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Growth Differentiation Factor 15 as a Useful Biomarker for Mitochondrial Disorders

Shuichi Yatsuga, MD, PhD,1 Yasunori Fujita, PhD,2 Akiko Ishii, MD, PhD,3 Yoshihiro Fukumoto, MD, PhD,4 Hajime Arahata, MD, PhD,5 Tatsuyuki Kakuma, PhD,6 Toshio Kojima, MD, PhD,7 Masafumi Ito, PhD,2 Masashi Tanaka, MD, PhD,8 Reo Saiki, MD,1 and Yasutoshi Koga, MD, PhD1

Objective: The diagnosis of mitochondrial disorders (MDs) is occasionally difficult because patients often present with solitary, or a combination of, symptoms caused by each organ insufficiency, which may be the result of respiratory chain enzyme deficiency. Growth differentiation factor 15 (GDF-15) has been reported to be elevated in serum of patients with MDs. In this study, we investigated whether GDF-15 is a more useful biomarker for MDs than several conventional biomarkers.

Methods: We measured the serum levels of GDF-15 and fibroblast growth factor 21 (FGF-21), as well as other biomarkers, in 48 MD patients and in 146 healthy controls in Japan. GDF-15 and FGF-21 concentrations were measured by enzyme-linked immunosorbant assay and compared with lactate, pyruvate, creatine kinase, and the lactate-to-pyruvate ratio. We calculated sensitivity and specificity and also evaluated the correlation based on two rating scales, including the Newcastle Mitochondrial Disease Rating Scale (NMDAS).

Results: Mean GDF-15 concentration was 6-fold higher in MD patients compared to healthy controls (2,711 ± 2,459 pg/ml vs 462.5 ± 141.0 pg/mL; p < 0.001). Using a receiver operating characteristic curve, the area under the curve was significantly higher for GDF-15 than FGF-21 and other conventional biomarkers. Our date suggest that GDF-15 is the most useful biomarker for MDs of the biomarkers examined, and it is associated with MD severity.

Interpretation: Our results suggest that measurement of GDF-15 is the most useful first-line test to indicate the patients who have the mitochondrial respiratory chain deficiency.

Mitochondrial disorders (MDs) are occasionally difficult to diagnose because patients present with one or a combination of symptoms, such as psychomotor developmental delay, failure to thrive, short stature, seizures, muscle weakness, hearing loss, heart failure, renal failure, or diabetes. Because the plasma levels of lactate and pyruvate are not always accurate biomarkers or MD diagnosis, a novel biomarker, which is more sensitive, specific, reproducible, and quantitative, is required. Minimizing the number of specimens examined for MD diagnosis is a very important issue for clinicians and/or diagnostic centers in terms of cost- and time-effectiveness.

No reliable biomarker was available for the screening or diagnosis of MDs until 2011.1 Because an MD
may present as “any symptom in any organ at any age.”\textsuperscript{2} we must develop an optionally useful biomarker for MD diagnosis and the evaluation of therapeutic efficacy. In 2011, serum fibroblast growth factor 21 (FGF-21) was reported to be a useful biomarker for the screening and diagnosis of muscle-manifested MDs.\textsuperscript{3,4} The proposed diagnostic process for MDs is as follows: (1) clinical assessment; (2) measurement of the FGF-21 level; (3) long-range polymerase chain reaction, next-generation sequencing, or whole-exome sequencing of nuclear DNA; and (4) with/without histopathological and biochemical analysis via muscle biopsy, which may indicate approximately 70% to 80% of clinically suspected MDs.\textsuperscript{5} FGF-21 appears to be a useful novel biomarker for the specific screening of MDs because the measurement of FGF-21 involves a less-invasive approach than muscle biopsy.

Growth differentiation factor 15 (GDF-15) is a member of the transforming growth factor beta superfamily. Expression of GDF-15 in nearly all tissues suggests its general importance in essential cellular functions. GDF-15 has been proposed as a biomarker of heart disease, kidney disease, and cancers; however, its exact biological functions remain poorly understood. It is hypothesized that GDF-15 is elevated by activating transcription factor 4 (ATF4), which is a stress-responsive gene induced by various stresses.\textsuperscript{6} GDF-15 is currently under investigation as a marker of the clinical risk factors, mortality, morbidity, and effectiveness of treatments for heart disease, kidney disease, and some cancers; however, GDF-15 does not appear to play a useful role as a diagnostic marker.\textsuperscript{7} In 2014, Kalko et al investigated the gene expression profile of TK2-deficient human skeletal muscle using complementary DNA microarrays and demonstrated that serum GDF-15 (GDF-15) may be a novel biomarker for MDs.\textsuperscript{8} We performed a microarray analysis of 2SD cybrid cells that harbor a mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes (MELAS)-causing mutation and control cells and demonstrated that expression and secretion of GDF-15 were increased in 2SD cells loaded by lactate; these findings suggest that GDF-15 may serve as a useful marker for MD diagnosis and evaluation of the therapeutic efficacy.
of drugs.6 In this study, we measured GDF-15 concentrations from patients who suffered from MDs, disease controls, and healthy controls, and evaluated its usefulness for MD diagnosis.

Patients and Methods

Participants

All MD patients who fulfilled the clinical diagnostic criteria (MELAS; mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes; MELAS, for which the main symptom was muscle weakness; Leigh syndrome [LS]; overlapping MELAS/LS; and Kearns-Sayre syndrome [KSS]) and carried a known genetic abnormality were enrolled in this study from 2005 to 2013 (Fig 1A). DNA, enzyme, or muscle histopathological analyses were performed on all patients (Supplementary Table 1). The disease controls consisted of adult and child patients with non-MD, including 6 defined Duchenne muscular dystrophy (DMD), 9 multiple sclerosis (MS), 4 anti-AQP4 (aquaporin 4) antibody-positive optic neuritis, 10 limbic encephalitis (anti-GluR [glutamate receptor] antibody-positive, anti-NMDA [N-methyl-D-aspartate] receptor-positive, varicella zoster virus infection, and lung cancer), 1 brainstem encephalitis, 1 meningoencephalitis, 1 SLE (systemic lupus erythematosus) with CNS (central nervous system), 1 NMO (neuromyelitis optica), 1 HUS (hemolytic uremic syndrome) encephalitis, and 8 defined heart failure (HF) patients. All DMD patients were diagnosed with a large deletion of the dystrophin gene by multiplex ligation-dependent probe amplification (MLPA). All MS, optic neuritis, limbic encephalitis, brainstem encephalitis, meningoencephalitis, NMO, and HUS encephalitis patients were diagnosed with fulfilled criteria. All HF patients were diagnosed based on an ejection fraction (EF) below 50% in this study (Supplementary Table 1). Healthy adult and child volunteers without treatment or ambulatory medical care were also recruited from Kurume University Hospital, Fukuoka, Japan (Fig 1B). Mean age, BMI, and % males are shown in Table 1. The study protocol was approved by the institutional review boards of all centers (coordinator: Kurume University Hospital, #13099). Written informed consent was obtained from all subjects before enrollment.

For the healthy controls, we calculated the body mass index (BMI: kg/m²) of each individual and recruited healthy adults (≥20 years old) who exhibited a BMI between 18 and 25 and children (<20 years old) who exhibited a BMI within ±2 standard deviations (SDs) of the average for each age in the Japanese population.

Procedures

The serum samples used in this study were collected for analysis from 2012 to 2014. All samples were stored at –80°C until analysis. We measured the GDF-15 (R&D Systems, Minneapolis, USA) and FGF-21 (FGF-21; BioVendor, Brno, Czech Republic) concentrations in duplicate samples by enzyme-linked immunosorbent assay (ELISA). The samples for which the concentration of GDF-15 or FGF-21 was higher than the range of detection were diluted 10-fold with dilution buffer and reanalyzed. The value from each assay was compared with reference GDF-15 and FGF-21 concentrations. We also measured the levels of conventional biomarkers, such as lactate, pyruvate, the lactate/pyruvate (L/P) ratio, and creatine kinase (CK). In addition, we recorded the height, body weight, BMI, and blood sugar levels. The results of each assay were compared with a linear calibration curve. All assays were performed by a trained scientist (S.Y. and R.S.).

The enrolled MD patients were clinically evaluated for disease severity using two rating systems: the Japanese Mitochondrial Disease Rating Scale (JMDRS)9 and the Newcastle Mitochondrial Disease Rating Scale (NMDAS).10 All scores were also evaluated with respect to the levels of GDF-15 or FGF-21 using correlation coefficients.

Statistical Analysis

The diagnostic test for MD using GDF-15 was evaluated by receive operating characteristic (ROC) curve analyses. The threshold value was determined using the Youden index. Ninety-five percent confidence intervals (95% CIs) for the area

<p>| Table 1. Demographics of the Number, Mean Age, Mean BMI, and % Males for MD Patients and Non-MD Patients |
|---------------------------------|-------|----------------|------------------|</p>
<table>
<thead>
<tr>
<th><strong>Number</strong></th>
<th><strong>Controls</strong></th>
<th><strong>MD Patients</strong></th>
<th><strong>Disease Controls</strong></th>
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<td>Age, years</td>
<td>23.6 ± 13.7</td>
<td>33.6 ± 18.7**</td>
<td>41.4 ± 17.2***</td>
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<tr>
<td>BMI, kg/m² (n)</td>
<td>19.1 ± 3.0 (146)</td>
<td>17.4 ± 3.3 (48)**</td>
<td>21.7 ± 3.8 (41)**</td>
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<tr>
<td>% males (n)</td>
<td>48.0 (70)</td>
<td>48.0 (23)</td>
<td>54.7 (23)</td>
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Mean age was significantly higher in MDs patients (33.6 ± 18.7 years old; p < 0.01) and disease controls (41.4 ± 17.2 years old; p < 0.001) than in healthy controls (23.6 ± 13.7 years old). Mean BMI was significantly lower in MDs patients (17.4 ± 3.3 kg/m²; p < 0.001) than in healthy controls (19.1 ± 3.0 kg/m²; p < 0.001) than in healthy controls (19.1 ± 3.0 kg/m²). Mann–Whitney U test: **p < 0.01; ***p < 0.001. BMI = body mass index; MD = mitochondrial disorder.
under the curve (AUC) of the ROC were obtained. Leave-one-out cross-validation of GDF-15 and FGF-21 was performed. Sensitivity and specificity of GDF-15 were compared with a measurement of FGF-21 using a McNemar test. Comparison of two correlated nonparametric AUCs was also performed. Spearman correlation coefficients were used to examine the associations between the biomarkers.

Role of the Funding Source
This work was supported, in part, by grant nos. 25461571 (to Y.K.), 25860891 (to S.Y.), and 25242062 (to M.T.) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) and by Grant-in-Aid no. 24-Nanchi-Ippan-005 (to Y.K.) for Research on Intractable Diseases from the Ministry of Health, Labor and Welfare of Japan.

All patients and control individuals had perceived adequate information concerning to this study according to the guidelines of the Declaration of Helsinki.

Results
We analyzed samples from 48 patients who fulfilled the clinicopathological and genetic criteria of MD. Of these 48 MD patients, 27 patients suffered from MELAS and carried the m.3243A>G mutation in mitochondrial DNA (mtDNA), with the exception of 3 patients whose
molecular defects had not been identified. Eleven patients suffered from MELA and carried the m.3243A>G mutation. Three patients were diagnosed as LS, including 1 patient who exhibited a cytochrome c oxidase deficiency, 1 patient who carried the m.3243A>G mutation, and 1 patient who carried the m.8993T>C mutation. Two patients suffered from overlapping MELAS/LS and carried the m.3243A>G mutation, and 5 patients suffered from KSS and carried a common large deletion in mtDNA. The demographic characteristics of the patients in this study are shown in Table 1. We also recruited 149 healthy controls; however, 3 individuals were excluded because of abnormal thyroid function (Fig. 1B).

We evaluated the severity of each patient using two rating systems, the JMDRS\(^9\) and the NMDAS,\(^10\) as shown in Supplementary Table 1. The JMDRS has been used in a Japanese MELAS cohort study and has been demonstrated to be useful for the evaluation of disease progression at 5-year intervals.\(^7\) Mean scores of MD patients on the JMDRS and the NMDAS are shown in Table 2.

The concentrations of GDF-15 and FGF-21 did not differ by age, sex, or diurnal variation.\(^12\) The mean concentration of GDF-15 and FGF-21 is shown in Supplementary Table 2. The concentration range of GDF-15 in the MD patients was substantially higher compared to the controls. GDF-15 concentration did not overlap between MD patients and controls; however, FGF-21 concentration occasionally overlapped. In contrast, the GDF-15 reference range in healthy controls was markedly narrow, as shown in Figure 2. GDF-15 concentration was higher in HF patients compared to controls; however, FGF-21 was not different between the HF patients and controls. GDF-15 and FGF-21 concentrations were not significantly different between the DMD, the MS, optic neuritis, limbic encephalitis patients, and controls (Fig 3).

Among the various MD subtypes, GDF-15 and FGF-21 concentrations were the highest in LS and were 10-fold higher than controls. GDF-15 and FGF-21 concentrations in MELAS and MELA patients displayed broad distributions. GDF-15 and FGF-21 concentrations in KSS patients tended to be lower compared to LS, MELAS, and MELA patients; however, they were significantly higher than the controls (Supplementary Table 2).

The correlation coefficients between the concentrations of GDF-15 and FGF-21 and the JMDRS and NMDAS were positively correlated (GDF-15: \(r = 0.76\) \([p < 0.001]\) for the JMDRS and \(r = 0.67\) \([p < 0.001]\) for the NMDAS).

FIGURE 3: Comparison of biomarkers for MDs, and GDF-15 and FGF-21 concentrations for MDs and non-MDs. GDF-15 and FGF-21 concentrations in the patients who suffered from MD subtypes, DMD, MS, optic neuritis, limbic encephalitis, brainstem encephalitis, meningoencephalitis, SLE with CNS, NMO, and HUS encephalitis, and HF, and the control group are shown. Each MD subtype displayed significantly higher levels of GDF-15 and FGF-21 than the control group. HF displayed significantly higher levels of only GDF-15 than the control group. (A) GDF-15 and (B) FGF-21. CNS = central nervous system; DMD = Duchenne muscular dystrophy; FGF-21 = fibroblast growth factor 21; GDF-15 = growth differentiation factor 15; HUS = hemolytic uremic syndrome; KSS = Kearns-Sayre syndrome; LS = Leigh syndrome; MD = mitochondrial disorder; MS = multiple sclerosis; MELA = mitochondrial encephalopathy, lactic acidosis (mitochondrial myopathy); MELAS = mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes; NMO = neuromyelitis optica; SLE = systemic lupus erythematosus.
for the NMDAS; FGF-21: \( r = 0.69 \) \( p < 0.001 \) for the JMDRS and \( r = 0.59 \) \( p < 0.001 \) for the NMDAS, as shown in Figure 4. The concentration of GDF-15 and FGF-21 were positively correlated \( (r = 0.64; p < 0.001) \), as shown in Figure 5.

AUC analyses indicated that the sensitivity and specificity were significantly larger for GDF-15 than those for FGF-21, lactate, pyruvate, the L/P ratio, or CK. The AUC was 0.997 (95% CI: 0.993–1.000) for GDF-15, 0.889 (95% CI: 0.837–0.962) for FGF-21,
In general, LS is clinically recognized as the most severe phenotype of the MD subtypes examined in this study because LS patients typically die before 10 years of age. According to both scaling system scores, LS displayed the highest scores of all examined MD subtypes. We can distinguish between non-MD patients with HF and/or renal failure, and MDs without those complications, by the information of clinical findings and laboratory data. In these clinical conditions, we believe that we can distinguish MD patients from patients with MD-like symptoms by evaluation of GDF-15.
that ranged from normal function, easy fatigability, severe myopathy with sensorineural hearing loss, and diabetes mellitus to bed-ridden (terminal stage). On the other hand, we believe that MELA and KSS patients typically maintain normal or subnormal states with respect to the activities of daily living compared with LS and MELAS patients.

The correlations between the JMDRS and NMDAS scores and the GDF-15 and FGF-21 concentrations are shown in Figure 4. The scores according to both scale systems were the highest for LS; these scores are strongly influenced by clinical severity, and LS displayed the most severe phenotype in our study. The LS patients in our study were bed-ridden and tube-fed. In contrast, the scores for MELAS patients were highly variable for both scales and were lower than those for LS, but higher than those for KSS and MELA. The clinical scores of MELAS Patients 17 and 18, who were in the bed-ridden stage, were high on both the JMDRS and the NMDAS, which is consistent with the levels of GDF-15 and FGF-21. The overlapping MELAS/LS patients displayed highly variable scores among individuals. Thus, GDF-15 and FGF-21 may serve as quantitative biomarkers.

These results demonstrate that GDF-15 is the most useful biomarker for MDs compared with FGF-21, lactate, pyruvate, the L/P ratio, and CK. GDF-15 may serve as a useful tool to quantitatively monitor disease severity of our indexed MD subtypes. GDF-15 is a more useful biomarker for muscle-related MDs because of its substantially higher sensitivity and specificity than FGF-21 and other conventional biomarkers. FGF-21 was the best biomarker for muscle-manifested MDs, as reported in 2011. We originally used FGF-21 as a primary screening test for MDs; however, it was occasionally difficult to distinguish MD patients from non-MD individuals based on FGF-21 values because the reference range of FGF-21 was broad. In contrast, the reference range of GDF-15 was narrow and rarely overlapped between MD patients and non-MD individuals (Fig 2). Our reference range of GDF-15 was in accord with previous studies (Fig 7). GDF-15 may be especially useful for the diagnosis of LS and KSS because serum lactate is rarely elevated in these MDs. The present results revealed that GDF-15 was significantly elevated in MD patients, which is consistent with our recent report.

GDF-15 appeared to increase with the clinical severity of the MD (Fig 4). In general, MD severity is ranked in the following order: LS, overlapping MELAS/LS, MELAS, KSS, and MELA. In our study, mean GDF-15 levels were ranked in the following order: LS, MELAS, KSS, overlapping MELAS/LS, and MELA. These results suggest that GDF-15 may serve as a useful biomarker not only for the diagnosis, but also the determination of severity of MDs. GDF-15 may be potentially as useful as FGF-21 for the evaluation of drug efficacy; however, it should be verified by longitudinal studies in future.

The AUC was significantly larger for GDF-15 (0.997) compared with the conventional MD biomarkers, including FGF-21 (Fig 5). Our study demonstrated that the AUC was also larger for FGF-21 (0.889) compared with the other conventional MD biomarkers; however, the AUC was slightly larger for the L/P ratio (0.917) than for FGF-21 (0.889). The AUC for the L/P ratio (0.917) was within the 95% CI of a previous study (0.82–0.98). The AUC for sFGF-21 (0.889) was not within the 95% CI of a previous study (0.94–0.99). The calculated threshold values of GDF-15 and FGF-21 were 707.0 and 343.8 pg/mL, respectively. We evaluated the threshold values of 710.0 and 350.0 pg/mL for GDF-15 and FGF-21, respectively. The sensitivity and specificity of GDF-15 were 97.9% and 95.2%, respectively, and were substantially higher than FGF-21, which were 77.1% and 87.7%, respectively. Ultimately, GDF-15 was a more useful biomarker than FGF-21 whether we used our or other threshold values. The AUC was higher for FGF-21 than for the L/P ratio in a previous study; however, the AUC was comparable between FGF-21 and the L/P ratio in our study. We hypothesized that these differences in the AUC for FGF-21 were because of the different MDs and ages. Our MD patients were primarily typical and classical, and the FGF-21 levels may be lower in our study than in the previous study. However, the patients in the previous study were classified as MELAS, Alpers syndrome, MIRAS, and others; moreover, 26 of the 67 (38.8%) MD patients were children. In our study, 12 of the 48 (25%) MD patients were children. The child MD patients exhibited higher FGF-21 levels in both our study (LS and MELAS/LS overlapping) and the previous study (Alpers syndrome and patients with mtDNA mutations). Further assessment of the various MD subtypes is required.

On the other hand, GDF-15 in HF patients was significantly elevated compared to controls as well as MDs. However, FGF-21 in HF patients was not significantly elevated with controls. It is indicated that only GDF-15 does not completely distinguish HF with MDs from HF without MDs. We calculated that GDF-15 and FGF-21 were combined to analyze the severity and sensitivity for MDs, however, the severity and sensitivity in MDs were lower in combination than only GDF-15 (data not shown). Furthermore, the patients' number was too small to evaluate the severity and sensitivity.
The mechanism of elevation of GDF-15 is not clear in MDs patients. ATF4 is a master regulator of upstream of stress signals. GDF-15 was upregulated by ATF4 in mouse embryonic fibroblast. FGF-21 was also induced by ATF4 in mouse. We revealed that GDF-15 and FGF-21 were elevated in MDs compared to controls, and both GDF-15 and FGF-21 levels were correlated with MDs. Therefore, we suggested that the mechanism of elevation of GDF-15 in MDs is derived from ATF4 signaling and occurred from various stresses during mitochondrial dysfunctions. More investigations are requested to clarify what kinds of interactions exist in the disease condition such as HF, kidney disease, and cancers.

In conclusion, our data suggest that the measurement of GDF-15 in combination with FGF-21 by ELISA serves as the most useful first-line test in patients with suspected MDs to prioritize patients for muscle biopsy. Using new biomarkers, we may be able to diagnose MDs at a non-specialist hospital and reduce the unnecessary invasive biopsy procedures in children and adults.

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Authorship
Conception and design of the study: SY, YF, TK, MI, MT, YK. Acquisition, analysis & interpretation of data: SY, AI, YF, HA, TK, MT, RS, YK. Drafting the manuscript: SY, YF, TK, MI, MT, TK, MT, RS, YK.

Potential Conflicts of Interest
The authors declare that there is no conflict of interests regarding the publication of this paper.


