

—Original—

# Administration of an Antioxidant Prevents Lymphoma Development in Transmitochondrial Mice Overproducing Reactive Oxygen Species

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**Abstract:** Because of the difficulty to exclude possible involvement of nuclear DNA mutations, it has been a controversial issue whether pathogenic mutations in mitochondrial DNA (mtDNA) and the resultant respiration defects are involved in tumor development. To address this issue, our previous study generated transmitochondrial mice (mito-mice-ND6<sup>13997</sup>), which possess the nuclear and mtDNA backgrounds derived from C57BL/6J (B6) strain mice except that they carry B6 mtDNA with a G13997A mutation in the *mt-Nd6* gene. Because aged mito-mice-ND6<sup>13997</sup> simultaneously showed overproduction of reactive oxygen species (ROS) in bone marrow cells and high frequency of lymphoma development, current study examined the effects of administering a ROS scavenger on the frequency of lymphoma development. We used *N*-acetylcysteine (NAC) as a ROS scavenger, and showed that NAC administration prevented lymphoma development. Moreover, its administration induced longevity in mito-mice-ND6<sup>13997</sup>. The gene expression profiles in bone marrow cells indicated the upregulation of the *FasI* gene, which can be suppressed by NAC administration. Given that natural-killer (NK) cells mediate the apoptosis of various tumor cells via enhanced expression of genes encoding apoptotic ligands including *FasI* gene, its overexpression would reflect the frequent lymphoma development in bone marrow cells. These observations suggest that continuous administration of an antioxidant would be an effective therapeutics to prevent lymphoma development enhanced by ROS overproduction.

**Key words:** antioxidant, lymphoma prevention, mouse mtDNA mutation, ROS overproduction

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## Introduction

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Mitochondrial DNA (mtDNA) mutations that induce

mitochondrial respiration defects have been proposed to be involved in aging and age-associated disorders including tumor development [13, 14, 18, 23, 24]. Moreover,

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(Received 1 May 2014 / Accepted 27 May 2014 / Published online in J-STAGE 22 July 2014)

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mitochondrial respiration defects caused by nuclear DNA mutations and the resultant enhanced glycolysis under normoxic conditions, i.e. the Warburg effect, are reported to be responsible for tumor development [4, 7, 16, 19]. Therefore, it is also possible that mitochondrial respiration defects caused by an age-associated accumulation of mtDNA mutations induce the Warburg effect via compensatory upregulation of aerobic glycolysis, resulting in tumor development. In fact, somatic mutations were preferentially accumulated in tumor mtDNA [6, 10, 21]. However, there has been no direct evidence for the involvement of mtDNA mutations in the Warburg effect and in tumor development, because of the difficulty to exclude possible involvement of nuclear DNA mutations in these processes [2].

Our previous studies [1, 11, 12] resolved this issue by the use of intercellular mtDNA transfer technology between mouse cells expressing different phenotypes related to tumors. While mtDNA introduced from tumor cells into normal cells did not induce tumorigenicity [1], mtDNA exchange between poorly and highly metastatic lung carcinoma cells provided convincing evidence that a somatic G13997A mtDNA mutation in the *mt-Nd6* gene, which encodes subunit 6 of respiration complex I (NADH dehydrogenase), reversibly controls development of highly metastatic potentials [11]. In this case, the induction of high metastasis was not due to the Warburg effect, but to overproduction of the reactive oxygen species (ROS) [12]. Subsequently, G13997A mtDNA was transferred from highly metastatic carcinoma cells into mouse female germ line [26] to examine the effect of the G13997A mtDNA on tumor-related phenotypes. The resultant transmitochondrial mice possessing only G13997A mtDNA, named mito-mice-ND6<sup>13997</sup>, showed high frequency of lymphoma formation with aging [9], providing convincing evidence for the involvement of the mtDNA mutations in tumor development.

Based on these observations, this study examined whether continuous administration of an antioxidant prevents lymphoma development, and corresponds to an effective therapeutics to protect lymphoma development in mito-mice-ND6<sup>13997</sup>. We also examined the mechanisms of how ROS induce lymphoma formation in mito-mice-ND6<sup>13997</sup>.

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## Materials and Methods

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### *Ethical statement*

All animal experiments were performed in accordance with protocols approved by the experimental animal committee of the University of Tsukuba, Japan.

### *Mice*

Old inbred C57BL/6J Jcl (B6) mice were obtained from CLEA Japan. Mito-mice-ND6<sup>13997</sup> were generated in our previous work [26]. We maintained B6 mice and mito-mice-ND6<sup>13997</sup> sharing a common nuclear DNA background by repeated backcrossing of their females with B6 males. Animals were housed in groups of up to 5 in individually ventilated cages under standard conditions (22°C, 12 h light–dark cycle) receiving food and water *ad libitum*. Male mice were used in the experiments, and were monitored everyday for general health and signs of tumor burden such as hunched postures, ruffled coats and respiratory distress were humanely killed. Moribund mice were euthanized by cervical dislocation under general anesthesia (Avertin, 1.25%, 0.2 ml/20 g body weight, intraperitoneally).

### *NAC administration*

At 15 months old, sixteen mito-mice-ND6<sup>13997</sup> were divided at random into two groups. One group was given drinking water containing 10 g/l of NAC (SIGMA), and another group was given regular water. NAC supplemented water and regular water were prepared fresh everyday.

### *Histological analyses*

Formalin-fixed, paraffin-embedded serial sections (10  $\mu$ m) were used for histological analyses. Hematoxylin-and-eosin–stained sections were used for histopathological analysis to identify tumor tissues. The immunohistological analysis was performed with antibody to CD45 to determine whether the tumor tissues originated from leukocytes, and subsequently with antibodies to B220 and CD3 to determine whether the tumor tissues were of B-cell or T-cell origin, respectively. Deparaffinized slides were boiled for antigen retrieval, then incubated with rat anti-mouse CD45 (clone 30-F11; BD Biosciences, Cat. No. 550539) at a dilution of 1:200 or rat anti-mouse B220 (clone RA3-6B2; BD Biosciences, Cat. No. 553085) at a dilution of 1:50 or goat anti-mouse CD3 (clone M-20; Santa Cruz, Cat. No. sc-1127) at a

dilution of 1:50. Biotin-conjugated goat anti-rat IgG (BD Biosciences, Cat. No. 559286) and rabbit anti-goat IgG (Vector Laboratories, Cat. No. BA-5000) were used as secondary antibody at a dilution of 1:50 and 1:500, respectively. Detection of CD45, B220 and CD3 were performed using avidin-biotin complex methodologies (Vectastain Elite ABC Kit, Vector Laboratories) with DAB staining (Anti-Ig HRP Detection Kit, BD Pharmingen). Sections were counterstained with hematoxylin.

#### PCR array analysis

Mice were euthanized after 4-week administration of NAC and bone marrow cells were isolated from mice thighbones and shinbones. Total RNA was extracted by ISOGEN (Nippon Gene) from mouse bone marrow cells. RNA samples were subjected to DNase I treatment (Invitrogen) to eliminate DNA contaminants and reverse transcribed using Oligo (dT)<sub>12-18</sub> primer, 10 mM dNTP Mix, 0.1 M DTT, RNase Out Recombinant Ribonuclease Inhibitor, and SuperScript II-Reverse Transcriptase (Invitrogen). cDNA samples were subjected to RNase H treatment (Invitrogen), and applied according to the manufacturer's protocol to a RT<sup>2</sup> profiler PCR array real-time PCR reaction. Real-time monitoring PCR was performed with SYBR Green PCR Master Mix (QIAGEN) and an ABI PRISM 7900HT sequence detection system (Applied Biosystems). Mouse Cancer Pathway Finder RT<sup>2</sup> Profiler PCR Array PAMM-033Z (SABiosciences) was performed to profile the expression of 84 genes related to the cancer pathway (n=3). The expression profiling was performed using  $\Delta\Delta\text{Ct}$  methods according to manufacturer's protocols. The relative expression level for each gene was represented as cycle threshold (Ct). Normalized expression level was calculated as  $\Delta\text{Ct} = \text{Ct}(\text{Gene of interest}) - \text{Ct}(\text{control})$ . The average  $\Delta\text{Ct}$  from three mice was calculated as the relative expression level of each gene. Differential expression was calculated as  $\Delta\Delta\text{Ct} = \text{Ave } \Delta\text{Ct}(\text{sample mice}) - \text{Ave } \Delta\text{Ct}(\text{control mice})$ . Fold change was calculated as  $2^{-\Delta\Delta\text{Ct}}$ . The *P* values are calculated based on a Student *t*-test of the replicate  $2^{-\Delta\text{Ct}}$  values for each gene in the control group and treatment groups.

#### Statistical analysis

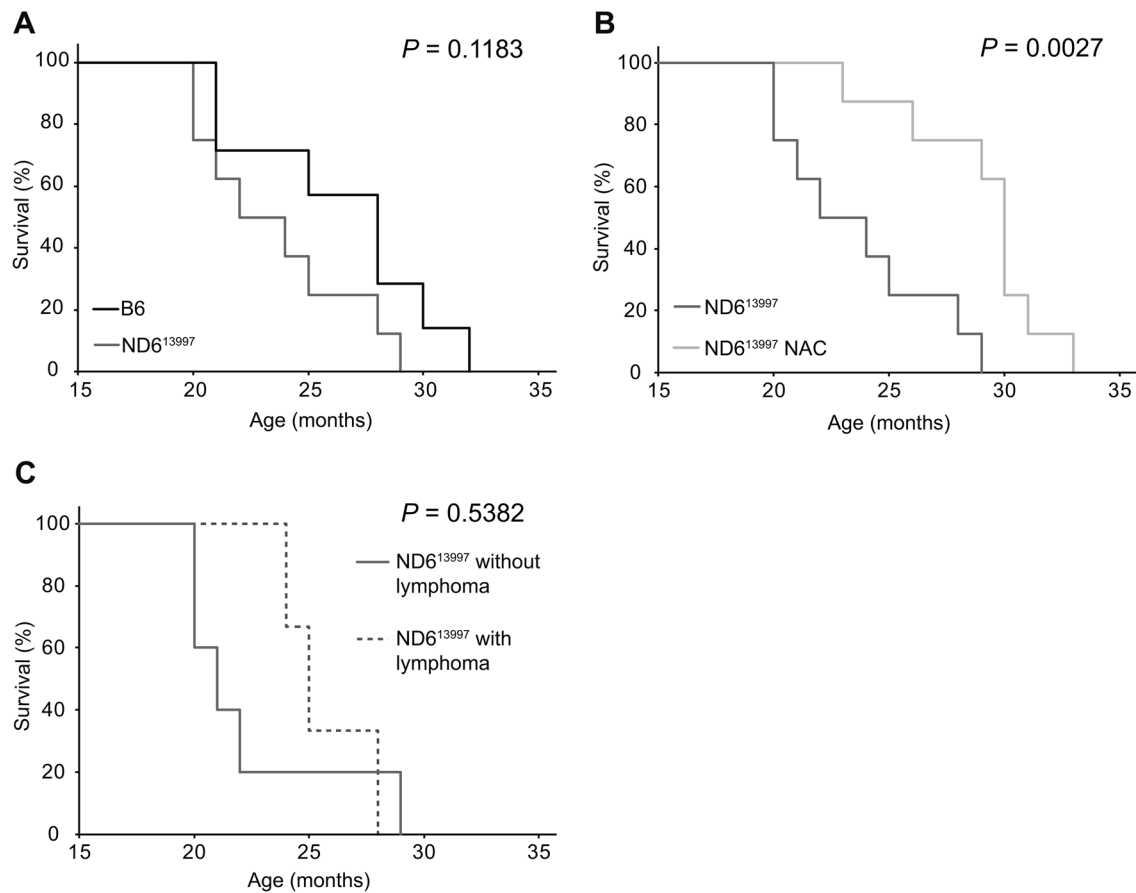
We analyzed data with the Student's *t*-test. Kaplan-Meier curves were assessed with the log-rank test. Values with *P*<0.05 were considered significant.

## Results

This study examined the idea that the frequent lymphoma development in the aged male mito-mice-ND6<sup>13997</sup> could be prevented by the administration of an antioxidant, if ROS overproduction is responsible for the lymphoma development. Since most mito-mice-ND6<sup>13997</sup> began to develop lymphoma more than 18 months after the birth [9], we used 15-month-old males for continuous administration of *N*-acetylcysteine (NAC) as a ROS scavenger. Eight of 16 mito-mice-ND6<sup>13997</sup> were treated with NAC from 15 months after the birth by oral administration based on the procedure reported previously [5]. The remaining eight mito-mice-ND6<sup>13997</sup> were not treated with NAC. Seven 15-month-old B6 males untreated with NAC were used as controls.

First, we examined whether the NAC treatment would affect the frequency of lymphoma development and the lifespan. Median survival times of NAC-treated mito-mice-ND6<sup>13997</sup>, untreated mito-mice-ND6<sup>13997</sup> and untreated B6 mice were 29.3, 22.5 and 25.8 months, respectively (Figs. 1A and B). No statistical significance of median survival times was observed between untreated mito-mice-ND6<sup>13997</sup> and untreated B6 mice (Fig. 1A). These results are consistent with our previous observations [9]. In contrast, NAC-treated mito-mice-ND6<sup>13997</sup> showed longer survival times than untreated mito-mice-ND6<sup>13997</sup> with statistical significance (Fig. 1B), while no statistical significance was present between NAC-treated mito-mice-ND6<sup>13997</sup> and untreated B6 mice (Figs. 1A and B). The median survival times of untreated mito-mice-ND6<sup>13997</sup> with and without lymphoma-like abnormalities were 24.5 and 20.5, respectively, and were not different with statistical significance (Fig. 1C).

Gross necropsy of all dead or euthanized moribund mice showed that three of eight mito-mice-ND6<sup>13997</sup> (38%) and one of seven B6 mice (14%) had macroscopic lymphoma-like abnormalities, including enlarged spleen, liver, and nodular tumors, but none of eight NAC-treated mito-mice-ND6<sup>13997</sup> had these abnormalities (Table 1). Histological analyses of abnormal tissues revealed that all were hematopoietic neoplasms and positive for the pan-leukocyte marker CD45 (Table 1 and Fig. 2). These observations suggest that these hematological neoplasms may correspond to lymphoma cells, although current study did not examine copy-number variations (CNVs) in nuclear genomes to show chromosomal instability [9].



**Fig. 1.** Kaplan-Meier survival curves of mito-miceND6<sup>13997</sup>. (A) Comparison of lifespans between B6 mice and mito-miceND6<sup>13997</sup>. Median survival times of B6 mice (n=7) and mito-miceND6<sup>13997</sup>(n=8) were 25.8 and 22.0 months, respectively. (B) Comparison of lifespans of between NAC-treated and untreated mito-miceND6<sup>13997</sup>. NAC administration was started from 15 months after the birth to the end of their lives. Median survival times of mito-miceND6<sup>13997</sup>(n=8) and mito-miceND6<sup>13997</sup> treated with NAC (n=8) were 22.0 and 29.3 months, respectively. (C) Comparison of lifespans between mito-miceND6<sup>13997</sup> that died with and without detectable lymphoma. Median survival times of mito-miceND6<sup>13997</sup> with detectable lymphoma (n=3) and mito-miceND6<sup>13997</sup> without detectable lymphoma (n=5) were 24.5 and 20.5 months, respectively.

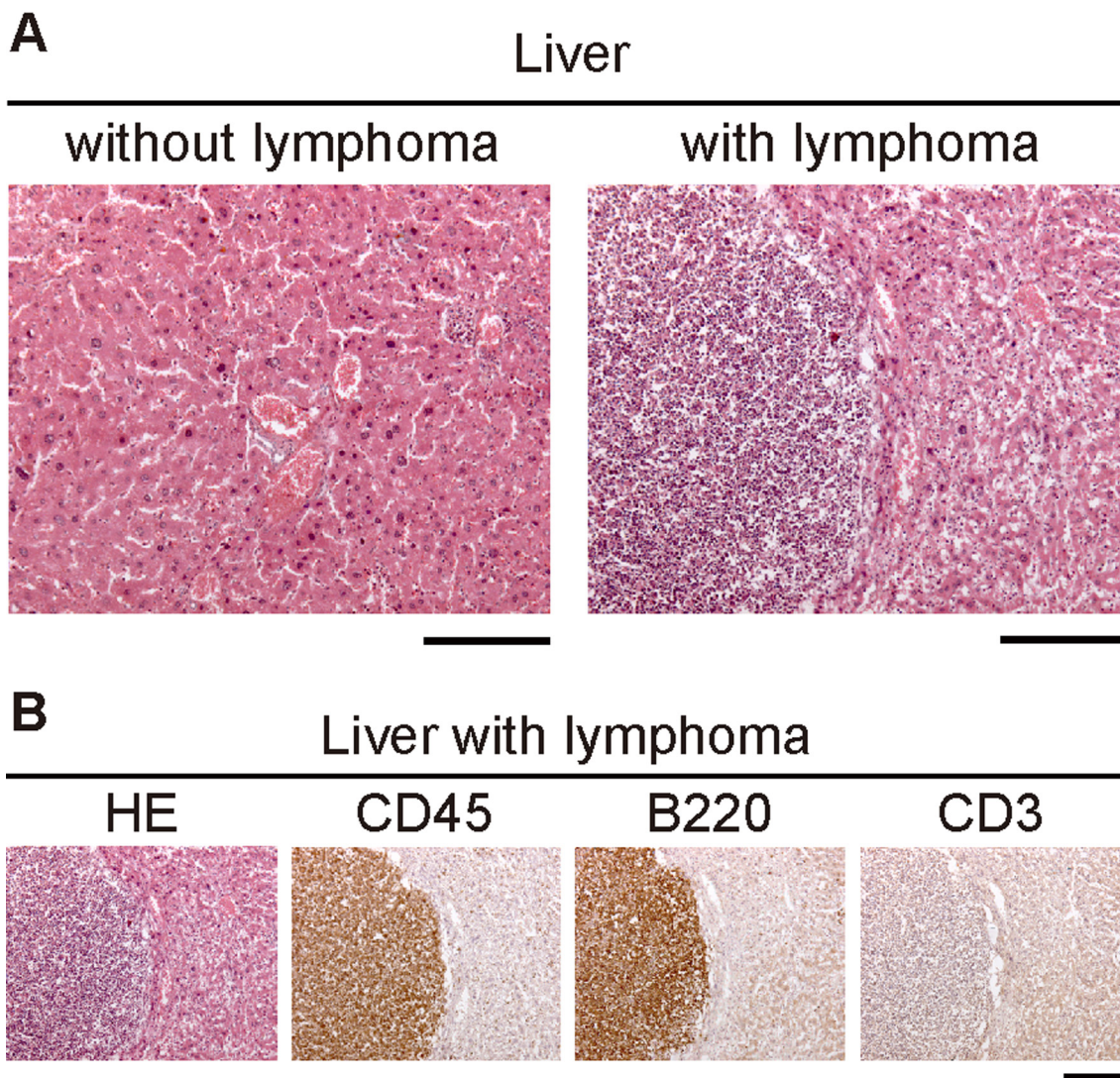
**Table 1.** Frequencies of lymphoma development in mice

Mouse strains	No. of mice	No. of mice with tumor	Tissues with tumor			Histological analysis			Cell lineage
			Spleen	Liver	Lymph node	CD45	B220	CD3	
B6	7	1	+	+	+	+	+	-	B-cell
Mito-mice-ND6 <sup>13997</sup>	8	3	+	-	+	+	+	-	B-cell
			+	-	-	+	-	+	T-cell
			+	+	+	+	+	-	B-cell
Mito-mice-ND6 <sup>13997</sup> (NAC-treated)	8	0							

Three tumors were of B-cell origin, expressing the B-cell maker B220, while one mito-mouse-ND6<sup>13997</sup> developed T-cell lymphoma staining positive for the T-cell marker CD3 (Table 1). These data indicate that NAC administra-

tion is an effective therapeutics to prevent lymphoma development caused by ROS overproduction. However, further works using more numbers of animals than those used in this study are required to confirm this idea.

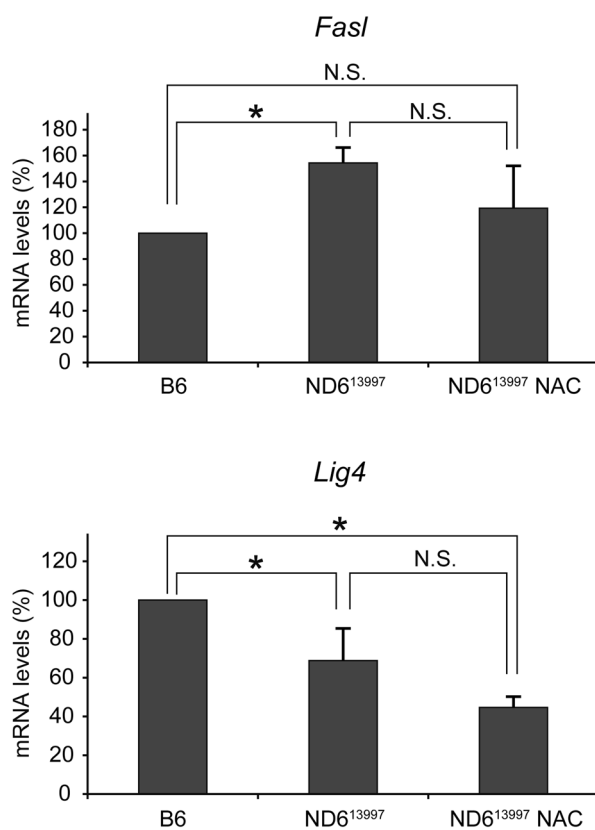




**Fig. 2.** Histological analyses for identification of lymphoma cells in the liver from mito-miceND6<sup>13997</sup>. (A) Hematoxylin and eosin (HE) staining of the liver sections from mito-miceND6<sup>13997</sup>. HE staining of the liver sections was carried out to identify tumor-like abnormalities in the liver. Left and right panels represent normal liver and liver with tumor-like abnormalities, respectively. (B) Histological analysis of serial sections of the liver with tumor-like abnormalities. HE, hematoxylin-eosin staining to show tumor formation; CD45, immunohistochemistry using antibody to CD45 to detect leukocytes; B220, immunohistochemistry using antibody to B220 to detect B cells; CD3, immunohistochemistry using antibody to CD3 to detect T cells. Because this tissue was stained positively with CD45 and B220, but not with CD3, the results represent abnormal growth of B cell-lymphoma, but not T cell-lymphoma, in the liver. (Scale bars, 200  $\mu$ m).

Then, we examined the mechanism of frequent lymphoma development, which can be prevented by administration of an antioxidant to mito-mouse-ND6<sup>13997</sup>. Our previous study showed that no tumors other than lymphoma were developed, and that ROS were overproduced in bone marrow cells but not in splenocytes [9]. It is therefore likely that the ROS overproduction in the bone marrow cells of mito-mouse-ND6<sup>13997</sup> is crucial

for lymphoma development. Thus, we used bone marrow cells to identify genes that would be responsible for frequent lymphoma development in mito-mice-ND6<sup>13997</sup>, and compared the expression of the genes related to cancer pathway (transformation and tumorigenesis) between mito-mice-ND6<sup>13997</sup> and B6 mice using PCR array (Mouse Cancer Pathway Finder RT<sup>2</sup> Profiler PCR Array). Because most mito-mice-ND6<sup>13997</sup> began to develop



**Fig. 3.** Cancer related gene expression profiles of bone marrow cells. RT<sup>2</sup> Profiler mouse cancer pathway finder PCR array was performed on bone marrow cells. Two genes of 84 cancer pathway-related genes, *FasI* and *Lig4*, were differentially expressed between bone marrow cells of B6 mice and mito-mice-ND6<sup>13997</sup>. However, only the *FasI* gene expression was reversibly regulated to normal levels by administration of NAC. Values were normalized to a pool of housekeeping genes on the array by  $\Delta\Delta C_t$  and are reported as the fold change in gene expression relative to healthy B6 bone marrow cells. Data are represented as mean values with SD (n=3). \*,  $P < 0.05$ .

lymphoma 18 months after the birth, we used 15-month-old male mice for this experiment to exclude the influence of the developed lymphoma cells.

Out of 84 cancer pathway-related genes represented on Mouse Cancer Pathway Finder RT<sup>2</sup> Profiler PCR Array (Supplemental table 1), only two genes, *FasI* and *Lig4* related to apoptosis and DNA repair, respectively, were selected as genes being differentially expressed between bone marrow cells of B6 and mito-mice-ND6<sup>13997</sup> (Fig. 3). Then, we examined whether expression levels of these genes can be changed to the levels of B6 mice by administration of NAC for 4 weeks to mito-mice-ND6<sup>13997</sup>. The results showed that *FasI* overexpres-

sion in mito-mice-ND6<sup>13997</sup> was suppressed to B6 levels by NAC administration, while suppressed expression of *Lig4* levels did not restore to B6 levels (Fig. 3). These results indicated the association of *FasI* overexpression to frequent development of lymphoma in mito-mice-ND6<sup>13997</sup>.

## Discussion

This study addressed two important issues of whether treatment of the mito-mice-ND6<sup>13997</sup> with an antioxidant could be an effective therapeutics to prevent lymphoma development mito-mice-ND6<sup>13997</sup>, and how ROS overproduction caused by G13997A mtDNA introduced from highly metastatic lung carcinoma cells induces lymphoma development in mito-mice-ND6<sup>13997</sup>.

Concerning the first issue on the therapeutics of lymphoma development, the results in this study showed that administration of NAC, one of the frequently used antioxidants, is very effective to prevent lymphoma development in mito-mice-ND6<sup>13997</sup>. Our previous study proposed the idea that ROS overproduction, but not lactate overproduction, is responsible for frequent development of lymphoma in mito-mice-ND6<sup>13997</sup> with G13997A mtDNA based on the observations that lymphoma was not developed in different mito-mice-COI<sup>6589</sup> that showed lactate overproduction but did not show ROS overproduction [9]. If this idea is correct, it can be predicted that administration of the antioxidants would prevent lymphoma development in mito-mice-ND6<sup>13997</sup>. Current study examined this prediction and showed that NAC administration is effective to prevent lymphoma development caused by ROS in mito-mice-ND6<sup>13997</sup>. However, further works are required to show that NAC administration prevents ROS overproduction in bone marrow cells of mito-mice-ND6<sup>13997</sup>.

While NAC administration prevents lymphoma development (Table 1), it also induced longevity in mito-mice-ND6<sup>13997</sup> (Fig. 1B). Median survival times of B6 mice, mito-mice-ND6<sup>13997</sup>, and NAC-treated mito-mice-ND6<sup>13997</sup> were 25.8, 22.0, and 29.3, respectively (Figs. 1A and B). The longer median survival times of NAC-treated mito-mice-ND6<sup>13997</sup> would not be due to the prevention of lymphoma formation by NAC administration, because the median survival times of untreated mito-mice-ND6<sup>13997</sup> with and without lymphoma were 24.5 and 20.5, respectively, and were not different with statistical significance (Fig. 1C). Since it appears to be

controversial whether oxidative stress suppresses longevity or not [20, 25], we could not at present explain why NAC administration induced longevity in mito-mice-ND6<sup>13997</sup>. To examine this issue, we have to carry out NAC administration not only to mito-mice-ND6<sup>13997</sup> but also to B6 mice using more numbers of animals.

Concerning the second issue of how ROS overproduction caused by G13997A mtDNA induced lymphoma development, this study showed that overexpression of *Fasl* is related the process, because its overexpression in mito-mice-ND6<sup>13997</sup> was suppressed to normal levels by NAC administration (Fig. 3). Given that natural-killer (NK) cells mediate the apoptosis of various tumor cells via the expression of genes encoding tumor necrosis factor-family ligands including the *Fasl* gene [22], it would not be conceivable that its overexpression is responsible for lymphoma development. Probably, overexpression of the *Fasl* gene in NK cells simply reflects the progress of the frequent lymphoma development in bone marrow cells of mito-mice-ND6<sup>13997</sup>. Therefore, we could not at present explain the mechanisms of how ROS overproduction induces frequent lymphoma development in aged mito-mice-ND6<sup>13997</sup>.

Then, a question is why mito-mice-ND6<sup>13997</sup> preferentially develop lymphoma [9] (Table 1), even though ROS induces various types of cellular damages, leading to genomic instability that can result in the development of various types of tumors [15]. One answer to this question would be that nuclear genetic background of B6 strain we used for generation of mito-mice-ND6<sup>13997</sup> is prone to develop lymphoma [3, 8, 17]. Based on these observations, we would like to propose an idea that G13997A mtDNA alone would not induce lymphoma development in the absence of B6 nuclear genetic background. To examine this idea, we are going to generate new mito-mice-ND6<sup>13997</sup> carrying G13997A mtDNA but carrying different nuclear genetic background derived from different mouse strains that are not prone to develop lymphoma by backcrossing female mito-mice-ND6<sup>13997</sup> to males from different mouse strains.

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### Acknowledgments

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This work was supported by Grants-in-Aid for Scientific Research A 25250011 (to J.-I.H.), Scientific Research A 23240058 (to K.N.), and Scientific Research on Innovative Areas 24117503 (to J.-I.H.) from the Japan Society for the Promotion of Science. This work was supported

also by the World Premier International Research Center Initiative; Ministry of Education, Culture, Sports, Science and Technology–Japan (to K.N. and J.-I.H.).

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