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Pathological similarities between low birth weight-related nephropathy and nephropathy associated with mitochondrial cytopathy

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Abstract

Background: Individuals born with a low birth weight (LBW) have a higher risk of developing kidney dysfunction during their lifetime and sometimes exhibit focal segmental glomerulosclerosis (FSGS) lesions in their glomeruli. We herein try to obtain other pathological characteristics of LBW-related nephropathy.

Methods: We retrospectively evaluated the renal pathology of four patients demonstrating FSGS with a history of LBW. Two mitochondrial cytopathy patients were also analyzed. DNA mutations were surveyed using a PCR-Luminex assay.

Results: In all four FSGS patients with a history of LBW, focal segmental glomerulosclerosis were detected. Interestingly, granular swollen epithelial cells (GSECs), which have previously been reported exclusively in patients with mitochondrial cytopathy, were also observed in the distal tubules and/or collecting ducts of all four patients with a history of low birth weight in this study. Electron microscopy revealed that these granular swollen epithelial cells included an increased number of enlarged mitochondria. Furthermore, cytochrome c oxidase subunit IV staining of patients with a history of low birth weight and patients with mitochondrial DNA mutations showed unbalanced expression patterns in glomeruli and a part of tubular cells. However, no mitochondrial gene mutations were detected in any of our four patients with low birth weight-related nephropathy.

Conclusions: This is the first report to show the pathological similarities not only in glomeruli but also tubuli between nephropathy with a LBW history and nephropathy with mitochondrial cytopathy.

Virtual Slides: The virtual slide(s) for this article can be found here: http://www.diagnosticpathology.diagnomx.eu/vs/13000_2014_181

Keywords: Focal segmental glomerulosclerosis, Low birth weight, Mitochondria, Granular swollen epithelial cell

Background

Low-birth-weight (LBW) is also associated with an increased risk of end-stage renal disease (ESRD) [1]. Studies of both humans and animals have shown LBW to be significantly associated with a decreased number of nephrons [2-4]. Brenner proposed the glomerular hyperfiltration theory in which adaptive mechanisms activated in response to nephron loss increases the capillary pressure and the incidence of glomerular hypertrophy [5,6]. This intraglomerular hypertension results in accelerated damage to nephrons with further nephron loss. This vicious cycle promotes the further progression of chronic kidney disease (CKD).

In particular, it is well known that representative glomerular changes in LBW individuals include focal segmental glomerulosclerosis (FSGS) [7]. Although intraglomerular hypertension is associated with the pathogenesis of FSGS, the clear mechanisms by which FSGS lesions are formed have not been clarified. Because detail pathological analysis sometimes gives a clue to assess the pathogenesis, we

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herein evaluated LBW-related nephropathy (LBWN) in order to obtain more detail pathological characteristics.

Methods

Patients

From January 2006 to December 2011, we performed 472 kidney biopsies in our division. In these cases, there were four cases of FSGS among patients born with a birth weight under 2,500 g (according to the WHO definition of LBW). These four patients (LBW 1–4) were retrospectively evaluated pathologically and genetically. In addition, two patients (Mt 1 and 2) who were found to have mitochondrial DNA (mtDNA) mutations and underwent kidney biopsies to investigate the cause of their proteinuria were also evaluated. As normal controls for staining (N = 3), kidneys dissected because of kidney cancer were used.

Histological analysis

Briefly, kidney specimens fixed in 10% neutral buffer formalin followed by embedding in paraffin were used for a light microscopy analysis with routine staining, as follows: hematoxylin and eosin (HE), periodic acid-Schiff (PAS), Masson's trichrome stain and periodic acid-methenamine-silver (PAM)-HE stain. The kidney specimens used for the electron microscopy analysis were fixed in 2% glutaraldehyde (pH 7.3-7.4) followed by 2% osmium tetroxide (pH 7.3-7.4). For cytochrome c oxidase subunit IV (COX IV) staining, paraffin-embedded specimens were used. Following deparaffinization and antigen activation using 10 mM of boiled citrate buffer (pH 6.0), rabbit anti-COX-IV antibodies (clone 3E11) (Cell Signaling Technology, Inc., Danvers, MA) were used as the primary antibody. SignalStain® Boost IHC Detection Reagent (HRP, Rabbit) (Cell Signaling Technology, Inc.) was used to detect the rabbit primary antibody, according to the manufacturer's instructions.

Assay for detecting mitochondrial gene mutations

Blood samples (1 ml/each analysis) were collected in EDTA-2Na tubes. Following centrifugation, the pellets were used for the analysis. For the analysis of urine sediments, over 100 ml of urine was collected from each patient, and the pellets obtained after centrifugation were used for the analysis. Five slices (4 μm) of frozen kidney sections were used for the genetic analysis. A PCR-Luminex assay, which can be used to survey 61 different pathogenic mtDNA mutations, was performed according to our previously reported method [8].

Ethical considerations

This study was conducted in accordance with the "Ethical Guidelines for Clinical Studies" (Revised on December 28, 2004, Ministry of Health, Labour and Welfare of Japan). All medical professionals involved in this study were required to comply with these ethical standards. The local Ethics Committee of Chiba-East Hospital approved the study protocol (No. 22), and all subjects provided their informed consent to participate in this study.

Results

All four LBW-related nephropathy patients exhibited perihilar variants of FSGS in their glomeruli

The background characteristics of the patients are summarized in Table 1. All four LBWN patients underwent kidney biopsies in order to determine the etiology of their persistent proteinuria. All four LBWN patients were male, with a mean birth weight of 1,692 g. The gestational ages and the reasons for the low birth weight could not be fully assessed in these cases (The gestational age of LBW1 was 28 weeks and that of LBW2 was 38 weeks). No subjects had symptoms of myopathy or encephalopathy, a history of stroke-like episodes or difficulty hearing. Although all patients had no subjective symptoms, they were found to have proteinuria on periodic medical examinations. Only one (LBW 3) of the LBWN patients suffered from diabetes mellitus (with a five-year history). The kidney size tended to be smaller than normal, except in LBW 3 (Table 1). The light microscopy analysis revealed that the glomeruli of all LBWN patients exhibited FSGS (Figure 1). Patient Mt 1 was initially found to have proteinuria at 39 weeks of gestation and was subsequently referred to our hospital because the proteinuria persisted for one year after delivery. Although she had no symptoms of MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis, stroke-like episodes), we found increased morphologically abnormal mitochondria in her podocytes (Figure 2A). Therefore, we evaluated her mtDNA at our hospital and detected an mtDNA mutation (3243 A > G). Patient Mt 2 was found to have proteinuria on a periodic medical checkup at 19 years of age. She also had no symptoms of MELAS. She refused an assessment of her serum lactate level. However, she had a brother with MELAS (3243 A > G), and a kidney biopsy revealed that her podocytes included increased mitochondria (data not shown). Her mtDNA mutation (3243 A > G) was diagnosed at another hospital. Although the characteristic glomerular change in patients with mitochondrial cytopathy is FSGS, as previously described in several reports [9-11], neither of our two patients with mtDNA mutations had apparent FSGS lesions in the evaluated glomeruli (data not shown). An electron microscopy analysis of the two patients with mtDNA mutations revealed that the glomerular epithelial cells contained an increased number of enlarged mitochondria and mild foot process effacement. On the other hand, we found no morphological abnormalities or increments in the number of mitochondria in any of the observed sections obtained from the LBWN patients (Figure 2B-D). However, the

Table 1 Summary of patients

Case	Age	Sex	BMI[1]	Birth weight (g)	BP[2] (mmHg)	eGFR[3] (ml/min/1.73m^2)	Urinary protein[4] (g/gCr)	Serum lactate[5] (mg/dl)	Long axis of kidney[6] (mm)	Number of total glomeruli	Global sclerosis[7] (%)	Segmental sclerosis[8] (%)	Glomerular hypertrophy[9]	Foot process effacement	mtDNA mutation[10]
LBW1	21	M	22.0	978	120/65	98	0.34	15.2	880	24	8.3	16.7	+	+	none
LBW2	20	M	21.0	1900	126/82	46	0.56	n.d.	848	4	0	25	+	+	none
LBW3	36	M	31.2	1400	125/75	74	1.35	n.d.	1208	14	42.9	7.1	+	+	none
LBW4	41	M	24.5	2465	121/70	63	0.83	15.8	988	20	15	10	+	+	none
Mt1	34	F	17.6	2720	115/63	48	0.29	9.1	936	11	45.5	0	-	+	3243 A>G
Mt2	28	F	17.5	3630	96/63	118	1.39	n.d.	953	16	0	0	-	+	3243 A>G

[1]BMI, body max index at the time of kidney biopsy.
[2]BP, blood pressure: BP at the time of kidney biopsy is expressed as systolic/diastolic.
[3]eGFR, estimated glomerular filtration: eGFR is calculated by Japanese GFR equation [12] from serum creatinine value, age, and sex at the kidney biopsy.
[4]The data of urinary protein is based on a urinalysis on the day of the kidney biopsy.
[5]Serum lactate is measured by an enzymatic assay (normal range: 3-17mg/dl). We did not measure the lactate values in three of six patients (expressed as "n.d.").
[6]The long axis of the left kidney was measured by an echogram just before the kidney biopsy.
[7]The rate of the number of globally sclerosed glomeruli/total glomeruli in the observed section is expressed.
[8] The rate of the number of segmentally sclerosed glomeruli/remnant glomeruli (total glomerular number minus GS glomeruli) is expressed.
[9]The existence of glomerular hypertrophy is defined when the diameter of capillary area is over 250μm [13].
[10]mtDNA mutation was surveyed in blood, urine, and kidney specimens by the PCR-Luminex method [11] except Mit2 patient, who was already diagnosed by a gene analysis in the other hospital.

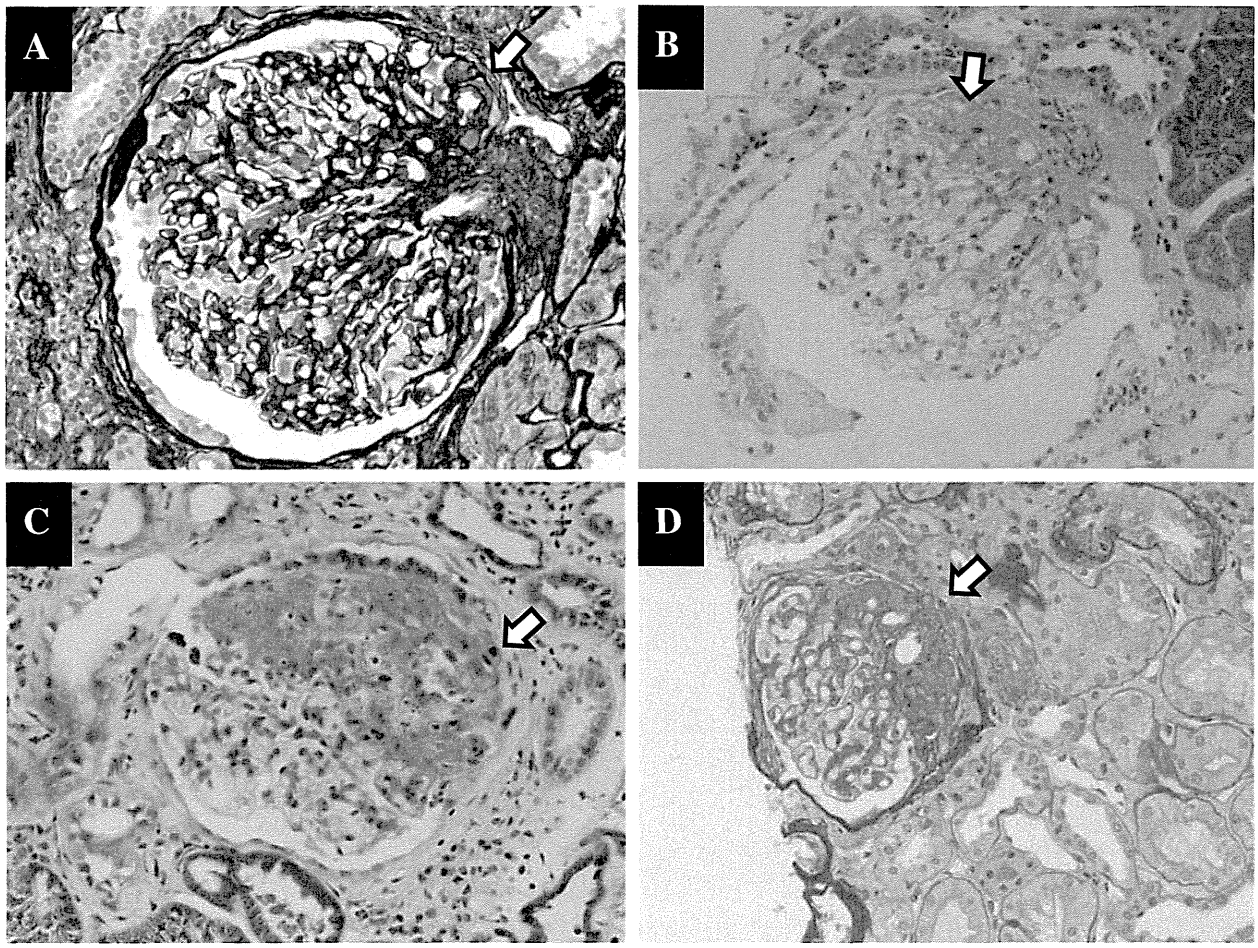

Figure 1 Representative photos of glomeruli in the FSGS patients born with LBW. (A): Exudative and sclerotic lesions are indicated in the perihilar area by arrows (LBW 1, PAM-HE stain). **(B)**: Segmental sclerosis in the perihilar area is indicated by the arrow (LBW 2, Masson's Trichrome stain). **(C)**: Segmental sclerosis is indicated by the arrow (LBW 3, Masson's Trichrome stain). **(D)**: Segmental sclerosis in the perihilar area is indicated by the arrow (LBW 4, PAS stain).

podocytes of the four LBWN patients showed mild foot process effacement (Figure 2B-D), similar to that observed in the patients with mtDNA mutations (Figure 2A).

All four patients with LBW-related nephropathy and the two patients with mtDNA mutations had granular swollen epithelial cells in their collecting ducts or distal tubules

The presence of granular swollen epithelial cells (GSECs) in the collecting ducts or distal tubules was recently reported to be a characteristic pathological change in patients with mitochondria cytopathy [14]. All four patients with LBWN as well as the two patients with mtDNA mutations had GSECs in their collecting ducts and/or distal tubules (Figure 3A-F). In addition, a portion of these GSECs had dropped out of the arrangement of the tubules. The electron microscopy analysis revealed that these GSECs contained an increased number of enlarged mitochondria (Figure 3G, H), and the nuclei occasionally seemed to be condensed.

The expression of Complex IV exhibited a mosaic pattern of staining in the glomeruli and tubules of both the LBW-related nephropathy patients and mtDNA mutation patients

Complex IV, which is composed of 13 subunits, is the terminal of the electron transfer chain in mitochondria. It was reported that the complex IV activity of podocytes changes in pathological conditions [15]. Therefore, we here stained for COX IV, which is one of the subunits. A COX IV expression analysis of normal controls showed that podocytes should be almost equally positive in the glomerulus (Figure 4A) and that tubular cells are also equally positive at the same luminal level (Figure 4B). However, in the LBWN patients, the expression of COX IV in the podocytes was unbalanced. Only a few podocytes appeared to intensely express COX IV, although the others expressed less COX IV (Figure 4C, E). In addition, the COX IV staining in a part of tubules of the LBWN patients exhibited a mosaic pattern, even at the same luminal level

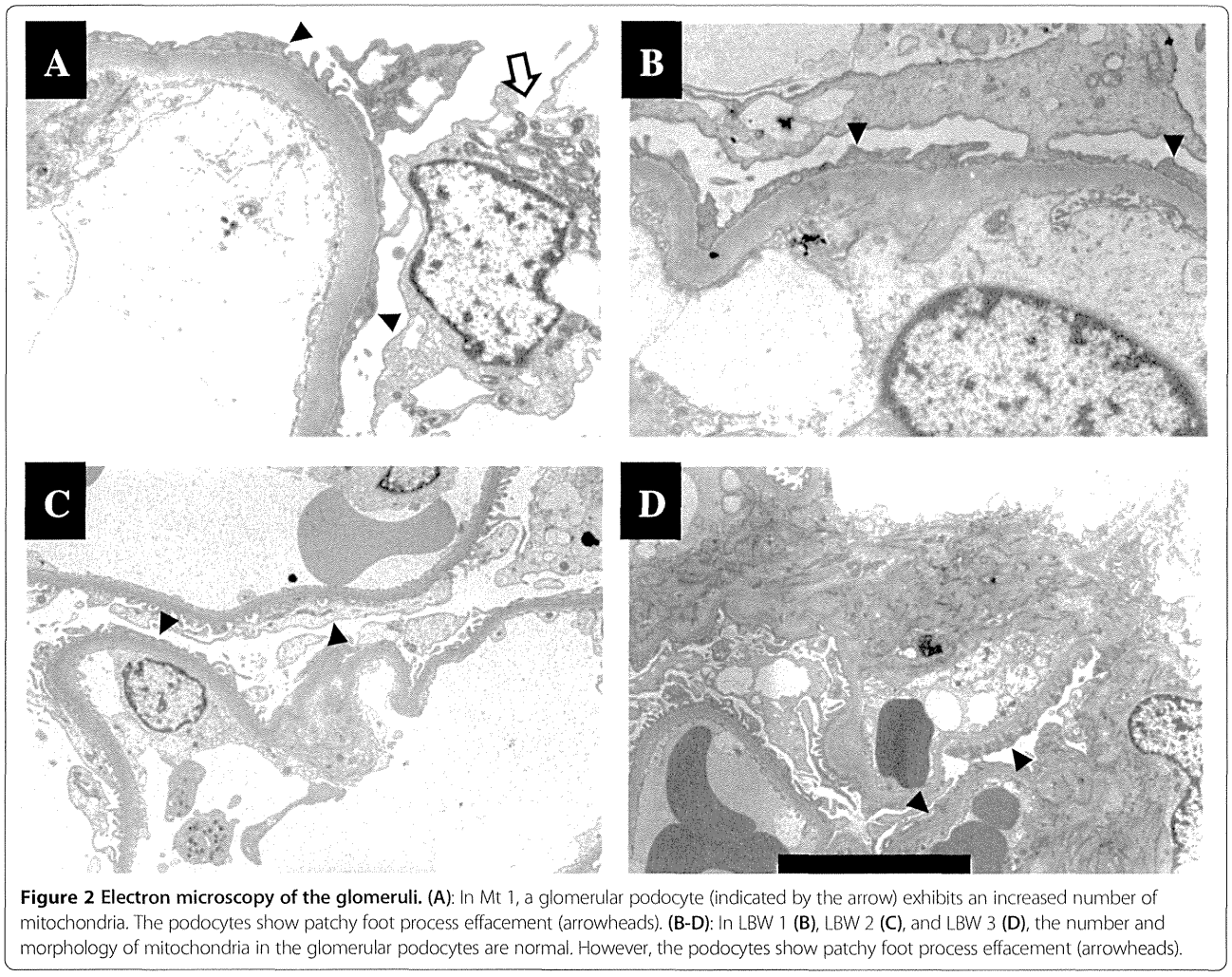

Figure 2 Electron microscopy of the glomeruli. (A): In Mt 1, a glomerular podocyte (indicated by the arrow) exhibits an increased number of mitochondria. The podocytes show patchy foot process effacement (arrowheads). **(B-D)**: In LBW 1 **(B)**, LBW 2 **(C)**, and LBW 3 **(D)**, the number and morphology of mitochondria in the glomerular podocytes are normal. However, the podocytes show patchy foot process effacement (arrowheads).

(Figure 4D,F). The cells strongly expressing COX IV appeared to correspond to GSECs. The expression patterns both of the glomerular podocytes and tubules in the patients with mtDNA mutations showed a mosaic pattern (Figure 4G, H), similar to that observed in the LBWN patients. The degree of the mosaic patterns in glomeruli and tubular cells should be more intense in patients with mtDNA mutations compared with LBWN patients.

The LBW-related nephropathy patients did not have any mitochondrial gene mutations

According to the PCR-Luminex assay, none of the 61 mtDNA mutations were detected in the blood, urine or kidney sections of the four LBWN patients (Table 1). However, an mtDNA mutation (3243 A > G) was clearly detected in all samples of blood, urine sediment and kidney sections in patient Mit 1. Because another patient (Mit 2) had already been found to have 3243 A > G mtDNA at another hospital, we did not perform an mtDNA mutation analysis using RCR-Luminex due to ethical considerations.

Discussion

Table 2 presents a summary of our results. The characteristic feature of glomerular involvement observed in patients with mitochondrial cytopathy is focal segmental glomerulosclerosis (FSGS), as previously described in a considerable number of reports [9-11]. In addition, it was recently reported that the presence of granular swollen epithelial cells (GSECs), in which enlarged mitochondria increase, in the distal tubules or collecting ducts is a specific pathological change in patients with mitochondrial cytopathy [14]. On the other hand, adults born with LBW have a high risk of kidney damage [1,16] and sometimes exhibit FSGS lesions in their glomeruli [7]. In this report, we showed that the pathological findings of kidney biopsy specimens were similar between patients with mtDNA mutations and those with LBWN with respect to the following three points (Table 2): 1. glomerular changes involve the presence of FSGS lesions and podocytes with foot process effacement; 2. a portion of tubular cells display characteristics of GSECs, in which the number of enlarged mitochondria is increased; and 3. The complex IV

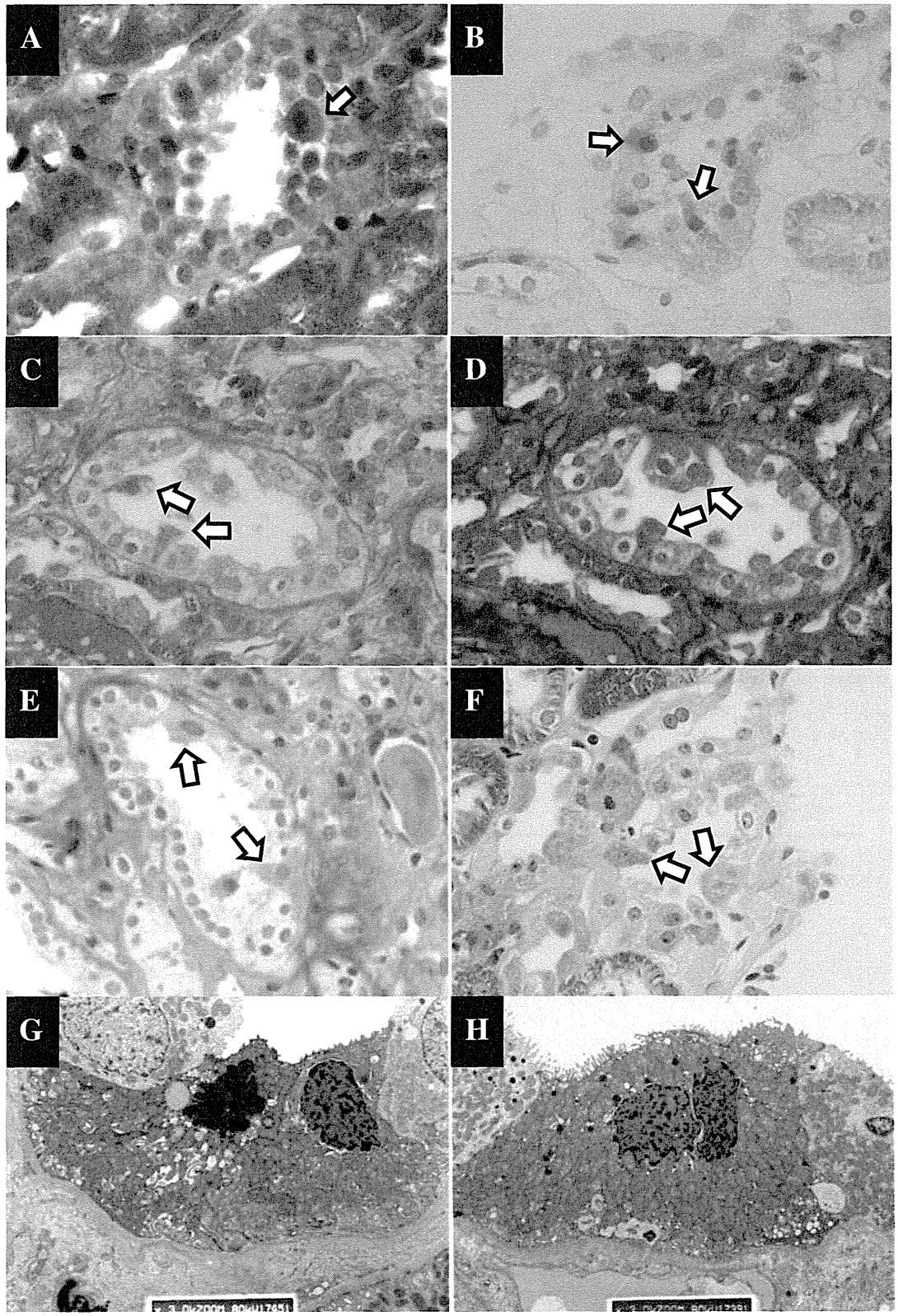

Figure 3 (See legend on next page.)

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Figure 3 Photos of tubules in patients of LBW-related nephropathy and patients with mtDNA mutation; (A)-(F): Masson's Trichrome stain, (G) and (H): electron microscopy (original magnification: × 3000). GESCs are present in collecting ducts or distal tubules of LBW 1 **(A)**, LBW 2 **(B)**, LBW 3 **(C)**, LBW 4 **(D)**, Mt 1 **(E)**, and Mt 2 **(F)**. In LBW 4 **(G)**, the number of mitochondria with morphological abnormalities is increased in the collecting duct. One nucleus appears to be condensed. In Mt 2 **(H)**, increased mitochondria are also observed in the cytoplasm of collecting duct.

expression shows an unbalanced expression pattern in a portion of podocytes and tubular cells. In fact, although our two patients with mtDNA mutations had no FSGS lesions, there is a possibility of sampling error. As another possibility, because these two patients had no other symptoms of mitochondrial cytopathy, the glomerular lesions could be slight. Furthermore, although we evaluated 5 obesity-related nephropathy with FSGS lesions (all patients are under 40-year-old), there were no GSECs.

Because the presence of GSECs in the tubules was previously described to be an exclusive characteristic change in patients with mitochondrial cytopathy (12), we initially suspected that the four patients with LBWN had certain mtDNA mutations. However, based on the PCR-Luminex method [8], which can be used to detect 61 different pathogenic mtDNA mutations, no mtDNA mutations were detected in the blood, urine sediment or kidney sections of the four patients with LBWN. Although these 61 mtDNA mutations include most mtDNA mutations previously reported in Japan [8], we cannot completely deny the existence of other mtDNA mutations in the four LBWN patients. The sensitivity of this method for surveying mtDNA mutations has previously been documented [8]. In addition, this method of detecting mtDNA mutations using blood, urine sediment and kidney sections was able to clearly detect the 3243 A > G mtDNA mutation in patient Mt 1. This report also provides a new strategy for detecting mtDNA mutations in nephropathy patients carrying mtDNA mutations using urine samples. This method is based on the observation that urine sediment consists of certain components derived from podocytes and/or tubular cells.

Why do patients with LBWN exhibit similar pathological changes to those observed in nephropathy patients with mtDNA mutations? It appears that the mitochondrial function of podocytes is involved in the formation of FSGS lesions, as we and others also recently proposed [17-21]. Although we cannot exactly evaluate the degree of foot process effacement by the limitation of sampling, mild foot process effacement were observed all our cases of LBWN. In the present study, we did not observe any increments in the number of mitochondria in the podocytes of the patients with LBWN, which is indeed a different pathology from that observed in mitochondrial cytopathy

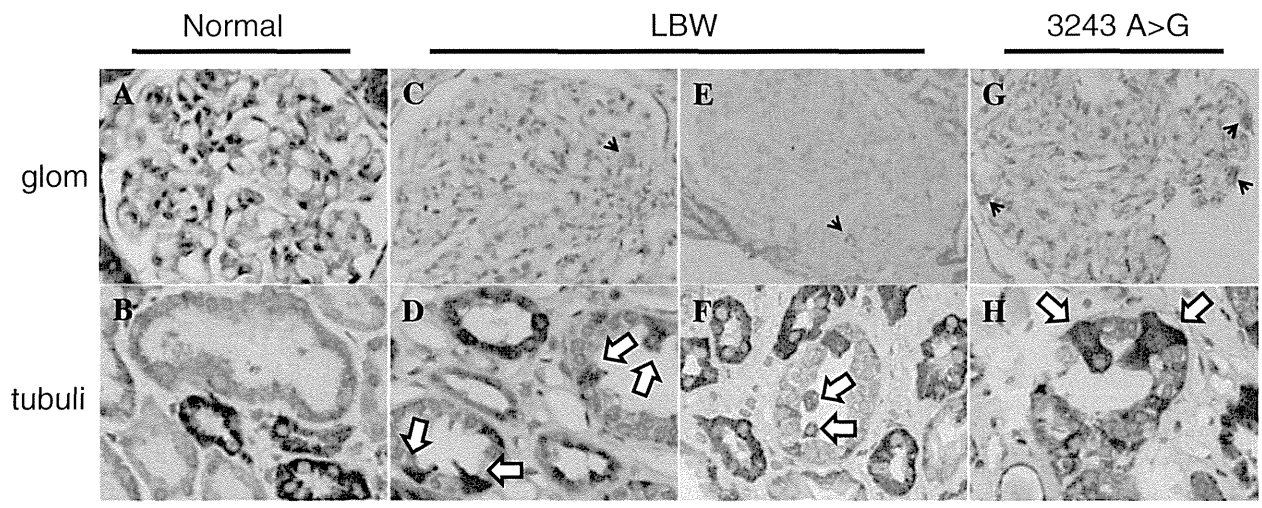

Figure 4 COX IV staining of the glomeruli and tubules. A: In the glomeruli of a normal control, the podocytes almost equally express COX IV. **B**. In the tubules of a normal control, the tubular cells at the same luminal level almost equally express COX IV. **C**. In the glomeruli of LBW 1, only one podocyte (arrow) expresses more COX IV than the other podocytes. **D**. In the tubules of LBW 1, unbalanced strong staining for COX IV is detected (arrows). Only a part of tubular cells express extremely intense COX IV compared with other tubular cells at the same luminal level. **E**: In the glomeruli of LBW 4, only one podocyte (arrow) expresses more COX IV than the other podocytes. **F**. In the tubules of LBW 4, staining for COX IV in a part of tubular lumens exhibits a mosaic pattern. The GSECs should express intense COX IV staining (arrows). **G**: In the glomeruli of Mt 1, a portion of podocytes (arrows) express more COX IV than the other podocytes. **H**: In the tubules of Mt 1, a portion of tubular cells are intensely stained (arrows) compared with the other cells in the same lumen. These cells should coincide with GSECs002E.

Table 2 Summary of the findings of nephropathy in the mitochondrial cytopathy and LBW-related nephropathy patients

	Normal	Mitochondrial cytopathy	LBW-related nephropathy
mtDNA mutation	No	Yes	No
FSGS lesion	No	Yes[1]	Yes
Glomerular hypertrophy	No	Yes or No	Yes
Foot process effacement	No	Yes	Yes
Increased mitochondria in podocyte	No	Yes	No
GSECs in tubules	No	Yes	Yes
COX IV expression in glomeruli	uniform	mosaic pattern	weak (but intense in a few podocytes)
COX IV expression in tubules	uniform	partially mosaic pattern	partially mosaic pattern

[1]Although the two patients with mitochondrial cytopathy had no FSGS lesions in the glomeruli, the presence of FSGS lesions is a characteristic glomerular change in mitochondrial cytopathy patients, as reported in many previous reports.

patients. However, an increased number of mitochondria is only one phenomenon underlying mitochondrial abnormalities and is easily detected pathologically. In fact, mitochondrial biogenesis is controlled by complex mechanisms [22]; therefore, we cannot deny the possibility of mitochondrial dysfunction in LBWN patients, even when an increased number of mitochondria is not observed. COX IV is one of the major components of complex IV, the last enzyme in the respiratory electron transport chain in mitochondria [23]. When we stained the kidney sections using anti-COX-IV antibodies, the staining intensity of podocytes and tubular cells ay the same luminal levels were uniform. However, in patients with mtDNA mutations, COX IV expression, which was mainly expressed by podocytes in glomeruli, showed mosaic patterns. Furthermore, a part of tubular cells with mtDNA mutations extremely expressed COX IV compared with other tubular cells at the same luminal levels (Figure 4). COX IV expression was not uniform in glomeruli of LBWN patients, too. A few podocytes expressed stronger COX IV compared with most of other podocytes. Only a part of tubular cells in LBWN patients expressed extremely intense COX IV as like those in patients with mtDNA mutations (Figure 4). Although we cannot judge whether an unbalanced COX IV expression directly affects the pathogenesis of kidney disease, our results suggest the possibility that the mitochondrial function, not mitochondrial DNA mutations, is associated with the etiopathogenesis of low birth weight-related nephropathy.

In addition, GSECs could be occasionally observed in aged patients. Therefore, GSECs might not be specific findings. However, because all of LBWN patients had GSECs in a part of their tubular cells, we think that GSECs in LBWN should be "meaningful" pathological changes. Furthermore, if GSECs are observed, especially in young patients, the analysis of mtDNA might be needed [24]. Now, we cannot explain why GSECs partially appear. Furthermore, it is obscure that a part of tubular cells show intense COX IV expression although most of tubular cells uniformly express COX IV at the same luminal levels. Although we now hypothesize that unbalance between high energy demand and low energy supply might result in mitochondrial dysfunction, further studies must be needed.

We cannot exclude the possibility that our four patients with LBWN had similar pathological lesions to those observed in the patients with mitochondrial cytopathy "by chance". In spite of these limitations, some specific cases with characteristic pathological findings should provide new aspects [25-27]. This report provides new clues regarding mitochondria to prompt investigations of the pathomechanisms underlying the renal dysfunction associated with LBW.

Conclusion

This is the first report to show the pathological similarities not only in glomeruli but also tubuli between nephropathy with a LBW history and nephropathy with mitochondrial cytopathy.

Abbreviations

LBW: Low-birth-weight; ESRD: End-stage renal disease; CKD: Chronic kidney disease; FSGS: Focal segmental glomerulosclerosis; LBWN: LBW-related nephropathy; COX IV: Cytochrome c oxidase subunit IV; GSECs: Granular swollen epithelial cells; MELAS: Mitochondrial myopathy, encephalopathy, lactic acidosis, stroke-like episodes.

Competing interests

All authors declared that they have no competing interest.

Authors' contributions

TI formed the study concept and organized this study. TM analyzed mtDNA mutations. NM, TK, RR and MN provided their efforts for making data and critical suggestions to this study. YY and HK analyzed renal pathology and provided expertise as renal pathologists. All authors read and approved the final manuscript.

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GDF15 is a novel biomarker to evaluate efficacy of pyruvate therapy for mitochondrial diseases

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ABSTRACT

Pyruvate therapy is a promising approach for the treatment of mitochondrial diseases. To identify novel biomarkers for diagnosis and to evaluate therapeutic efficacy, we performed microarray analysis of 2SD cybrid cells harboring a MELAS-causing mutation and control cells treated with either lactate or pyruvate. We found that expression and secretion of growth differentiation factor 15 (GDF15) were increased in 2SD cells treated with lactate and that serum GDF15 levels were significantly higher in patients with mitochondrial diseases than in those with other diseases, suggesting that GDF15 could be a useful marker for diagnosis and evaluating the therapeutic efficacy of pyruvate.

1. Introduction

Mitochondrial diseases are caused by mitochondrial or nuclear genome mutations that affect the functions of mitochondria. The symptoms are caused by impaired energy metabolism due to mitochondrial dysfunction and manifest mostly in tissues with a high energy demand such as brain, heart, and muscle. Mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) is one of the most common of the mitochondrial diseases (Pavlakis et al., 1984). The A-to-G transition at the 3243 position of the mitochondrial DNA (m.3243A > G) located in the mitochondrial $tRNA^{Leu\ (UUR)}$ gene is a MELAS-causing mutation, and it is detected in approximately 80% of patients with MELAS (Goto et al., 1990, 1992; Kirino et al., 2004; Yasukawa et al., 2000).

These pathogenic mutations typically result in defective ATP synthesis in mitochondria, and therefore ATP production depends on the glycolytic pathway. Since lactate production is aberrantly increased by the acceleration of glycolysis when energy demand is elevated, the lactate to pyruvate (L/P) ratio in serum is often increased in patients with mitochondrial diseases and has been clinically used for estimating the dysfunction of mitochondrial respiration. It is well known that the L/P ratio reflects the intracellular NADH/NAD^+ ratio. Since NAD^+ is indispensable for oxidation of glyceraldehyde 3-phosphate (GAP) to 1,3-bisphosphoglycerate (BPG) by glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in the glycolytic pathway, a shortage of NAD^+ interrupts this reaction, resulting in decreased ATP biosynthesis. Tanaka et al. (2007) proposed that the addition of pyruvate would facilitate oxidation of NADH to NAD^+ via the lactate dehydrogenase reaction, which would restore ATP production by the glycolytic pathway even under defective respiratory conditions. Indeed, positive effects of sodium pyruvate on clinical manifestations of mitochondrial diseases have been reported (Koga et al., 2012; Saito et al., 2012). However, useful biomarkers for evaluating the therapeutic efficacy of pyruvate remain to be developed.

Cybrid cell lines established by the fusion of enucleated myoblast cells from a patient with a cultured cell line depleted of mtDNA have been used to elucidate the pathogenesis and underlying molecular mechanisms of mitochondrial diseases. We previously reported increased expression of amino acid starvation-responsive genes in cybrid cells with MELAS and NARP (neuropathy, ataxia, and retinitis pigmentosa) mutations (Fujita et al., 2007). In our earlier study (Kami et al., 2012), we found that exposure to excessive sodium lactate significantly increases the intracellular L/P and NADH/NAD^+ ratios in cybrid cells harboring the MELAS mutation (m.3243A > G), which implies worsening of lactic acidosis and NAD^+ shortage. On the other hand, we found that treatment with sodium pyruvate facilitates the ATP production and improves the energy status, as indicated by a decrease in the L/P ratio and retention of the NADH/NAD^+ ratio. Taken together, we considered that these experimental conditions would be ideal for identifying biomarker candidate genes, whose expression levels reflect

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the intracellular energy deficiency and the effect of pyruvate on energy metabolism.

In the present study, we performed a global gene expression analysis of cybrid cells with the MELAS mutation (m.3243A > G: 2SD cells) and control cybrid cells (2SA cells) treated or not with lactate or pyruvate. We identified several biomarker candidate genes, among which we focused on growth differentiation factor 15 (GDF15). The level of GDF15 in the conditioned medium was significantly higher in 2SD cells than in 2SA cells, which level was further increased by lactate but was not affected by pyruvate in 2SD cells. We also demonstrated that the concentration of GDF15 in the serum was markedly elevated in patients with mitochondrial diseases compared with that in those with other pediatric diseases. Thus, we identified GDF15 as a novel serum marker for the diagnosis of mitochondrial diseases and possibly for monitoring the disease status and progression and for evaluating the therapeutic efficacy of pyruvate.

2. Materials and methods

2.1. Cell culture

The 2SA and 2SD cybrid cell lines were previously established by Chomyn et al. (1992). Briefly, 14 cybrid clones were isolated after the fusion of enucleated myoblasts derived from a MELAS patient with mtDNA-deficient $\rho^0$206 cells generated from a human 143B osteosarcoma cell line. Among those clones, 10 clones had homoplasmic wild-type mtDNA, and 4 clones harbored strongly predominant mutant mtDNA. For our experiments, we chose two clones, 2SA and 2SD cybrid cell lines carrying 100% wild-type mtDNA and 94% m.3243A > G mutant mtDNA, respectively. The 2SD but not 2SA cybrid cells were shown to be defective in mitochondrial protein synthesis and respiratory capacity (Chomyn et al., 1992). Cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, and 0.4 mM uridine at 37 °C under a humidified atmosphere of 5% CO_2.

2.2. Microarray analysis

Total RNA was isolated from cells by using a miRNeasy mini kit (Qiagen, Venlo, Netherlands). One hundred nanograms of total RNA was labeled and amplified with a low input quick amp labeling kit (Agilent Technologies, Santa Clara, CA, USA) used according to the manufacturer's instructions. The labeled cRNA was hybridized to the Agilent SurePrint G3 Human GE 8x60K Microarray in a rotating hybridization oven at 10 rpm for 20 h at 65 °C. After hybridization, the microarrays were washed according to the manufacturer's instructions and scanned on an Agilent DNA Microarray Scanner with Scan Control software. The resulting images were processed, and raw data were collected by using Agilent Feature Extraction software. Expression data were analyzed by using GeneSpring GX 11 (Agilent Technologies). The signal intensity of each probe was normalized by a percentile shift, in which each value was divided by the 75th percentile of all values in its array. For pairwise comparison analysis, only the probes that had expression flags present under at least one condition were considered. The list was analyzed with Ingenuity Pathways Analysis software (Ingenuity Systems, Redwood, CA, USA)

2.3. Quantitative RT-PCR

Total RNA was reverse transcribed to cDNA with a High Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA, USA) used according to the manufacturer's protocols. Real-time PCR was performed on the StepOnePlus Real-Time PCR System (Life Technologies) using Power SYBR Green PCR Master Mix. 18S rRNA gene was used as an internal control for normalization. The sequences of primers are listed in Supplementary Table 1.

2.4. Patients

A written informed consent was obtained from all patients or their legal guardians. Enrolled patients were diagnosed with mitochondrial diseases by medical doctors in Kurume University Hospital over the period of 2005–2013. Seventeen patients diagnosed at this hospital as having mitochondrial diseases were recruited for this study. As a control group, 13 patients diagnosed as having other pediatric diseases such as dwarfism were also recruited. The clinical information of the patients is listed in Supplementary Table 2. This study was approved by the Institutional Review Board (Kurume University #13099).

2.5. ELISA and multiplex suspension array

Cells were placed on 60-mm dishes 1 day before replacing the medium with fresh medium. Conditioned medium cultured for 24 h was collected, and the particulates were removed by centrifugation (at 500 ×g for 10 min, at 10,000 ×g for 30 min). The GDF15 and INHBE concentrations in the supernatants and in the sera of patients were determined in duplicate by using a Human GDF-15 Immunoassay (R&D Systems, Minneapolis, MN, USA) and enzyme-linked immunosorbent assay kit for Inhibin Beta E (Uscn Life Science, Wuhan, Hubei, PRC) according to the manufacturer's instructions. For measuring other cytokine concentrations, the sera were subjected to a multiplex suspension array, Bio-Plex Pro Human Cytokine Grp II Panel 21-Plex (Bio-Rad, Hercules, CA, USA). The cytokines measured by use of this array were the following: IL-1α, IL-2Rα, IL-3, IL-12 (p40), IL-16, IL-18, CTACK, GRO-α, HGF, IFN-α2, LIF, MCP-3, M-CSF, MIF, MIG, β-NGF, SCF, SCGF-β, SDF-1α, TNF-β, and TRAIL. We measured the FGF21 (BioVendor, Czech Republic) concentration in duplicate samples by ELISA. Unmeasurable high-concentration samples of FGF21 and GDF15 were diluted 10-fold prior to measurement. The value from each assay was determined by reference to the linear portion of the standard curves for FGF21 and GDF15. All assays were performed by a trained scientist or technical staff.

2.6. Statistical analysis

Statistical analyses were performed by using IBM SPSS statistics (IBM, Armonk, NY, USA). We used the nonparametric Mann–Whitney *U* test to validate differences in cytokine levels in serum between mitochondrial disease patients and controls. The correlation between GDF15 and FGF21 concentrations in serum was assessed by Spearman correlation analysis. We plotted the receiver operating characteristics (ROC) curve for GDF15, HGF, SCF, SCGF-β, and FGF21 and calculated the area under the curve (AUC). The data for the sensitivity and 100 minus the specificity were plotted on a continuous scale.

3. Results

3.1. Gene expression changes in response to intracellular energy deficiency in 2SD cells

We performed microarray analysis of 2SD cybrid cells harboring the MELAS mutation (m.3243A > G) and 2SA control cybrid cells treated with 10 mM lactate or 10 mM pyruvate for 0, 4 or 8 h (Fig. 1A). The numbers of gene probes whose signal intensities were altered by 2-fold for each comparison are given in Supplementary Tables 3–6. We found remarkable changes in gene expression in 2SD cells, but not in 2SA cells, treated with lactate for 8 h. As shown in Supplementary Fig. 1A, we then selected gene probes that were increased by lactate treatment for 8 h compared with those without treatment and concurrently up-regulated by lactate but not by pyruvate at 8 h after treatment and thereby identified 313 probes that were specifically up-regulated by lactate in 2SD cells at 8 h

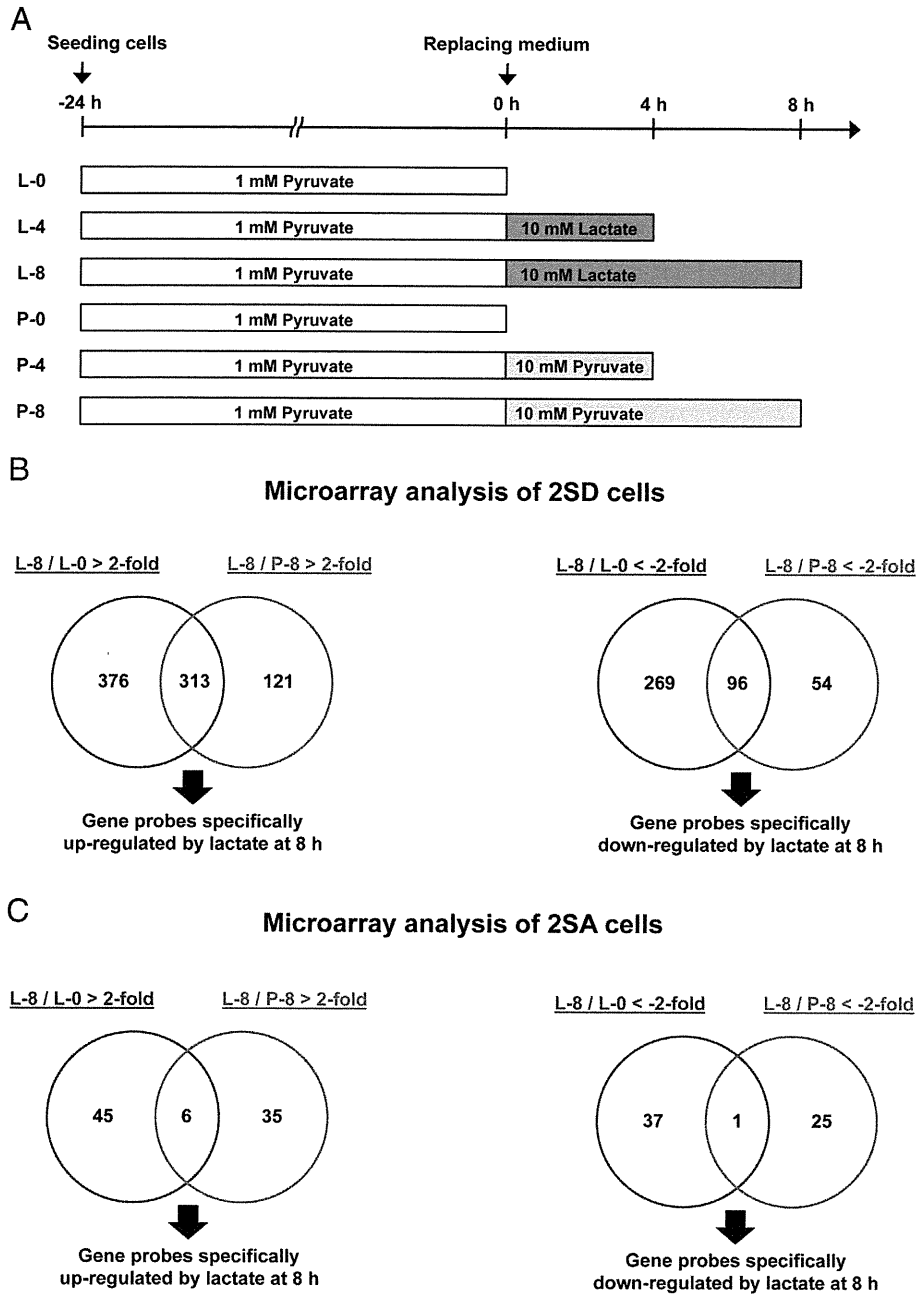

Fig. 1. Microarray analysis of 2SD and 2SA cells (A) Diagram of treatment protocols. Total RNA isolated from 2SD and 2SA cells treated with 10 mM lactate or 10 mM pyruvate for 0, 4, or 8 h were subjected to microarray analysis ($n = 2$). (B, C) Venn diagrams show the number of probes for genes in 2SD cells (B) or 2SA cells (C) that were increased (left panels) or decreased (right panels) in expression by lactate treatment for 8 h compared with their expression at 0 h and concurrently up-regulated by lactate but not by pyruvate after 8-h treatment. (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

(Fig. 1B). Using similar criteria (Supplementary Fig. 1B), we also identified 96 probes that were specifically down-regulated in 2SD cells by lactate treatment for 8 h (Fig. 1B). In 2SA cells, having normal mitochondrial function, the numbers of gene probes that responded to lactate treatment were limited (Fig. 1C). The clustering analysis of the 313 up-regulated (corresponding to 231 genes) and 96 down-regulated (corresponding to 75 genes) gene probes highlighted significant differences in gene expression patterns between 2SD and 2SA cells and also between lactate and pyruvate treatments (Fig. 2). These results suggest that a defective energy metabolism caused by exposure to a high dose of lactate resulted in significant changes in gene expression in 2SD cells.

3.2. Gene networks associated with intracellular energy deficiency in 2SD cells

In order to identify gene networks associated with a defective energy metabolism in the lactate-treated 2SD cells, a gene network analysis was performed on 231 up-regulated genes and 75 down-regulated ones. This analysis identified 11 and 5 gene networks for up- and down-regulated genes, respectively (Fig. 3 and Supplementary Figs. 2 and 3). The top-ranked gene network identified for the up-regulated genes contained those related to the amino-acid starvation response, such as ASNS, ATF3, NUPR1, DDIT3, CTH, TRIB3, STC2, and PCK2 (Fig. 3A). It is worth noting that GDF15, on which we focused in the

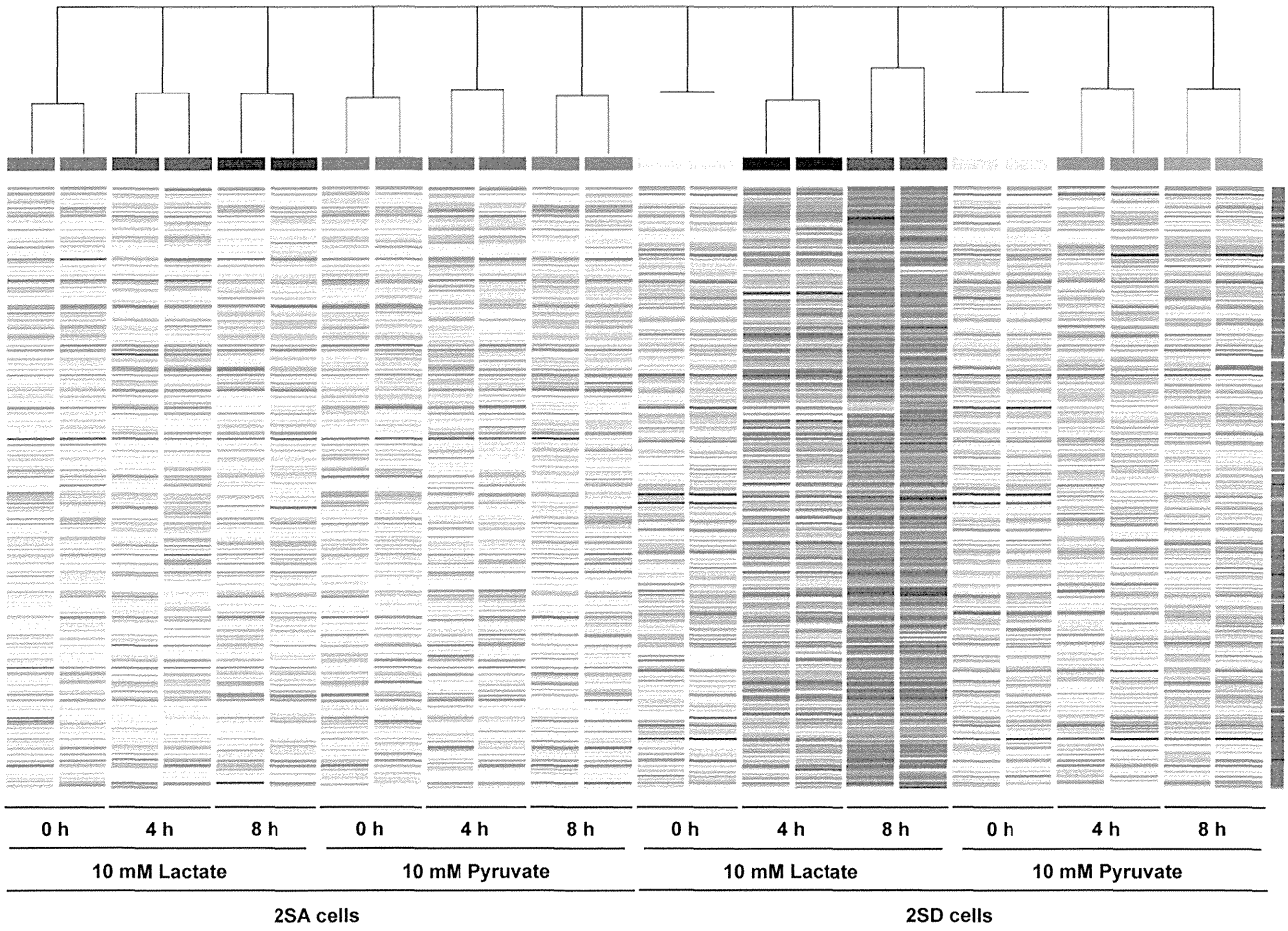

Fig. 2. Clustering analysis of the microarray data The gene probes up-regulated ($n = 313$) and down-regulated ($n = 96$) at 8 h after lactate treatment were subjected to clustering analysis. Part of the data are shown. (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

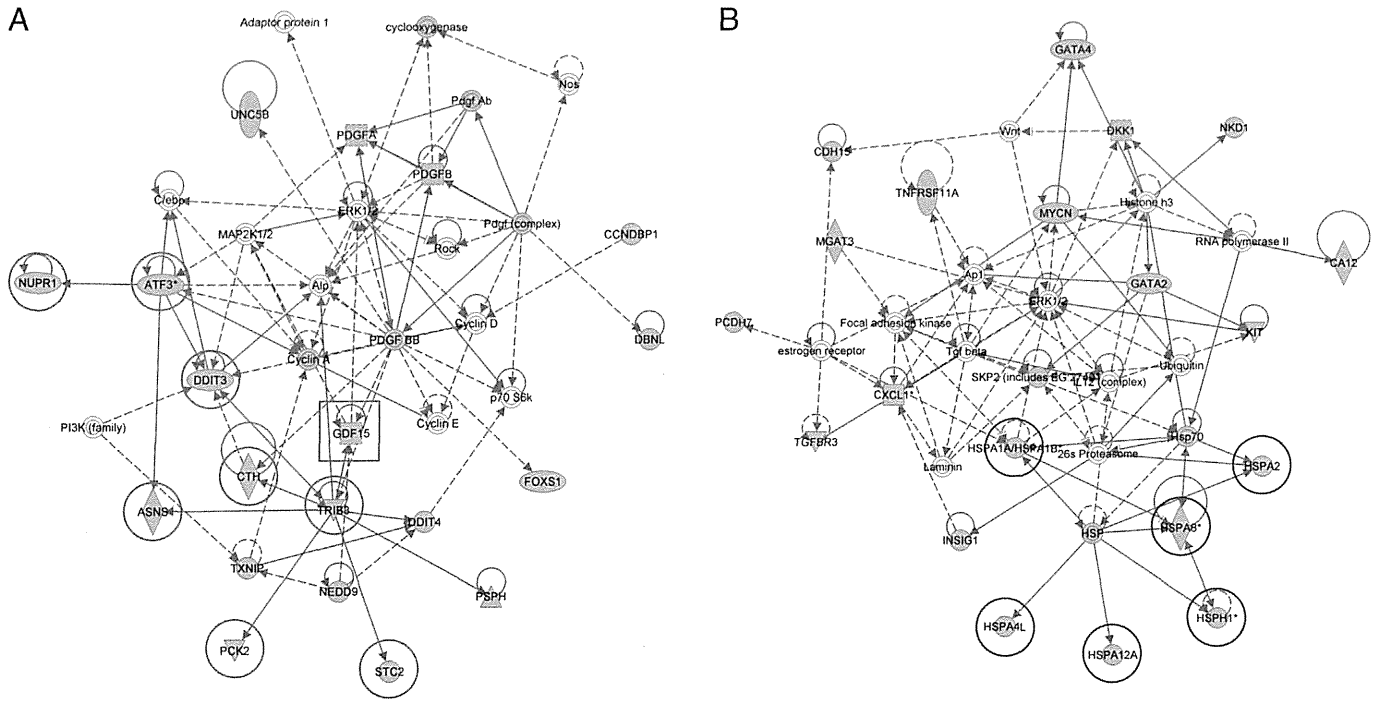

Fig. 3. Gene network analysis of the microarray data The genes specifically up-regulated ($n = 231$) and down-regulated ($n = 75$) at 8 h after lactate treatment were subjected to gene network analysis. The top-ranked gene networks in terms of the number of genes included are shown for up-regulated (A) and down-regulated (B) genes. Genes involved in the amino-acid starvation response (red circles) and heat-shock response (blue circles) as well as GDF15 (red square) are denoted. (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

present study, was included in this network. On the other hand, the gene network for down-regulated genes included those linked to the heat-shock protein response, such as HSPA1A, HSPA2, HSPA4L, HSPA8, HSPA12A, and HSPH1 (Fig. 3B).

3.3. GDF15 as a potential biomarker for diagnosis and evaluating the therapeutic efficacy of pyruvate

Proteins encoded by genes related to intracellular energy deficiency in 2SD cells and secreted into the medium could be potential biomarkers for mitochondrial diseases. Gene annotation analysis revealed the location of gene products that were specifically up- and down-regulated by lactate at 8 h (231 and 75 genes, respectively) (Table 1). Twenty-three up-regulated genes and 4 down-regulated genes were annotated to the extracellular space, each of which is listed in Tables 2 and 3. Among them, we focused on the top 2 ranked up-regulated genes, growth differentiation factor 15 (GDF15) and inhibin beta E (INHBE).

To validate the intracellular expression levels of these genes, we performed quantitative RT-PCR for GDF15 and INHBE. The expression levels of GDF15 (Fig. 4A) and INHBE (Fig. 4B) in the 2SD cells were increased by treatment with 10 mM lactate, but not with 10 mM pyruvate, for 4 or 8 h. Furthermore, GDF15 expression at 0 h was higher in 2SD cells than in 2SA cells. These results confirmed the reproducibility of our microarray data and identified GDF15 and INHBE as candidate biomarkers. To determine whether the secretion of GDF15 and INHBE proteins was increased in 2SD cells in response to lactate treatment, we measured their concentrations in medium from 2SA and 2SD cells cultured for 24 h in the presence of 1 mM pyruvate, 10 mM lactate, or 10 mM pyruvate. ELISA showed that the GDF15 levels were higher in the conditioned medium of 2SD cells than in that of 2SA cells under all of the culture conditions (Fig. 4C). Moreover, treatment with 10 mM lactate, but not with 10 mM pyruvate, promoted secretion of GDF15 in 2SD cells in comparison with treatment with 1 mM pyruvate, whereas 2SA cells did not respond to the high dose of lactate and pyruvate treatment. In contrast, INHBE protein was not detectable by ELISA in the conditioned medium of either 2SD or 2SA cells under any culture conditions (data not shown). These results indicate that GDF15 could be a potential biomarker for diagnosis and monitoring the disease status and progression as well as for assessing the therapeutic efficacy of pyruvate for the treatment of mitochondrial diseases.

3.4. GDF15 as a biomarker for diagnosis of mitochondrial diseases

In order to validate the feasibility of GDF15 as a serum biomarker, we measured its concentration in the serum of 17 patients with mitochondrial diseases as well as in that of 13 patients with other pediatric diseases as a control (Supplementary Table 2). ELISA showed that the average concentration of GDF15 in the serum of mitochondrial disease patients was 2632.9 pg/mL, whereas that for other pediatric disease patients was 285.2 pg/mL, suggesting that GDF15 levels were significantly increased in the serum of mitochondrial disease patients and could clearly distinguish mitochondrial disease patients from control patients (Fig. 5A).

Table 1
The location of probes (genes) up- and down-regulated in 2SD cells with lactate treatment for 8 h.

Location	Up-regulated		Down-regulated	
	Probe number	Gene number	Probe number	Gene number
Nucleus	39	35	14	14
Cytoplasm	51	47	25	19
Plasma membrane	37	33	16	16
Extracellular space	26	23	5	4
Unknown	160	93	36	22

Since fibroblast growth factor 21 (FGF21) was recently proposed as a diagnostic marker for mitochondrial diseases (Davis et al., 2013; Suomalainen et al., 2011), we also measured the FGF21 levels in the serum of the same mitochondrial disease patients and control patients (Fig. 5B). The serum FGF21 levels were higher in patients with mitochondrial diseases than in those with other diseases. Furthermore, there was a good correlation between the serum GDF15 and FGF21 levels (Fig. 5C).

In an attempt to find additional biomarkers, we determined the serum levels of 21 cytokines in the same patients by using the multiplex suspension array. As shown in Supplementary Fig. 4A, the serum concentrations of HGF and SCF were higher in patients with mitochondrial diseases than in control patients, whereas the serum levels of SCGF-β were lower in the former than in the latter.

Finally, we performed ROC curve analysis of GDF15, HGF, SCF, SCGF-β, and FGF21. As shown in Fig. 5D, the area under the curves (AUC) for GDF15 (0.986) was higher than that for FGF21 (0.787). The AUC for FGF21 was similar to those for HGF (0.747), SCF (0.729), and SCGF-β (0.837) (Supplementary Fig. 4B), indicating that GDF15 had the maximum sensitivity and specificity for diagnosis of mitochondrial diseases. These results suggest that GDF15 has the greatest potential as a novel diagnostic marker for MELAS and other mitochondrial diseases.

4. Discussion

Based on the global gene expression analysis of cybrid cells with mitochondrial dysfunction, we identified GDF15 as a potential biomarker whose expression and secretion reflected the intracellular energy deficiency and the effect of pyruvate therapy on the energy metabolism. We then determined the serum levels of GDF15 in patients with mitochondrial diseases and other diseases and identified GDF15 as a novel diagnostic marker for mitochondrial diseases. Although additional clinical studies are needed, the serum GDF15 concentration may be a useful biomarker not only for diagnosis of mitochondrial diseases but also for monitoring the disease status and progression as well as for determining the efficacy of pyruvate therapy.

GDF15 is a member of the transforming growth factor-β (TGF-β) superfamily and is widely expressed in mammalian tissues (Unsicker et al., 2013). GDF15 plays important roles in multiple pathologies including cardiovascular diseases, cancer, and inflammation. It has been shown that GDF15 is up-regulated by tumor suppressor p53 in response to high glucose or treatment with anti-cancer compounds (Baek et al., 2002; Li et al., 2013; Yang et al., 2003). The p53 protein is a transcription factor that responds to a variety of stresses such as DNA damage, oxidative stress, hypoxia, and metabolic stress, and it activates the expression of genes to induce cell cycle arrest, DNA repair, senescence, and cell death (Sermeus and Michiels, 2011; Sperka et al., 2012; Zhang et al., 2010). CDKN1A (p21), a potent cyclin-dependent kinase inhibitor, is a major downstream effector of p53, which induces cell-cycle arrest (Sperka et al., 2012). In our microarray data, the CDKN1A expression level was 3.5-fold increased by lactate treatment of 2SD cells (data not shown). Previous reports demonstrated increased expression of CDKN1A in the skeletal muscle of patients with mitochondrial diseases and a cell line depleted of mitochondrial DNA (Behan et al., 2005; Crimi et al., 2005). Besides CDKN1A, we found other p53 effector genes in the list of genes up-regulated in the lactate-treated 2SD cells, including GADD45A, EGR2, DDIT3, CHMP4C, SESN2, ULBP1, DDIT4, and NUPR1 (data not shown). These results suggest that p53 activation may have played an important role in the induction of GDF15 expression in 2SD cells treated with lactate. It has been also demonstrated that p53 activation caused by metabolic stress is mediated by AMP-activated protein kinase (AMPK; Zhang et al., 2010). Our previous metabolomic profiling revealed that the ATP level drops but that the ADP and AMP levels are increased in lactate-treated 2SD cells (Kami et al., 2012), implying that elevation of the AMP/ATP ratio may activate p53 through AMPK activation. Taken together, it is possible that p53 induced GDF15 expression in

Table 2
Genes annotated to the extracellular space among those specifically up-regulated by lactate treatment for 8 h.

Gene symbol	Accession number	Entrez gene name	Fold change	
			L-8/L-0[a]	L-8/P-8[b]
GDF15	NM_004864	Growth differentiation factor 15	27.4	14.8
INHBE	NM_031479	Inhibin, beta E	15.0	9.4
AREG	NM_001657	Amphiregulin	14.0	2.2
ECM2	NM_001393	Extracellular matrix protein 2, female organ and adipocyte specific	11.8	9.0
ADM2	NM_024866	Adrenomedullin 2	10.3	3.0
MMP3	NM_002422	Matrix metallopeptidase 3 (stromelysin 1, progelatinase)	9.8	4.2
IL1A	NM_000575	Interleukin 1, alpha	7.6	6.0
C12orf39	ENST00000256969	Chromosome 12 open reading frame 39	6.3	6.7
APOL6	NM_030641	Apolipoprotein L, 6	6.2	3.8
SCG5	NM_003020	Secretogranin V (7B2 protein)	5.2	3.0
SPOCK2	NM_014767	Sparc/osteonectin, cwcv and kazal-like domains proteoglycan (testican) 2	5.1	6.6
AMTN	NM_212557	Amelotin	5.0	3.9
IL23A	NM_016584	Interleukin 23, alpha subunit p19	4.4	2.8
ADAMTS17	NM_139057	ADAM metallopeptidase with thrombospondin type 1 motif, 17	3.5	2.2
VEGFA	NM_001025370	Vascular endothelial growth factor A	3.4	2.5
STC2	NM_003714	Stanniocalcin 2	3.4	2.6
PDGFB	NM_002608	Platelet-derived growth factor beta polypeptide	2.8	3.8
C1QTNF1	NM_198594	C1q and tumor necrosis factor related protein 1	2.6	2.9
HECW2	NM_020760	HECT, C2 and WW domain containing E3 ubiquitin protein ligase 2	2.4	2.1
IGFALS	NM_004970	Insulin-like growth factor binding protein, acid labile subunit	2.3	2.5
IGFBP1	NM_000596	Insulin-like growth factor binding protein 1	2.3	2.1
PDGFA	NM_002607	Platelet-derived growth factor alpha polypeptide	2.2	2.2
CLEC3B	NM_003278	C-type lectin domain family 3, member B	2.1	2.2

[a]Fold change between 8 h and 0 h after lactate treatment
[b]Fold change between lactate treatment and pyruvate treatment at 8 h

response to AMPK activation caused by the intracellular energy deficiency. However, it remains to be determined whether other stresses such as oxidative stress may also have participated in p53 activation and GDF15 induction in the lactate-treated 2SD cells.

Gene network analysis demonstrated that the top-ranked network contained not only genes associated with the amino-acid starvation response but also the GDF15 gene (Fig. 3A). In a mouse model of late-onset mitochondrial myopathy, the expression of amino-acid starvation-responsive genes was shown to be elevated (Tyynismaa et al., 2010). The asparagine synthetase (ASNS), which is a representative gene involved in the amino-acid starvation response, has been reported to be up-regulated in the skeletal muscle of patients with mitochondrial diseases and in cybrid cells established from a mitochondrial disease patient (Crimi et al., 2005; Fujita et al., 2007). Activating transcription factor 4 (ATF4) is a master regulator of integrated stress responses (ISR), in which a variety of stresses, including amino-acid starvation as well as glucose starvation, ER stress, hypoxia, and oxidative stress, induce phosphorylation of eIF2α followed by up-regulation of ATF4 to activate expression of stress-responsive genes (Harding et al., 2003; Jiang et al., 2004; Rouschop et al., 2010; Rzymski et al., 2010; Teske et al., 2011). It is noteworthy to point out that GDF15 has been shown to be up-regulated by ATF4 in mouse embryonic fibroblasts (Jousse et al., 2007). Taken together, such findings suggest that the ISR pathway may also contribute to the induction of GDF15 in response to defective energy metabolism and play a role in the pathogenesis of mitochondrial diseases.

In the present study, we validated the clinical usefulness of GDF15 as a diagnostic marker by determining the serum GDF15 levels in patients with mitochondrial diseases and in those with other pediatric diseases. The results showed that serum GDF15 levels were significantly elevated in patients with mitochondrial diseases, which finding is consistent with a recent report (Kalko et al., 2014). We also demonstrated that GDF15 had higher sensitivity and specificity than FGF21, which was recently identified as a sensitive and specific blood biomarker for muscle pathology in a wide range of mitochondrial diseases in adults and children (Suomalainen et al., 2011). Our small-scale study, however, may have underestimated the clinical usefulness of FGF21, because the AUC for FGF21 reported by 2 independent groups (0.95 and 0.91) was higher than that in the present study (0.787).

Using the multiplex suspension array, we also identified HGF, SCF, and SCGF-β as potential diagnostic markers for mitochondrial diseases. The ROC curve analysis, however, revealed that GDF15 had the maximum sensitivity and specificity for diagnosis of mitochondrial diseases compared with HGF, SCF, SCGF-β, or FGF21. Based on the microarray analysis, we also selected INHBE as the next best candidate gene (Table 2). INHBE is a member of the activin beta family, which has been reported to be primarily expressed in the liver and up-regulated by drug-induced ER stress, cysteine deprivation, and insulin treatment (Bruning et al., 2012; Dombroski et al., 2010; Hashimoto et al., 2009; Lee et al., 2008). Although secreted INHBE protein was not detectable in the conditioned medium from the cell cultures, we are currently investigating the clinical usefulness of INHBE as a biomarker for diagnosis and monitoring of the disease status and progression.

Table 3
Genes annotated to the extracellular space among those specifically down-regulated by lactate treatment for 8 h.

Gene symbol	Accession number	Entrez gene name	Fold change	
			L-8/L-0[a]	L-8/P-8[b]
CXCL1	NM_001511	Chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	−3.4	−2.6
PDZRN3	NM_015009	PDZ domain containing ring finger 3	−2.4	−2.0
SLC39A10	NM_020342	Solute carrier family 39 (zinc transporter), member 10	−2.3	−2.9
DKK1	NM_012242	Dickkopf 1 homolog (Xenopus laevis)	−2.1	−2.3

[a]Fold change between 8 h and 0 h after lactate treatment
[b]Fold change between lactate treatment and pyruvate treatment at 8 h

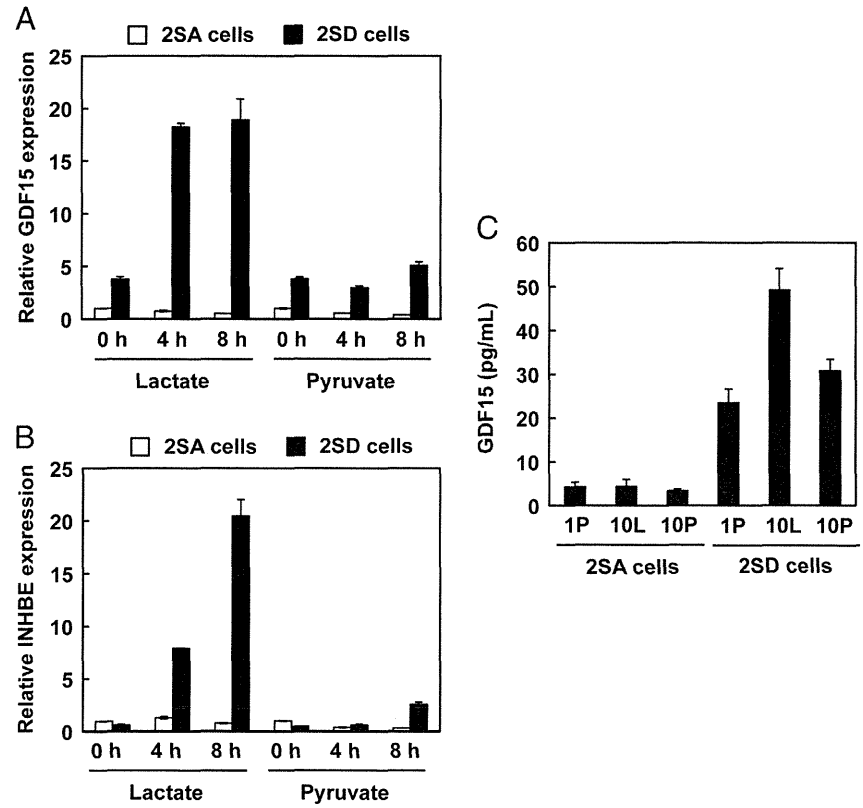

Fig. 4. Quantitative RT-PCR and ELISA for GDF15 and INHBE Total RNA isolated from 2SA and 2SD cells treated with 10 mM lactate or 10 mM pyruvate for 0, 4 or 8 h (n = 3) were subjected to quantitative RT-PCR for GDF15 (A) and INHBE (B). (C) The conditioned medium collected from 2SA and 2SD cell cultures treated with 10 mM lactate (10 L), 10 mM pyruvate (10P) or 1 mM pyruvate (1P) for 24 h was subjected to ELISA for GDF15 protein ($n = 3$).

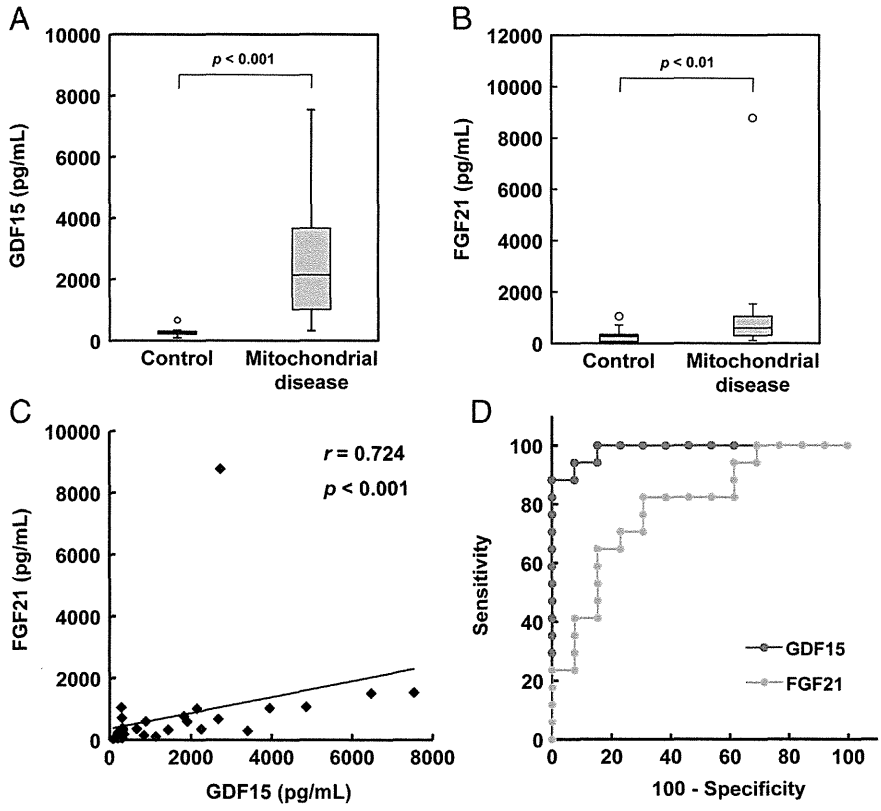

Fig. 5. Measurement of the GDF15 and FGF21 concentrations in the serum of patients. The serum GDF15 (A) and FGF21 (B) concentrations in 17 patients with mitochondrial diseases as well as those in 13 patients with other pediatric diseases were determined by ELISA. The outlier is shown with an open symbol. (C) A correlation analysis between the serum GDF15 and FGF21 levels was performed for the patients described above by use of IBM SPSS statistics. (D) The ROC curve analysis for GDF15 and FGF21 was performed. Areas under the curves (AUC) for GDF15 and FGF21 were 0.986 (95% CI 0.957-1.000) and 0.787 (95% CI 0.621-0.953), respectively.

It is well known that mitochondrial dysfunction is associated with the pathology of various diseases such as Parkinson's disease, Alzheimer's disease, diabetes, and aging (Exner et al., 2012; Lopez-Otin et al., 2013; Martin and McGee, 2014). GDF15, which may reflect mitochondria dysfunction, could be a useful marker for those diseases and the aging process. In support of this idea, the serum GDF15 level was reported to be elevated under various pathological conditions such as cancers, cardiovascular diseases, diabetes, and obesity (Dostalova et al., 2009; Kempf et al., 2007; Welsh et al., 2003); however, in most cases, it was not as high as that observed in mitochondrial diseases. Recent cohort studies also demonstrated that the serum GDF15 level is a novel predictor of all-cause mortality and is associated with cognitive performance and cognitive decline (Fuchs et al., 2013; Wiklund et al., 2010). We thus anticipate that GDF15 will attract more interest with respect to a variety of diseases and aging associated with mitochondrial dysfunction.

In conclusion, we identified GDF15 as a novel serum marker for the diagnosis of mitochondrial diseases and possibly both for monitoring the disease status and progression and for evaluating the therapeutic efficacy of pyruvate. Large-scale clinical trials including combined use of other markers such as FGF21 should confirm the clinical usefulness of GDF15.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.mito.2014.10.006.

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Review Article

Oxidative Stresses and Mitochondrial Dysfunction in Age-Related Hearing Loss

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Age-related hearing loss (ARHL), the progressive loss of hearing associated with aging, is the most common sensory disorder in the elderly population. The pathology of ARHL includes the hair cells of the organ of Corti, stria vascularis, and afferent spiral ganglion neurons as well as the central auditory pathways. Many studies have suggested that the accumulation of mitochondrial DNA damage, the production of reactive oxygen species, and decreased antioxidant function are associated with subsequent cochlear senescence in response to aging stress. Mitochondria play a crucial role in the induction of intrinsic apoptosis in cochlear cells. ARHL can be prevented in laboratory animals by certain interventions, such as caloric restriction and supplementation with antioxidants. In this review, we will focus on previous research concerning the role of the oxidative stress and mitochondrial dysfunction in the pathology of ARHL in both animal models and humans and introduce concepts that have recently emerged regarding the mechanisms of the development of ARHL.

1. Introduction

Oxidative stress represents an imbalance between the production of reactive oxygen species (ROS) and the detoxification of their reactive intermediates. ROS, such as hydroxyl radicals, superoxide anions, hydrogen peroxide, and singlet oxygen, are primarily generated by mitochondria in most mammalian cells and are generally regarded as the toxic side-products of cellular metabolism [1–3]. ROS are normally detoxified by a variety of antioxidant enzymatic scavengers, including superoxide dismutase (SOD), catalase, glutathione S-transferase (GST), and glutathione peroxidase (GPX) [4].

Mitochondria are a major site of ROS-induced oxidative damage [5, 6]. ROS generated by mitochondria are hypothesized to damage key mitochondrial components such as mitochondrial DNA (mtDNA), mitochondrial membranes, and respiratory chain proteins and nuclear DNA that affect mitochondrial function. mtDNA is a circular, closed, double-stranded molecule and is not protected by histones. Therefore, mtDNA is more susceptible to DNA insults in comparison with nuclear DNA. Most of mtDNA mutations are characterized by heteroplasmy, which is defined as the presence of more than one an organellar genome within a cell or tissue from a single individual. As the percentage of mutant alleles increases, the mitochondrial bioenergetic defect becomes more severe. The expression of disease depends on the percentage of mutant alleles.

It has been widely considered that aging is the process of accumulated oxidative damage caused by ROS [7, 8]. This damage accumulates over time, causing mitochondrial dysfunction and an associated decrease of energy production, and results in tissue dysfunction. ROS production increases with age and it is known that oxidative stress and associated mitochondrial dysfunction play an important role in aging and age-related diseases [1, 2].

Age-related hearing loss (ARHL), which is also called presbycusis, is the progressive loss of hearing associated with aging and is the most common sensory disorder in the elderly population [9–11]. ARHL afflicts approximately half of the people over 65 years of age in the United States [12]. The prevalence of the ARHL is expected to increase as the elderly population grows [9, 13, 14]. It has been proposed that ARHL is associated with many factors, including environmental,

medical, and hereditary factors [12, 15]. So far, no effective treatment has been found for this age-related disorder.

Many studies have been conducted based on the assumption that age-related oxidative stress and mitochondrial dysfunction could be an underlying pathology of ARHL as well as other age-related diseases. In this review, we will focus on previous research concerning the role of the oxidative stress and mitochondrial dysfunction in the pathology of ARHL in both animal models and humans and introduce concepts that have recently emerged as potential mechanisms for the development of ARHL.

2. Pathological Findings in ARHL

Sound waves travel down the external ear canal and cause the tympanic membrane to vibrate. The ossicles in the middle ear link the vibrating tympanic membrane to the cochlea, the auditory end organ of the inner ear. The cochlea is filled with fluid that vibrates in response to the movement of the ossicles. The inner and outer sensory hair cells are located within a core component of the cochlea, the organ of Corti. When a sound pressure wave travels from the basal turn to the apical turn of the cochlea, the basilar membrane vibrates [16]. Displacement of stereocilia, the mechanosensing organelles of the hair cell, in association with the vibration of the basilar membrane, opens transduction ion channels, allowing entry of potassium ions from the endolymph produced by the stria vascularis. This transduction current then activates voltage-dependent calcium channels along the hair cell lateral wall and base [17]. The inner hair cells release the neurotransmitter glutamate to encode acoustic signals for the adjacent spiral ganglion neurons (SGNs), which are the primary auditory neurons [18].

Based on postmortem pathological analysis, ARHL in humans is generally classified into 3 types: sensory hearing loss (loss of sensory hair cells), neuronal hearing loss (loss of SGNs), and metabolic hearing loss (atrophy of the stria vascularis) [9, 19], although it is now well established that most cases of ARHL exhibit mixed pathological changes [9]. This idea is supported by the observation that the progressive loss of hair cells and SGNs leads to ARHL because these two cell types do not regenerate in mammals.

3. Candidate Genes for ARHL Associated with Oxidative Stress and Mitochondrial Dysfunction

Many genetic investigations of ARHL, such as genome-wide association studies and candidate-gene-based association studies, have been performed recently [20]. With regard to oxidative stress and mitochondrial function, several genes and loci have been proposed as a result of candidate-gene-based association studies, which are based on hypotheses about the relationship between specific known loci and phenotypes.

The superoxide dismutases (SODs), which catalyze the dismutation of superoxide into oxygen and hydrogen peroxide, are an important part of the antioxidant defense system against ROS. Recently, evidence from the London ARHL cohort suggested an effect of common superoxide dismutase 2 (SOD2, also known as manganese SOD or mitochondrial SOD) promoter variation, −38 C > G, on SOD2 promoter regulation and linked it to ARHL risk in men; however, this association was only suggestive due to a lack of replication [21].

The glutathione S-transferases (GSTs) catalyze the detoxification of electrophilic substrates by conjugation with reduced glutathione and participate in intracellular binding and transport of lipophilic substances. Decreased glutathione and GST activity levels cause an increase in susceptibility to cell damage. A previous study investigated the association between ARHL and genes related to oxidative stress using a large set of samples from two population groups, a general European group and a Finnish group [22]. Although an association between the polymorphisms of glutathione S-transferase, mu 1 (*GSTM1*) or glutathione S-transferase, theta 1 (*GSTT1*), and ARHL was not detected in the former population, there were significant associations between both genes and ARHL in the latter population.

Mitochondrial uncoupling proteins (UCPs), which are members of the larger family of mitochondrial anion carrier proteins, facilitate the transfer of anions from the inner to the outer mitochondrial membrane and the return transfer of protons from the outer to the inner mitochondrial membrane. UCPs reduce the mitochondrial membrane potential in mammalian cells. The main function of uncoupling protein 2 (UCP2) is the control of mitochondria-derived ROS [23]. *UCP2* Ala55Val polymorphisms exhibited a significant association with ARHL in a Japanese population [24].

4. Deletions and Mutations of mtDNA in the Peripheral Auditory System of ARHL Patients

Acquired mtDNA defects have been proposed as important factors in aging. Increases in deletions, mutations, or both, in mtDNA have been reported in human temporal bone studies from ARHL patients in comparison with normal-hearing control tissues. A 4977-base pair deletion of mtDNA from celloidin-embedded temporal bone sections was significantly more frequent in cochlear tissue from ARHL patients in comparison to those with normal hearing [25]. Another study reported that quantitative analysis of the mtDNA in archival cochlear tissue samples revealed a mean common deletion level of $32 \pm 14\%$ in ARHL patients, in comparison with a level of $12 \pm 2\%$ in age-matched controls with normal hearing, and showed a significant correlation between the common deletion level and the severity of hearing loss [26]. Cytochrome c oxidase subunit 3 (COX3) expression was significantly diminished in SGNs from ARHL patients in comparison with age-matched normal-hearing individuals. In addition to the mtDNA common deletion, other deletions involving the mtDNA major arc contributed to the observed deficit in COX3 expression [27]. Mutations within the cytochrome c oxidase subunit 2 (*COX2*) gene in the spiral ganglion and