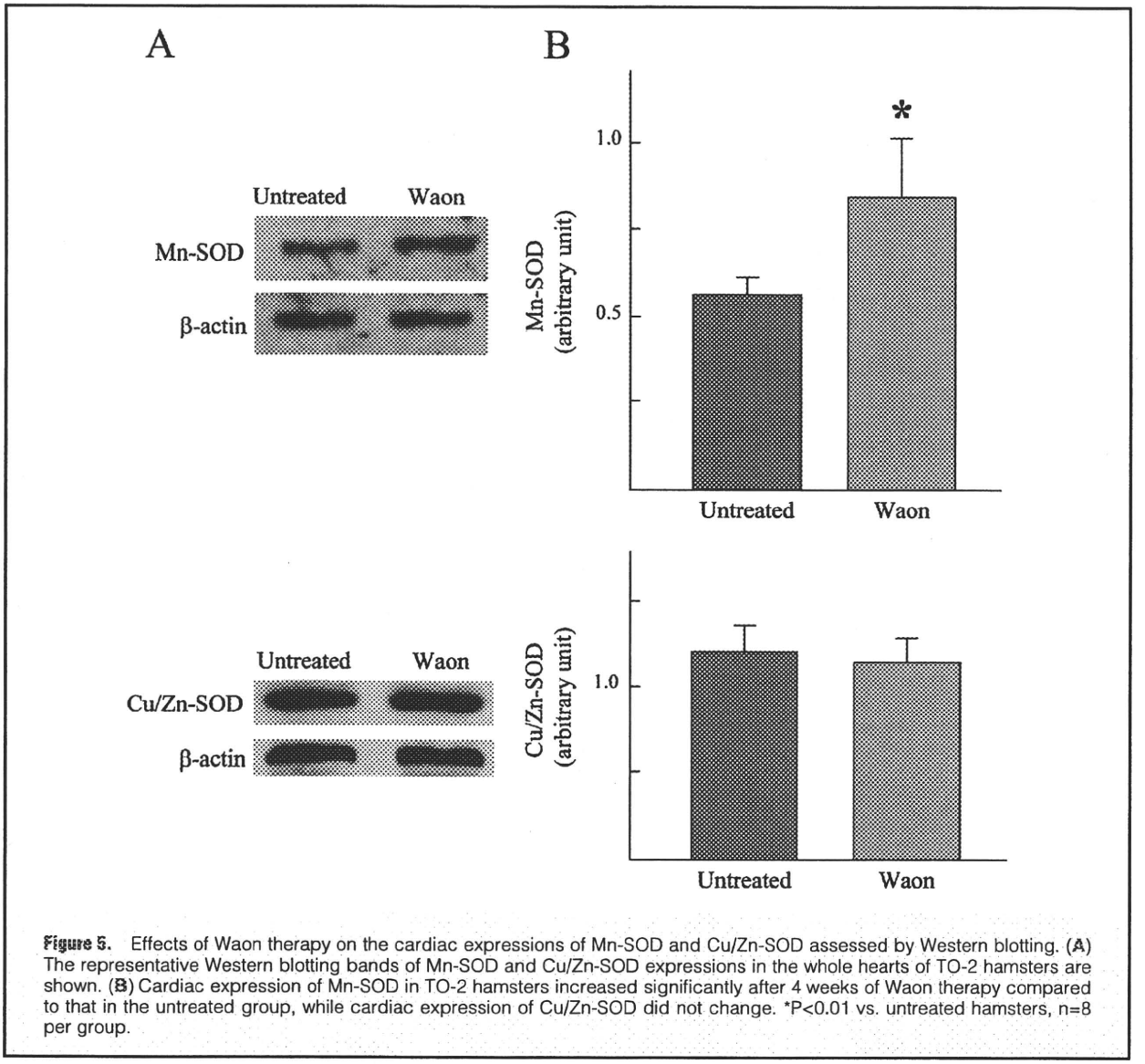

Figure 5. Effects of Waon therapy on the cardiac expressions of Mn-SOD and Cu/Zn-SOD assessed by Western blotting. (**A**) The representative Western blotting bands of Mn-SOD and Cu/Zn-SOD expressions in the whole hearts of TO-2 hamsters are shown. (**B**) Cardiac expression of Mn-SOD in TO-2 hamsters increased significantly after 4 weeks of Waon therapy compared to that in the untreated group, while cardiac expression of Cu/Zn-SOD did not change. *P<0.01 vs. untreated hamsters, n=8 per group.

Plasma Concentrations of Nitrate and Nitrite The nitric oxide metabolites, nitrate and nitrite, were measured at baseline and 4 weeks after treatment (Figure 2). The plasma concentration of nitric oxide metabolites increased significantly in the Waon therapy group, but did not change in the control group (Waon therapy group, 71.2±35.4 to 92.0±40.5 μmol/L, P<0.05; control group, 96.2±90.2 to 77.4±43.5 μmol/L, P=0.23). There was no significant difference between the 2 groups at baseline (P=0.26).

Animal Experiments

Effect of Waon Therapy on Cardiac Function In order to examine the effect of Waon therapy on oxidative stress in CHF, animal experiments using TO-2 cardiomyopathic hamsters were performed. First of all, the effect of Waon therapy on cardiac function in TO-2 hamsters was confirmed. Waon therapy significantly increased the LV +dP/dt of TO-2 hamsters compared to untreated hamsters (LV +dP/dt: Waon therapy group, 5,880±1,640 vs. untreated group, 4,180±660 mmHg/s, P<0.01, n=11 per group, %FS: Waon therapy group, 23.3±4.3 vs. untreated group, 16.5±4.2%, P<0.01, n=11 per group).

Effect of Waon Therapy on Oxidative Stress Immunohistochemistry using 4-HNE antibody, which is a marker of oxidative stress, was performed to analyze the effect of Waon therapy on oxidative stress in the failing heart. Cardiac 4-HNE immunoreactivities were lower in TO-2 hamsters following 4-week Waon therapy than in untreated hamsters (Figure 3A), which indicates that Waon therapy decreases oxidative stress in the failing heart.

ELISA of 4-HNE also revealed that 4-week Waon therapy decreased the concentration of cardiac 4-HNE of TO-2 hamsters significantly compared to untreated hamsters (Waon therapy group, 3.57±0.96 vs. untreated group, 5.53±1.17 μg/ml, P<0.05, n=8 per group, Figure 3B).

Effect of Waon Therapy on HSP27 and HSP32 Expressions The representative Western blotting bands of HSP27 and HSP32 expression in the whole hearts of TO-2 hamsters are shown in Figure 4. Cardiac expressions of HSP27 and 32 were significantly upregulated in hamsters treated with 4-week

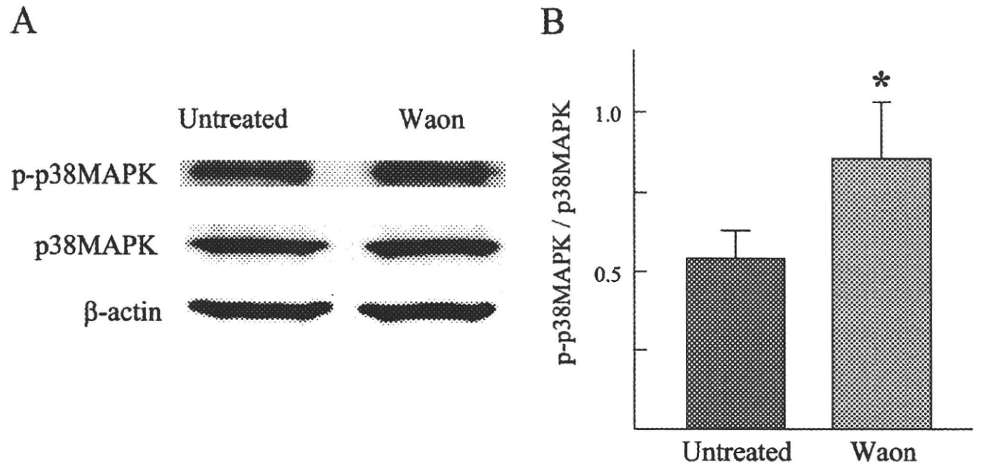

Figure 5. Effects of Waon therapy on the activation of cardiac p38MAPK signaling assessed by Western blotting. (A) The representative Western blotting bands of p38MAPK and p-p38MAPK expressions in the whole hearts of TO-2 hamsters are shown. (B) Cardiac expression of p-p38MAPK in TO-2 hamsters increased after 4 weeks of Waon therapy compared to that in the untreated group, while cardiac expression of p38MAPK did not change. The ratio of p-p38MAPK for p38MAPK in TO-2 hamsters was significantly higher in Waon therapy group than that in the untreated group. *P<0.01 vs. untreated hamsters, n=8 per group.

Waon therapy compared to untreated hamsters (HSP27, Waon therapy group, 1.25±0.30 vs. untreated group, 0.27±0.16 arbitrary units, n=8 per group, P<0.01; HSP32, Waon therapy group, 0.83±0.18 vs. untreated group, 0.54±0.12 arbitrary units, n=8 per group, P<0.01).

Effect of Waon Therapy on SOD Expression Cardiac expression of Mn-SOD was significantly upregulated in hamsters treated with 4-week Waon therapy compared to untreated hamsters (Waon therapy group, 0.81±0.20 vs. untreated group, 0.52±0.16 arbitrary units, n=8 per group, P<0.01; Figure 5). However, cardiac expression of Cu/Zn-SOD was not changed by Waon therapy (Cu/Zn-SOD, Waon therapy group, 1.14± 0.08 vs. untreated group, 1.21±0.16 arbitrary units, n=8 per group, P=0.29, Figure 5).

Effect of Waon Therapy on the Activation of p38MAPK Waon therapy did not change cardiac expression of p38MAPK. However, cardiac expression of p-p38MAPK was upregulated in hamsters treated with 4-week Waon therapy compared to untreated hamsters. The ratio of p-p38MAPK for p38MAPK in TO-2 hamsters was significantly higher in the Waon therapy group than that in the untreated group. (Waon therapy group, 0.84±0.18 vs. untreated group, 0.53±0.14 arbitrary units, n=8 per group, P<0.01; Figure 6.)

Discussion

The present clinical study demonstrated that 4 weeks of Waon therapy improved cardiac function, decreased plasma hydroperoxide concentrations and increased plasma nitrite and nitrate concentrations. In addition, 4 weeks of Waon therapy improved cardiac function and decreased oxidative stress in failing hearts of TO-2 hamsters. Furthermore, it appears that Waon therapy reduces the oxidative stress through HSP27, HSP32 and Mn-SOD.

Oxidative stress is implicated in the pathogenesis of heart failure. Reactive oxygen species (ROS) are produced in the failing myocardium, and ROS causes progression of heart failure.[37] Increased ROS in CHF impairs vascular endothelial function, which is represented by the endothelium-dependent vasodilatory response, through decreased NO bioavailability induced by decreased eNOS activity, including decreased eNOS expression and increased eNOS uncoupling.[38–43] As vascular endothelial function is one of the most important factors affecting clinical symptoms in CHF, therapy that improves vascular endothelial dysfunction is considered to be an ideal treatment for CHF. Furthermore, increased oxidative stress induces apoptosis of cardiomyocytes, resulting in further impairment of the failing myocardium.[37] Therefore, therapies that decrease oxidative stress are important to improve vascular endothelial function and cardiac function in CHF.

SOD is well known as a key anti-oxidant enzyme, and some kinds of HSP, such as HSP27 and HSP32, reduce oxidative stress.[33–37] It has been reported that overexpression of HSP27 attenuated doxorubicin-induced cardiac dysfunction through the decreases of oxidative stress and apoptosis in hearts of HSP27 transgenic mice.[44] We demonstrated that Waon therapy upregulated the cardiac expressions of HSP27 and Mn-SOD in TO-2 cardiomyopathic hamsters. In addition, Waon therapy was shown to increase cardiac expression of HSP32 in TO-2 hamsters. HSP32 is also known as Heme Oxygenase-1, and it plays a role in cellular protection against injury caused by ROS. HSP32 degrades the pro-oxidant heme and catalyzes it into biliverdin and bilirubin, which function as anti-oxidants.[35,36] Hearts of heterozygous HSP32 knockout mice, subjected to ischemia/reperfusion injury, had increased oxidative stress and infarct size compared to wild type mice.[45] In contrast, hearts of HSP32 transgenic mice had reduced oxidative stress and infarct size compared to wild type mice when they were subjected to ischemia/reperfusion injury.[46] These results clarified the cardio-protective effect of HSP32. Given the results of the clinical study and the animal experiments presented in this paper, the increases of HSP27, Mn-SOD and HSP32 by Waon therapy appear to reduce oxidative stress and improve cardiac function in CHF.

It is reported that whole-body hyperthermia with 15 min

42°C hot water bathing increases cardiac Mn-SOD through the production of TNF-α and IL-1β of normal rats.[47] TNF-α activates the translocation of NF-κB and increases Mn-SOD.[48] However, Waon therapy did not modulate the cardiac expression of TNF-α and NF-κB signaling in TO-2 hamsters with heart failure (data not shown). We think that TNF-α and NF-κB have already increased in heart failure, therefore, the mechanisms by which Waon therapy increases Mn-SOD in failing hearts might not involve TNF-α/NF-κB pathway. It is reported that p38MAPK is activated by several stress factors, and is involved in the induction of HSP and Mn-SOD.[49] In this study, we demonstrated that Waon therapy increased cardiac p-p38MAPK in TO-2 hamsters with heart failure. We believe that Waon therapy activates p38MAPK signaling, which leads to the induction of HSP and Mn-SOD.

Oxidative stress is also involved in the pathogenesis of arteriosclerosis and major cardiovascular diseases.[50] We reported that Waon therapy for 2 weeks improved impaired vascular endothelial function in the setting of atherosclerotic risk factors, such as hypertension, hypercholesterolemia, diabetes mellitus, obesity, and smoking.[12] In addition, we demonstrated that 2 weeks of Waon therapy significantly decreased urinary 8-epi-PGF2α levels in patients with at least 1 atherosclerotic risk factor, when compared to those of patients who did not undergo Waon therapy.[13] Thus, Waon therapy reduces oxidative stress and improves vascular function in patients with atherosclerotic risk factors.

Although the plasma concentrations of nitrite and nitrate were significantly lower in patients with atrial fibrillation than in the control subjects,[51] there is no significant difference in the incidence of atrial fibrillation between 2 groups in the present study.

There are limitations in this study. It is difficult to get rid of the bias in data, because this study is not blind or a cross over test.

Conclusion

Waon therapy decreases oxidative stress and is an innovative non-pharmacological therapy for patients with CHF.

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A novel algorithm from personal genome to the pathogenic mutant causing mitochondrial cardiomyopathy

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Key words for each page

Page 4, personal genome, novel algorithm,

Page 5, genetic background, mitochondrial cardiomyopathy, oxidative phosphorylation,

Page 6, phenotype, heteroplasmy, whole genome,

Page 7, gene screening, microarray, revised Cambridge resequencing system (rCRS),

Page 8, pathogenic mutant, open reading frame (ORF),

Page 9, mitochondrial genome, tRNA,

Page 10, higher structure, transcript, transgene,

Page 11, mitochondriosis, electron microscopy,

Page 12, magnetic resonance spectroscopy, risk factor, comprehensive analysis.

Abstract

Amazing progresses in both human genome analysis and bioinformatics *in silico* have made it possible to reach whole genome profiling in a short period with a reasonable cost and time. In this review, we have introduced the next step after reading the full genome sequence of both nuclear and mitochondrial genomes to identify the pathogenic site(s) in several cardiomyopathies. Considering ~3 million sites of SNP per person, it is difficult to reach not a personal variant but a pathogenic site. The current algorithm might be promising for the identification of responsible gene, even in the case of polygenic nature.

Introduction

Human whole genome was reported just 10 years before and the aim of next decades is addressed to the clinical translation of personal genetic background of each patient, searching for the precise mechanism of pathogenicity, gene counseling and/or tailored medicine to provide most suitable option for the treatment (1). For the assessment of genetic origin of heart failure and/or DCM, mitochondrial (mt) genome presents one of the most informative and cost-effective research, because of the *i*) the abundant rate of exons over introns, not like a nuclear genome, *ii*) short genome size to determine the whole DNA sequence (2) to profile for the progression of various diseases (3), *iii*) repeating the beating throughout life with consuming and producing huge amount of ATP in their own cells and *iv*) continuous exposure to reactive oxygen species (ROS) produced in the oxidative phosphorylation with much less protective actions than nuclear genome.

Mt-genome includes abundant variants not related to the pathogenicity but reflecting the haplogroup or phylogeny to adopt extracellular environment (1). Accordingly, mt-genome study is so meaningful and fascinating but it includes widespread problems, as follows. *i*) Ethical conflicts originated in a disclosure of patient's privacy (2), *ii*) Methodological arguments to sample considerable amount of living human cardiomyo-

cytes to evaluate the mutant's phenotype, *iii*) Changes in the hetroplasmy rate during tissue culture, as is convenient for the analysis and the amplification (3), *iv*) Environmental difference of cardiomyocytes *in situ* under mechanical and/or chemical stress(es) from cultured cells *in vitro*, and the resultant modification of the phenotype and *v*) The case with no identical variant in rodent to prepare transgenic models (4) and *vi*) Intrinsic problems to patents and licensing (5). In this short review, we present a new scheme to overcome these dilemmas and to clarify the pathogenic mechanism of various mt-disease, based on abundant source of bioinformatics *in silico*.

An algorithm to reach the pathogenic mutant in mtCM

1) Necessity for the full-sequencing of mt-Genome

In Out-patient section, the Tokyo University Hospital, we have followed ~80 cases with HCM and DCM, of which diagnosis was based on clinical and laboratory data including morphological, physiological, biochemical, serological and the most-importantly pathological characteristics of endomyocardial biopsy samples (6). For the conventional measurement of gene survey, we have employed gene polymorphism using PCR (polymerase chain reaction)-amplified SSCP (single stranded conformation polymorphism) or RFLP (restriction fragment length polymorphism, Fig. 1) for ~15

years. In the recent 5 years, we have shifted more time-saving and accurate modality, sequence- specific primer cycle elongation-fluorescence correlation spectroscopy (SSPCE-FCS) as described previously (7). As candidate genes, we have selected several variants popular in Japan (8) and detected 3 pedigrees with the identical mutations (9).

Because the classic methods to utilize PCR-based gene amplification often causes misreading of not the responsible, but the pseudogene(s) in other site. Particularly, nuclear genes preserve incredible amount of pseudogenes with the same sequence as the ancient mtDNA (NUMT, Ref. 10) in part, even when the original mtDNA has already altered adapting to a new environment (Fig. 2). Consequently, whole mt-genome sequencing is preferable over the classic methods and would be essential in future to avoid misdiagnosis.

2) New modality to read the whole mt-DNA sequence

The whole mt-DNA sequences of all three probands and 10 Japanese volunteer patients without CM or heart failure as an internal standard were determined with GeneChip® Human Mitochondrial Resequencing Array 2.0 (11, Toyo-oka et al, In submission). The DNA sequences different from the world-standard, rCRS were

confirmed with the classic dye-tterminator method (Sanger sequencing) equipped with size-separation in capillary using mitoSEQ™ Resequencing System for the Human Mitochondrial Genome.

3) Identification of pathogenic mutant in the ORF of mt-genome

For the evaluation of physiological significance of open reading frame (ORF), it would be reasonable to assume that the synonymous mutation has no or less meaning on the pathogenesis except the modification of codon usage in nuclear or mitochondrial genome (12-14). The mutation within ORF would directly cause the conformational change in the encoded protein (transgene) and accordingly result in the functional modification, if any. For the integration of all 13 polypeptides coded by the mt-gene into the internal membrane, most of mt-proteins abundantly include hydrophobic domain and buried in the phospholipid bilayer. Among the mt-proteins, ATPase 6 is the most hydrophobic peptide and mutation of the current case from alanine to threonine occurred in the midst of the hydrophobic rigid structure (Toyo-oka *et al,* In submission). Thus, it would be conceivable to assume that the present mutation causes a serious alteration in oxidative phosphorylation at the final step to synthesize ATP. Another mutation to cause NARP (Neuropathy, Ataxia and Retinitis Pigmentosa) confirmed the

scheme described above in the same *ATP6* gene (15).

The other mutations in ORF constitute main source of the mitochondrial gene-related diseases and the predicted structure of the transgene, *i.e.* LHON in *ND1*, *ND4* or *ND6* (16-19) or KSS with the large 5kb deletion spanning from *ATP8* to *ND5* of (20, 21) wait more fine analysis like an ionic charge of the constituent amino acids, modulation of helical structure and intrageneic suppressor action in LHON *ND1* gene (22). For the functional prediction, the higher ordered structure ND6 gene (23) or gene interference between nuclear and mt-genomes might be much informative to estimate the mutant function (Toyo-oka *et al,* In submission).

4) Pathogenic mutation in tRNA of mt-genome

The tRNA is another large source of mt-gene mutations, because tRNA is situated at the critical step of protein synthesis and the defect will course a serious problem in the production rate of each component protein in mitochondria. Several mutations have been reported on MELAS 3243 in $tRNA^{Leu(UUR)}$ (24, 25) and MERRF 8344 in $tRNA^{Lys}$ (26, 27) or dilated cardiomyopathy (DCM) in $tRNA^{Thr}$ (Toyo-oka *et al.,* In submission). McFarland *et al,* raised 5 criteria (28),

i) ~3/4 of mutation sites in stem regions of the secondary structure,

ii) pathogenic hot spots in both the acceptor and anticodon stems,

iii) the disruption of Watson-Crick base pairs,

iv) more common pathogenicity in C-G base pairing than A-T pairing secondary to the lower thermodynamic energy and

v) preferential pathogenicity in loop structure with unusual number of nucleotides that may affect the tertial structure.

To these criteria, we add here the following three items more for the pathogenicity,

vi) medical records describing the identical mutation in other mitochondrion-related diseases, especially in energy consuming tissues, like neurodegenerative diseases in brain, inner ear or retina, skeletal., or cardiac muscles like myopathy, HCM or DCM and endocrine organs like diabetes mellitus with or without angiopathy,

vii) conservation of the wild-type sequence in non-human primates, suggesting the biological significance and

viii) pathological features of mitochondriosis in the electron microscopy of biopsy samples.

When each criterion is precisely inspected, each item is not independent, but some overlap among these stratifications. Furthermore, each item may require scoring for the more exact prediction in future. Particularly, the morphological observation using fresh sample to avoid the postmortem degeneration is critical to proceed to advanced

step of an accurate diagnosis for the genetic diseases.

The endomyocardial biopsy samples provide several characteristic findings in mitochondria, involving accumulation of huge number of bizarre-formed mitochondria, *i.e.* mitochondriosis (Toyo-oka *et al,* In submission), concentric cristae (24), hypertrophic mitochondria within myocytes and vessel walls with or without paracrystalline mitochondrial inclusions (25, 26).

Other comments on gene analysis

The DNA sequence in rRNA is meaningful for the exact and efficient protein synthesis but the clinical significance of the mutant is still obscure except except rare case of A1555G mutation in 12S-rRNA with sensory hearing loss or DEAF gene (29, 30).

As the initial step of assignment of the pathogenic mutations coding mitochondrial gene, the nuclear gene should be separately or independently examined not to be mixed each other. Then, the combination of two analyses would yield unexpected results that show double or sometimes triple mutation and pathogenesis of the complex familial disease is clarified or the classic "penetrance" is explained by the multiple gene defect with the different time-course (Toyo-oka et al, In submission). The SNP microarray commercially available now is still at the primitive stage to cover pathogenic mutants or

variants near to the responsible locus, because even when 900k gene chip is used for the analysis (Fig. 3).

In addition, magnetic resonance spectroscopy (MRS) together with ^{1}H-magnetic resonance imaging (^{1}H-MRI) will be promising for the elucidation of an actual effect of mutant(s) on cardiac function, especially to detect the energy metabolism with ^{31}P-MRS in the case with mtCM (31, 32), though both bore size and magnetic intensity of super-conducting coils are insufficient for precise measurement of human hearts *in vivo* within a limited measuring-time.

It is very meaningful to determine the belonging haplogroup as a risk factor. From the world-wide survey (32-37), we identified that the present mutation belonged to B5b1a subhaplogroup (Toyo-oka *et al,* In submission). The same G15927A mutation in $tRNA^{Thr}$ has been reported to modify the pathogenesis of other neurological diseases (38). In addition, the current sequence was restricted to the Japanese and no similarity to Chinese or Koreans has been reported (9), as may imply that the new haplogroup has branched from East Asians after their ancestor left the Eurasian continent (Fig. 4). In part, the full resequencing together with comprehensive gene analysis would clarify the controversial results obtained from PCR analysis using a partial amplification in mitochondrial gene mutation.

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