation is described along with genotypes of the *NRF2* gene.
doi:10.1371/journal.pone.0073794.g002

MAF [22], it is anticipated that the homozygote –617A/A significantly attenuates the positive feedback loop of transcriptional activation of the *NRF2* gene.

It has recently been reported that NRF2 regulates the basal expression of the murine double minute-2 (*Mdm2*) gene [42]. Since human MDM2 is an oncoprotein that binds to p53 protein and inactivates the tumor suppressor activity of p53 [43], NRF2 can indirectly contribute to p53-mediated cell cycle control and/or apoptosis [44]. One SNP in the *MDM2* promoter region, a T-to-G change at nucleotide c.309 (rs2279744) in the first intron, increases the binding affinity toward stimulatory protein 1 (Sp1) and results in higher expression levels of MDM2 protein [45]. This, in turn, attenuates the p53 tumor suppressor pathway and accelerates tumor formation in humans [45]. Asians, including Japanese, have higher frequencies of the 309G allele as compared with African-Americans and Caucasians [46]. It has been reported that this polymorphism in the *MDM2* gene is associated with the prognosis for several types of tumors, including lung cancer [47].

As demonstrated in Table 6, the 309G (SNP) allele frequency of the *MDM2* gene was markedly lower (0.333) in the genotype group of *NRF2* –617A/A, as compared with those observed in the genotype groups of *NRF2* –617C/C and –617C/A. It is suggested that lung cancer patients harboring both the 309T (WT) allele in the *MDM2* gene and the –617A allele in the *NRF2* gene have better prognosis owing to well-controlled tumor suppression via

Table 6. Classification of primary lung cancer patients with respect to genotypes of *NRF2* and *MDM2* genes.

	NRF2 (–617)		
	C/C	C/A	A/A
Patients (*N*)	216	147	24
MDM2 (c.309)	N (%)	N (%)	N (%)
T/T	35 (16.2)	36 (24.5)	11 (45.8)
T/G	100 (46.3)	63 (42.9)	10 (41.7)
G/G	81 (37.5)	48 (32.6)	3 (12.5)

N, the number of patients; % in parentheses.
doi:10.1371/journal.pone.0073794.t006

Table 7. Frequencies of wild type (–617C) and SNP (–617A) alleles in the *NRF2* gene among different ethnic groups.

	Allele frequency		NRF2 (–617)				
Ethnic group	C	A	C/C	C/A	A/A	N	Data source
African	0.925	0.075	0.850	0.150	0.000	246	*
African-American	0.893	0.107	0.787	0.213	0.000	61	*
European	0.883	0.117	0.778	0.208	0.013	379	*
American in Utah	0.888	0.112	0.788	0.200	0.012	85	*
American mixed	0.862	0.138	0.757	0.210	0.033	181	*
Mexican in Los Angels	0.803	0.197	0.667	0.273	0.061	66	*
Japanese	0.775	0.225	0.618	0.315	0.067	89	*
Japanese (lung cancer)	0.748	0.252	0.558	0.380	0.062	387	This study
Taiwanese	0.726	0.274	0.524	0.405	0.071	168	This study
Chinese in Beijing	0.722	0.278	0.515	0.412	0.072	97	*
Southern Han Chinese	0.710	0.290	0.500	0.420	0.080	100	*

N, the number of subjects.
*1000 Genomes. http://browser.1000genomes.org/Homo_sapiens/Variation/Population?db=core;r=2:178129537–178130537;v=rs6721961;vdb=variation;vf=4574214.
doi:10.1371/journal.pone.0073794.t007