の機能評価についての解析を一部始めることが出来た。

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## G. 知的財産権の出願・登録状況

（予定を含む。）

１. 特許取得 なし

２. 実用新案登録 なし

３. その他 なし

別紙３

厚生労働科学研究費補助金（難治性疾患政策研究事業）
HSD10 病の発症形態と患者数の把握、診断基準の作成に関する研究（H26-難治等（難）一般-021）
研究分担報告書

# 研究題名

# HSD10 病の生化学診断の問題点の検討

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## 研究要旨

βケトチオラーゼ（BKT）欠損症と HSD10 欠損症は、イソロイシンの代謝過程において障害部位が隣接している。臨床症状は大きく異なるが、異常代謝産物の種類は類似している。いずれもアシルカルニチン分析では C5-OH と C5:1 の上昇がみられる。一方尿中有機酸分析では、2-メチル-3-ヒドロキシ酪酸（2M3HBA）とチグリルグリシン(TG)の増加がみられるが、HSD10 欠損症では 2-メチルアセト酢酸(2MAA)が検出されない。すなわち 2MAA が検出されるかどうかが生化学診断でのほぼ唯一の鑑別点となる。しかし実際には BKT 欠損症でも 2MAA の検出されな症例が多く、GC/MS による生化学診断が必ずしも容易ではない。

この理由を検討したところ、2MAA の不安定性を確認した。すなわち 2MAA が検出できなかった原因として、①凍結してあっても古い尿検体や、室温で乾燥した尿ろ紙検体では 2MAA は分解して検出困難な場合があること、②生化学的に軽症の BKT 欠損症では、2MAA が検出困難な可能性があることが明らかになった。

臨床症状と 2MAA の不安定性を加味しながら BKT 欠損症と HSD10 欠損症の生化学診断を行う必要がある。2MAA の有無によって鑑別する GC/MS 分析の自動診断アルゴリズムを開発した。これにより尿中有機酸分析による生化学診断、鑑別診断が容易になることが期待される。



## 研究協力者

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## A．研究目的

イソロイシン代謝過程のβケトチオラーゼ(BKT)欠損症と HSD10 欠損症は、図 1 に示すように代謝障害部位が近接しているため、異常代謝産物が類似している。尿中有機酸分析で、2-メチル-3-ヒドロキシブチリル-CoA 脱水素酵素（2M3HBD）より下流の 2-メチルアセト酢酸(2MAA)がは、βケトチオラーゼ（BKT）欠損症でみられるが HSD10 欠損症ではみられないという点が、両者の大きな鑑別点である。

しかし、これまでに診断した多くの BKT 欠損症患者の分析において、鑑別点となるべき 2MAA が検出されず、臨床経過から鑑別せざるを得ないことがあった。本研究において、尿中有機酸分析による 2MAA の検出の問題点を明らかにし、生化学的診断法の改良に関する研究を行った。

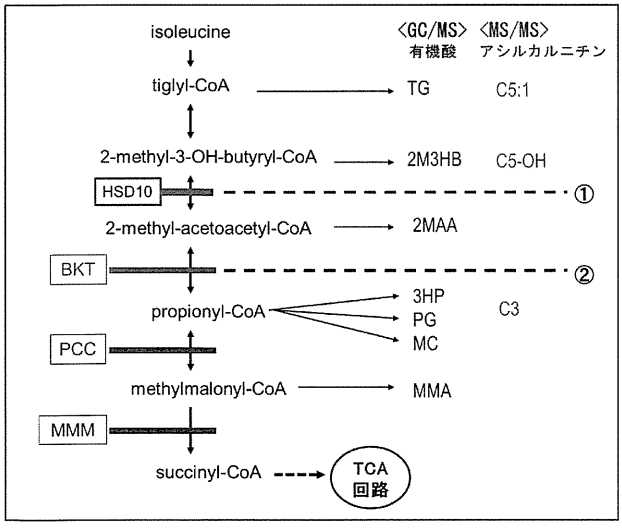


図 1．イソロイシンの代謝経路
略字：BKT＝βケトチオラーゼ（βケトチオラーゼ欠損症の欠損酵素，短鎖型チオラーゼ T2 と同義）
PCC＝プロピオニル-CoA カルボキシラーゼ（プロピオン酸血症の欠損酵素）
MMM＝メチルマロニル-CoA ムターゼ（メチルマロン酸血症の欠損酵素）
TG＝チグリルグリシン
2M3HBA=2-メチル-3-ヒドロキシ酪酸
2MAA=2-メチルアセト酢酸
3HP＝3-ヒドロキシプロピオン酸
PG=プロピオニルグリシン
MC＝メチルクエン酸；MMA=メチルマロン酸。

HSD10 欠損症では①より上の代謝産物が、βケトチオラーゼ欠損症では②より上の代謝産物が蓄積する。

## B．研究方法

### 1）血中アシルカルニチン分析

血液ろ紙、または血清をブチル誘導体化していたものをタンデムマスによって分析した。前処理はスタンダードな前処理法に準じた（Shigematsu Y, et al., J Chromat B 792: 63-72, 2003）。

### 2）尿中有機酸分析

溶媒抽出-オキシム・トリメチルシリル誘導体化（oxime-TMS 化）して GC/MS 分析を行った（Fu XW, et al., J Chromat B 758: 87-94, 2001）。インド、ベトナムおよび日本人のβケトチオラーゼ欠損症患者それぞれ 1 例ずつと、HSD10 欠損症の日本人症例 3 例の検体を対象とした。インドおよびベトナムの症例の検体はガスリーろ紙を用いた血液ろ紙、および尿ろ紙の形で送られた。日本人症例は、それぞれ血液濾紙と凍結された尿を用いて分析を行った。

### 3）アセト酢酸の安定性の検討

メチルアセト酢酸（2MAA）がコマーシャルで入手困難なことから、類似した構造を持つアセト酢酸（AAA）を濾紙にしみこませて室温に放置して、28 日間の安定性を検討した。

## C．研究結果

### 1）アセト酢酸の安定性の検討

**濾紙にしみこませたいくつかのαケト酸またはβケト酸の室温での安定性を検討した。図 2 に示すように、アセト酢酸（AAA）は 7 日後に半減し、2 週間後には 1/5 以下に低下していた。**

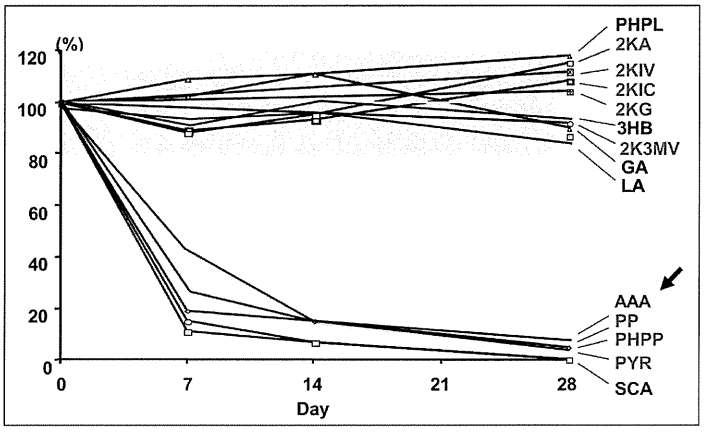


**図 2．尿ろ紙中の代謝産物の安定性**

Fu XW et al, J Chromat B 758: 87-94, 2001 より一部引用。尿ろ紙を室温に放置した。縦軸は%変化、横軸は放置した日数。略字：PHPL＝p-ヒドロキシフェニル乳酸；2KA＝αケトアジピン酸；2KIV＝αケトイソバレリン酸；2KIC=αケトイソカプロン酸；2KG=αケトグルタル酸；2K3MV=αケト-3-メチルバレリン酸；GA=グルタル酸；Pyr＝ピルビン酸；PP＝フェニルピルビン酸；PHPP＝p-ヒドロキシフェニルピルビン酸;SCA＝サクシニルアセトン；AAA＝アセト酢酸（矢印）。

### 2）尿中有機酸所見

GC/MS を用いた尿中有機酸分析において、HSD10 欠損症患者では（図 3A）乳酸の著増の他に 2M3HBA と TG の増加を認めた。βケトチオラーゼ欠損症の分析例は同一症例で 1986 年に採取された検体（図 3B）と、2013 年 12 月に採取された検体（図 3C）とを解析し、いずれも 2M3HBA と TG が検出されたが、図 1B では 2MAA は検出されず、図 1C では 2MAA が検出された。古い保存検体では 2MAA を検出しにくいことが明らかになった。

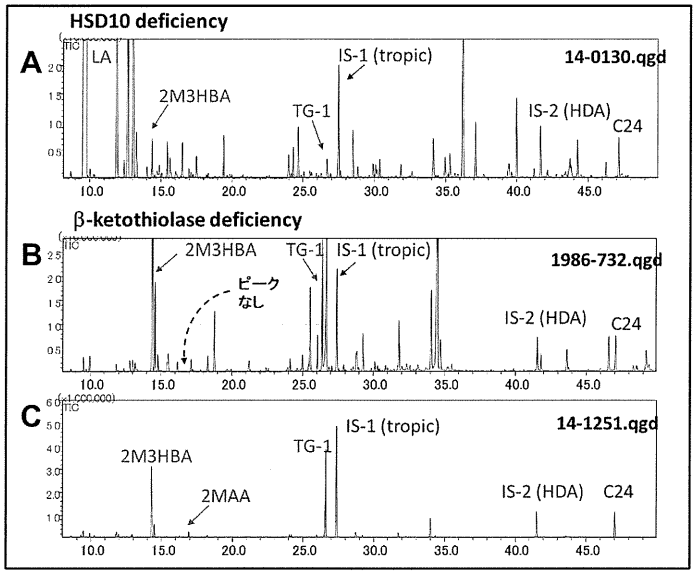


**図 3．尿中有機酸クロマトグラム**

A＝HSD10 欠損症；B＝βケトチオラーゼ欠損症（日本人症例、1980 年代の採取検体）；C＝B と同一症例で、2013 年 12 月の採取検体。

### 3）2MAA の GC/MS を用いた自動診断アルゴリズムによる同定

2MAA の oxim-TMS 誘導体の分子量は m/z 275 と推定された。この分子イオンを C-ion に、フラグメントイオンの中でも特徴的な m/z 260 を Q-ion とし、Q/C 比を 81.4 と設定した。このデータをガスクロマトグラフィー・マススペクトロメトリー（GC/MS）を用いた自動診断アルゴリズムに組み込み再解析を行うと、図 4 のように HSD10 欠損症では未検出だったが、BKT 欠損症では尿ろ紙も凍結尿もいずれでも 2MAA が検出可能であった。

診断アルゴリズムは、表 1 に示すように、2-methyk-3-OH-butyrate と tiglylglycine が同時に検出された時、診断アウトプットには、βケトチオラーゼ欠損症、または HSD10 欠損症が示される。さらにこの 2 者に加えて第 3 の化合物として methylacetoacetate が検出されれば、「βケトチオラーゼ欠損症」と診断されるようアルゴリズムを設定した。これらの生化学診断には、下流代謝産物の methylcitrate および methylmalonate が出ていないことを確認する必要がある。

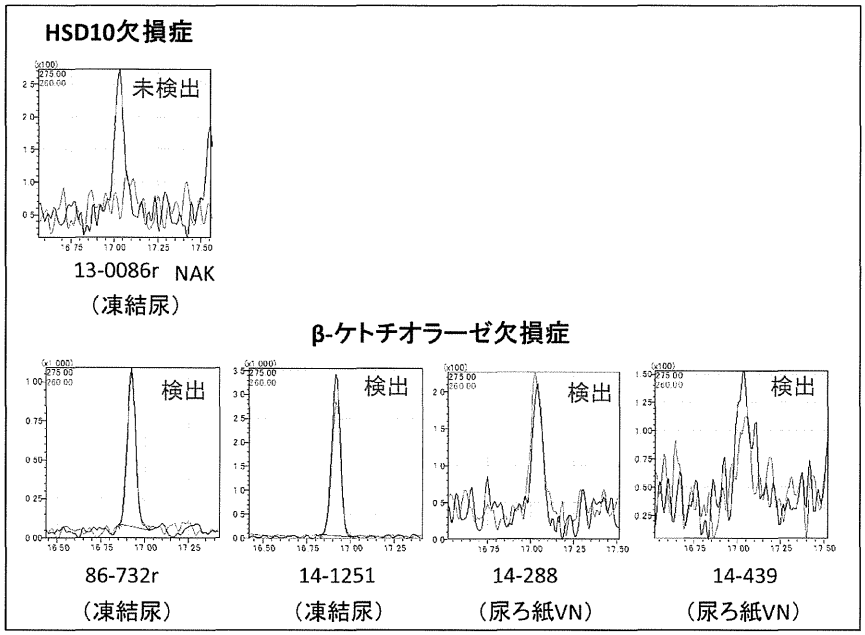


**図 4. 2-メチルアセト酢酸(2MAA)の同定**
HSD10 欠損症では検出できないが、βケトチオラーゼ欠損症では検出された。

表 1．βケトチオラーゼ欠損症と HSD10 欠損症の生化学診断アルゴリズム

| 同時に検出される代謝産物 | 出ていないことを確認すべき代謝産物 | 診断名アウトプット |
|---|---|---|
| 2-methyl-3-OH-butyrate<br>tiglylglycine | methylcitrate<br>methylmalonate | ・βケトチオラーゼ欠損症、または<br>・HSD10 欠損症 |
| 2-methyl-3-OH-butyrate<br>Tiglylglycine<br>methylacetoacetate | methylcitrate<br>methylmalonate | ・βケトチオラーゼ欠損症 |

## D．考察

当初、BKT 欠損症患者で 2MAA が検出されなかった理由として、2MAA の不安定さであることが分かった。尿ろ紙検体として室温におかれると 2 週間以内に 2MAA の大部分が分解することが分かった。また凍結されていても、長期に保存された検体では 2MAA が失われている可能性が高い。インドベトナムから送られる 3KT 欠損症患者の尿ろ紙検体で、2M3HBA と TG は検出されるものの、MAA が検出されなかった理由として明らかになった。この他にもαケト酸やβケト酸の一部は、アセト酢酸や MAA と同様に不安定になるものがあることも明らかになった。一定期間以上経過した古い検体では 2MAA が分解することも原因と考えられた。例えばβケト酸であるサクシニルアセトンは不安定なために、診断指標となるチロシン血症Ⅰ型の生化学診断には注意を払う必要がある。

2MAA の有無によって BKT 欠損症と HSD10 欠損症は鑑別される。2MAA が増加していれば BKT 欠損症と診断されるが、2MAA の不安定さのために 2MAA がどうかしてないからといって BKT 欠損症は否定できない。これらの所見を加味して、BKT 欠損症と HSD10 欠損症の自動診断アルゴリズムを作成することができた。

また生化学診断にあたっては、異常代謝産物の組み合わせのみならず、表 2 に示すように臨床所

見を加味する必要がある。

表 2. βケトチオラーゼ欠損症と HSD10 欠損症の鑑別点

| | βケトチオラーゼ欠損症 | HSD10 欠損症 |
|---|---|---|
| 遺伝形式<br>責任遺伝子の位置 | AR<br>11q22.3-23.1 | XR<br>Xp11.2 |
| 臨床症状 | ケトアシドーシス発作 | 男子<br>発達遅滞<br>神経変性<br>網膜変性 |
| 一般生化学 | ケトン陽性 | 高乳酸血症<br>低血糖<br>アシドーシス |
| アシルカルニチン分析所見 | C5:1<br>C5-OH | C5:1<br>C5-OH |
| 尿中有機酸分析所見 | TG<br>2M3HBA<br>3MAA | TG<br>2M3HBA<br>— |
| 酵素 | T2 | 2M3HBD |
| 遺伝子 | *ACAT1* | *HADH2 (HSD17B10)* |

略字：AR、常染色体性劣性遺伝；XR、X 連鎖性劣性遺伝；TG＝チグリルグリシン；2M3HBA＝2-メチル-3-ヒドロキシ酪酸；2MAA＝2-メチルアセト酢酸；T2＝アセトアセチル-CoA チオラーゼ；2M3HBD＝2-メチル-3-ヒドロキシブチリル-CoA 脱水素酵素。

## E．結論

BKT 欠損症と HSD10 欠損症は代謝障害部位が近接しているが、臨床症状が大きく異なる。アシルカルニチン所見は類似している。尿中有機酸分析による鑑別には 2MAA の有無が重要であるが、2MAA の検出は確実でなかった。この理由として 2MAA が室温では不安定であることが明らかになった。そこで 2MAA の不安定性を加味した BKT 欠損症と HSD10 欠損症の自動診断アルゴリズムを開発した。

## F．健康危険情報

特になし。

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## Ｈ．知的財産権の出願・登録状況

（予定を含む。）

1. 特許取得　　なし
2. 実用新案登録　なし
3. その他

別紙３

厚生労働科学研究費補助金（難治性疾患政策研究事業）
HSD10 病の発症形態と患者数の把握、診断基準の作成に関する研究（H26-難治等（難）-一般-021）
総合研究報告書

研究題名
# HSD10 病の予後を規程しうる神経ステロイド代謝測定系樹立

研究分担者　長谷川　行洋
東京都立小児総合医療センター　内分泌・代謝科

研究要旨　本邦初の HSD10 病を経験した。我々の経験した症例は臨床上、神経予後、生命予後ともに良好な非典型症例でる。この成因を解明するため、神経ステロイド代謝測定系樹立の第一段階を完了した。

研究協力者　　秋葉　和壽（都立小児総合医療センター）

A. 研究目的

我々は本邦初の HSD10 病を経験した。HSD10 病は世界で約 20 家系のみ報告されている X 染色体劣性遺伝の先天代謝異常症である。その原因はイソロイシン代謝、神経ステロイド代謝、ミトコンドリアの機能維持と多彩な機能をもつ蛋白である 2-methyl-3-hydroxybutryl-CoA 脱水素酵素(2M3HBD)の欠損である。典型例では重度の神経発達障害、心筋症をきたす予後不良な疾患である。我々の経験した症例は臨床上、神経予後、生命予後ともに良好な症例であり、8 歳現在重篤な神経症状や合併症を起こしていないという経過で過去の典型的報告と比べ予後に関して非典型例と位置づけられる。これまでの過去の研究によるとイソロイシン代謝は本疾患の神経予後に関与しないとされているが、この予後を規程する因子は不明である。候補となる成因として①神経ステロイドの代謝異常、②ミトコンドリアの機能異常の二つの仮説が報告されている。この両者の測定を簡便に行う方法は現在まで確立されていない。今回、我々は神経ステロイド代謝を評価する測定系を確立することを試みた。

B. 研究方法

1）野生型、変異型 cDNA クローニング；常法に従い行った。
2）発現させる細胞のスクリーニング；COS1, CHO, 293 細胞について行った。

C. 研究結果

1） 肝 cDNA ライブラリーから野生型 cDNA のクローニング、さらにそこから、変異体作成（我々の症例 A154T を含む4種類）をおこなった。
2）COS1 細胞、CHO 細胞については、RTPCR、ウエスタンブロットで内因性発現が確認された。
3）培養細胞上清を使い、タンデムマス法により神経ステロイド特に 3α,5α-THDOC、estrone、estradiol を測定する系を確立した。

D. 考察

我々と同様の仮説は Song-Yu Yang らの論文（PNAS.2009;106(35):14820-14824）に記載され、検討されているが、我々の方法論はタンデムマスで基質からの反応生成物（3α,5α-THDOC、estrone、estradiol）を評価する点が優れていると考える。

使用する細胞としては、内因性発現がないこと、さらに、理想的には神経系細胞株が望ましいがこういった条件を満たす細胞は現在までみつけることはできていない。

今後は患者自身から iPS 細胞を樹立し、本疾患原因遺伝子に変異を導入し、その後 Inoue らの方法（Cell Metabolsim 2013）を行い神経系細胞を誘導する研究を考えたい。詳細は示さないが変異を CRISPR/Cas9 法を用いて導入する技術まで確立している。

E. 結論

最終的な目的の第１段階が終了した。

G. 研究発表

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H. 知的財産権の出願・登録状況 （予定を含む。）
1. 特許取得　なし
2. 実用新案登録 なし

## Ⅱ．研究成果の刊行に関する一覧表

## Ⅲ．研究成果の刊行物・別刷

別紙４

研究成果の刊行に関する一覧表（分担報告書）

書籍

| 著者氏名 | 論文タイトル名 | 書籍全体の編集者名 | 書　籍　名 | 出版社名 | 出版地 | 出版年 | ページ |
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雑誌

| 発表者氏名 | 論文タイトル名 | 発表誌名 | 巻号 | ページ | 出版年 |
|---|---|---|---|---|---|
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Journal of Human Genetics (2014) 59, 609–614

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ORIGINAL ARTICLE

# The first case in Asia of 2-methyl-3-hydroxybutyryl-CoA dehydrogenase deficiency (HSD10 disease) with atypical presentation

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**2-Methyl-3-hydroxybutyryl-CoA dehydrogenase (2M3HBD) deficiency (HSD10 disease) is a rare inborn error of metabolism, and <30 cases have been reported worldwide. This disorder is typically characterized by progressive neurodegenerative disease from 6 to 18 months of age. Here, we report the first patient with this disorder in Asia, with atypical clinical presentation. A 6-year-old boy, who had been well, presented with severe ketoacidosis following a 5-day history of gastroenteritis. Urinary organic acid analysis showed elevated excretion of 2-methyl-3-hydroxybutyrate and tiglylglycine. He was tentatively diagnosed with β-ketothiolase (T2) deficiency. However, repeated enzyme assays using lymphocytes showed normal T2 activity and no T2 mutation was found. Instead, a hemizygous c.460G>A (p.A154T) mutation was identified in the *HSD17B10* gene. This mutation was not found in 258 alleles from Japanese subjects (controls). A normal level of the HSD17B10 protein was found by immunoblot analysis but no 2M3HBD enzyme activity was detected in enzyme assays using the patient's fibroblasts. These data confirmed that this patient was affected with HSD10 disease. He has had no neurological regression until now. His fibroblasts showed punctate and fragmented mitochondrial organization by MitoTracker staining and had relatively low respiratory chain complex IV activity to those of other complexes.**
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## INTRODUCTION

HSD10 disease, originally described as 2-methyl-3-hydroxybutyryl-CoA dehydrogenase (2M3HBD) deficiency,[1] is a rare X-linked recessive disorder caused by a mutation in the *HSD17B10* gene.[2–5] This gene encodes a multifunctional protein that has 17β-hydroxysteroid dehydrogenase activity as well as 2M3HBD activity,[3–5] and which is also an essential component of mitochondrial RNase P, being required for tRNA processing in mitochondria.[6]

This disorder was first identified in a patient with progressive infantile neurodegeneration whose urinary organic acid profile was suspected to be due to β-ketothiolase (mitochondrial acetoacetyl-CoA thiolase; T2) deficiency in isoleucine catabolism.[1] However, the clinical presentation of that patient was different from that of typical T2 deficiency, which is characterized by intermittent ketoacidosis and no clinical symptoms between crises, and typically normal development.[7,8] Fewer than 30 patients have been reported to date.[1,2,5,9–21] Typically, HSD10 disease is characterized by a progressive neurodegenerative course from 6 to 18 months of age, in conjunction with retinopathy and cardiomyopathy, leading to death at the age of 2–4 years or later.[5] However, clinical heterogeneity is noted in this disorder.[5] An atypical milder presentation was reported in three families.[13,14,17]

Here, we describe a 6-year-old Japanese boy with the HSD10 disease, who had no neurodegeneration and developed severe ketoacidosis at the age of 6 years. This is believed to be the first report of HSD10 disease in Asia.

## MATERIALS AND METHODS

### Case presentation

We report the case of a boy who had been well and achieved normal development until 6 years of age when he presented with severe ketoacidosis following

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a 5-day period of appetite loss and vomiting due to gastroenteritis. Physical examination at admission showed a height of 108 cm, body weight of 18.3 kg (2 kg loss), heart rate of 128 per min and respiratory rate of 32 per min. Unconsciousness was not noted. Laboratory testing showed blood gas pH 7.01, $pCO_2$ 9.2 mm Hg, $HCO_3^-$ 2.8 mEq $l^{-1}$, blood glucose 5.9 mmol $l^{-1}$, white blood cell count 16 180 $\mu l^{-1}$, hemoglobin 14.3 g $dl^{-1}$, blood urea nitrogen 14.5 mg $dl^{-1}$, aspartate aminotransferase 29 IU $l^{-1}$, alanine aminotransferase 17 IU $l^{-1}$, lactate dehydrogenase 238 IU $l^{-1}$, ammonia 65 $\mu g\ dl^{-1}$ and lactate 2.4 mmol $l^{-1}$.

After bolus infusion of 20 ml $kg^{-1}$ 5% glucose and electrolytes, blood total ketone body level was 14 mmol $l^{-1}$ and free fatty acid was 0.97 mmol $l^{-1}$. He responded to intravenous fluid infusion (including 5% glucose), and blood gas showed pH 7.48 and $HCO_3^-$ 23.7 mmol $l^{-1}$ on day 2 of hospitalization. He became well and started oral food intake on that day. He was discharged from the hospital on day 7 of hospitalization. Semiquantitative urinary organic acid analysis in the acute phase showed elevated excretion of 2-methyl-3-hydroxybutyrate and tiglylglycine, as well as ketones. He was tentatively diagnosed with T2 deficiency. One month later, he developed an episode of abdominal pain and lethargy in which hypoglycemia (1.4 mmol $l^{-1}$) and mild metabolic acidosis (blood pH 7.29, $pCO_2$ 36.4 mm Hg, $HCO_3^-$ 17.5 mmol $l^{-1}$ and lactate 5.5 mmol $l^{-1}$) were noted. He responded quickly to intravenous infusion of electrolytes and glucose. Urinary organic acid analysis at the acute phase of this episode showed elevated concentrations of 2-methyl-3-hydroxybutyrate but not of tiglylglycine and 2-methylacetoacetate (Table 1). Blood acylcarnitine analysis using tandem mass spectrometry showed elevated C5:1 carnitine but not C5-OH carnitine (Table 1). After this episode, he did not experience another metabolic event until now (6.5 years of age).

His mother claimed that his gross motor development was slow and he could walk alone after the age of 1 year and 6 months. He also had some clumsiness with fine motor skills. His growth was normal. His height and weight were 111.5 cm (−1.2 s.d.) and 22.2 kg (0 s.d.), respectively. His neurological development was slightly below normal with a verbal IQ of 112, performance IQ of 64 and a full scale IQ of 88 (Wechsler Intelligence Scale for Children). Cerebral magnetic resonance imaging and magnetic resonance spectroscopy yielded normal findings at the age of 6.5 years. No abnormal findings were identified in echocardiography and ophthalmological examinations at the age of 7 years.

### Enzyme assay and immunoblot analysis

Peripheral blood mononuclear cells were isolated from heparinized blood by gradient centrifugation in Ficoll–Paque medium (GE Healthcare, Uppsala, Sweden). The fibroblasts were cultured in Eagle's minimum essential medium containing 10% fetal calf serum. Acetoacetyl-CoA thiolase and succinyl-CoA:3-ketoacid CoA transferase were assayed in lymphocytes and fibroblasts, as described previously.[22] 2M3HBD activity in fibroblasts was measured as described previously.[1] Immunoblot analysis for 2M3HBD was done using anti-rat 2M3HBD antibody, which was originally made by us (TH) and anti-human glyceraldehyde 3-phosphate dehydrogenase antibody (sc-25778; Santa Cruz Biotechnology, Santa Cruz, CA, USA) as a reference. We used fibroblasts from an HSD10-deficient patient,[16] as a positive disease control.

### Mutation analysis

This study was approved by the Ethical Committee of the Graduate School of Medicine, Gifu University, Gifu, Japan. Genomic DNA was purified from the fibroblasts with Sepa Gene kits (Sanko Junyaku, Tokyo, Japan). Mutation screening was performed at the genomic level by PCR and direct sequencing, using primer sets for fragments including each exon and its intron boundaries. Primers and PCR conditions for *ACAT1* gene were as previously described.[23] For *HSD17B10*, we amplified each genomic region with the primer pairs shown in Supplementary Table S1.

### Screening of A154T mutation in the Japanese population

The presence of A145T mutations was screened using TaqMan triplet genotyping in 92 Japanese men and 83 women, according to the manufacturer's protocol (Life Technologies, Carlsbad, CA, USA).

### Mitochondrial morphology

Fibroblasts from HSD10 patients and control fibroblasts were cultured in Dulbecco's modified Eagle's medium (Life Technologies) supplemented with 10% fetal calf serum at 37 °C and 5% $CO_2$. The mitochondria in living fibroblasts were stained with 100 nM MitoTracker Red CMRXRos (Life Technologies) for 30 min at 37 °C. Fluorescent images were captured and analyzed with an LSM710 laser scanning confocal microscope equipped with an incubation system (Carl Zeiss, Oberkochen, Germany).

### Respiratory chain enzyme analysis

An *in vitro* respiratory chain enzyme activity assay[24] and blue native polyacrylamide gel electrophoresis[25,26] were used to quantify the activity and amount of respiratory chain enzyme complexes. The diagnostic criteria of Bernier *et al.*[26,27] were used to judge the activity.

### Structural analysis of the A154 mutation

The crystal structure of human HSD17B10 complexed with $NAD^+$ (PDB ID: 2O23, deposited in the RCSB protein databank; www.rcsb.org)[28] was used for

**Table 1 Urinary organic acid and serum acylcarnitine analyzes**

| | | *This patient* | | *T2D (severe)* | *T2D (mild)* |
|---|---|---|---|---|---|
| | *Mean (s.d.)* | *Hypoglycemic* | *Asymptomatic* | *Asymptomatic* | *Symptomatic* |
| *Urinary organic acids* | | | | | |
| Lactic acid | 37.9±28.1 | 7755.8 | 7.3 | 5.1 | 195.0 |
| 3-OH butyric acid | 27.8±21.5 | 17 116.1 | 3.0 | 5.4 | 6295.0 |
| Acetoacetic acid | 0.2±0.4 | 72.5 | 0.7 | 1.0[a] | 16.7[a] |
| 2-Me-3-OH butyric acid | 4.4±4.0 | 296.2 | 132.6 | 130.4 | 121.6 |
| 2-Methylacetoacetic acids | 0±0 | 0.0 | 0.7 | 69.4[a] | 2.8[a] |
| Tiglylglycine | 2.2±4.3 | 0.1 | 298.9 | 212.4 | 3.7 |
| *Serum acylcarnitines* | | | | | |
| C0 | 31.3±8.4 | 13.4 | | 67.4 | 79.2 |
| C2 | 6.2±2.1 | 16.2 | | 7.7[a] | 2.1[a] |
| C5:1 | 0.012±0.005 | 0.63 | | 0.72 | 0.079 |
| C5OH | 0.06±0.03 | 0.11 | | 0.34 | 0.06 |

T2D (severe) was GK01, and T2D (mild) was GK77.
Amounts of urinary organic acids are expressed as mmol per mol Cr.
Amounts of serum acylcarnitine are expressed as nnom $ml^{-1}$.
[a]Values may be low because of degradation due to long storage at −30 °C.

structural analysis. The program COOT was used to analyze the structure and PyMOL Molecular Graphics System, version 1.4.1 (Schrödinger, LLC; www.pymol.org/citing), was used to make the figures.

## RESULTS

### Exclusion of the diagnosis of T2 deficiency

We first made a tentative diagnosis of T2 deficiency, based on the severe ketoacidotic event with elevated 2-methyl-3-hydroxybutyrate and tiglylglycine in urinary organic acid analysis. However, repeated enzyme assays showed normal T2 activity (Supplementary Table S2). Furthermore, no T2 mutation was identified by genomic PCR followed by direct sequencing.

### Mutation analysis of HSD17B10 gene

Urinary organic acid analysis showed blockade at the T2 or 2M3HBD level in the isoleucine catabolic pathway. Therefore, we investigated the possibility of an *HSD17B10* gene mutation, although the clinical course of this patient was different from that of typical HSD10 patients. A hemizygous c.460G>A (p.A154T) mutation was identified in *HSD17B10* gene (Figure 1). His mother was a heterozygous carrier of this mutation. His maternal uncle did not have this mutation. Samples from maternal grandparents were not available for the study. TaqMan analysis showed that this mutation was not found in 258 alleles from Japanese subjects (controls).

### Enzyme assay and immunoblot analysis for 2M3HBD

We used a fibroblast cell line from a Dutch patient whose mutation was c.364C>G (p.L122V) as a positive disease control. He was classified with the infantile form of HSD10 disease because he had shown motor delay and spastic diplegia since infancy.[16] The patient was able to walk but had psychomotor retardation with spasticity and minimal language development (Bwee Tien Poll-The, personal communication), and hence his clinical manifestations were milder than for the typical infantile form of the disease.

2M3HBD activity was absent from the patient's fibroblasts, as well as HSD10-deficient fibroblasts with p.L122V mutation,[16] designated as L122V fibroblasts (Table 2). However, the control samples showed 2M3HBD activity, which was in accordance with reported control values for the assay.[1] Immunoblot analysis showed that fibroblasts from our patient and the previous HSD10-deficient patient had an almost similar amount of HSD17B10 protein to the controls (Supplementary Figure S1).

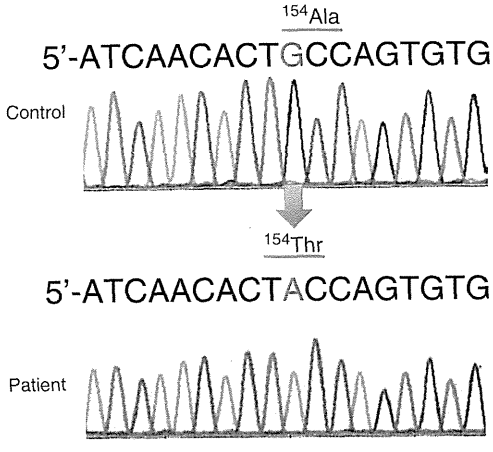


**Figure 1** HSD17B10 mutation. Genomic direct sequencing of exon 5. A hemizygous c.460G>A (p.A154T) substitution was identified. A full color version of this figure is available at the *Journal of Human Genetics* journal online.

### Mitochondrial staining

MitoTracker staining revealed a filamentous network-like structure of the mitochondria in control fibroblasts (Figure 2 and Supplementary Figure S2). Fibroblasts with the p.L122V and p.A154T mutations showed punctate and fragmented mitochondrial organization. This finding is the same as that previously reported in fibroblasts with R130C and D86G mutations.[17] Furthermore, mitochondria in A154T mutated cells had highly variable diameters, ranging from thin tubes to swollen bulbs.

### Respiratory chain enzyme assay

Respiratory chain enzyme assay of the patient's fibroblasts showed normal activity of complexes I, II and III (98–159% relative to citrate synthase) (Supplementary Table S3). Complex IV activity was also within the normal range but significantly lower than that of other complexes (51.6% relative to citrate synthase and 44.6% relative to complex II). In blue native polyacrylamide gel electrophoresis, the band corresponding to assembled complex IV was slightly decreased too (Supplementary Figure S3). These tendencies were also detected in fibroblasts with L122V mutation.

### Mutation site in the tertiary structure of human HSD17B10

HSD17B10 is a tetramer consisting of four identical subunits, each having the fold of short-chain dehydrogenase/reductase superfamily. Inspection of the human HSD17B10 structure (PDB ID: 2O23) revealed that residue Ala154 is close to the active site (Figure 3a). Ala154 is completely buried and the Cβ atom of Ala154 faces a hydrophobic (apolar) pocket created by residues such as Ile175, Val176 and Cγ of Thr195. The residue next to Ala154, Ser155, is one of the catalytic residues, and part of the catalytic triad formed by Ser155, Tyr168 and Lys172. The mutation of Ala154 to Thr154, that is, from a small, hydrophobic side chain to a larger, polar side chain results in steric clashes with residues Ile175, Val176 and Thr195 in the current conformation (Figure 3b). To avoid these steric clashes, main and side chain conformational changes are expected in the region around Ile175 and Ala/Thr154. The changes around Ile175 may also affect the catalytically competent conformation of the active site residue Lys172. In addition, the changes around Ala/Thr154 are expected to cause structural changes of the catalytic residue Ser155, which has to interact with the substrate for the reaction to occur. Therefore, all these rearrangements resulting in the non-optimal conformations of Ser155 and Lys172 may severely affect the catalytic capability of this enzyme. The substrate binding may not be affected as much because the catalytic triad is only at the beginning of the much larger substrate binding pocket[28] extending outward. Therefore, catalysis of both the steroid substrates such as allopregnanolone[21]

**Table 2 2M3HBD assay using fibroblasts**

| | *2M3HBD* | *AcAcCoA thiolase* |
|---|---|---|
| Control fibroblasts 1 | 0.75±0.40 | 15.6 |
| Control fibroblasts 2 | 0.90±0.58 | 28.1 |
| L122V fibroblasts | 0.19±0.08 | 28.0 |
| Patient's fibroblasts | 0.04±0.11 | 34.0 |

Acetoacetyl-CoA (AcAcCoA) thiolase activity was measured in the presence of potassium ion at 37 °C.

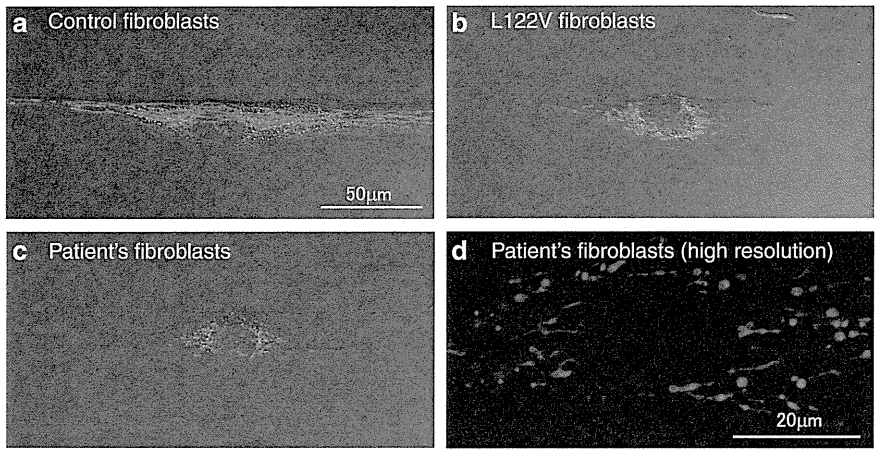


**Figure 2** Mitochondrial morphology. (**a**–**c**) Merged images from differential interference contrast (DIC) and MitoTracker Red. (**a**) Control fibroblast. (**b**) Fibroblast with the p.L122V mutation. (**c**) Fibroblast with the p.A154T mutation. (**d**) Fluorescent image of MitoTracker Red from the p.A154T mutated cell. Bars: **a**–**c**, 50 μm; **d**, 20 μm. A full color version of this figure is available at the *Journal of Human Genetics* journal online.

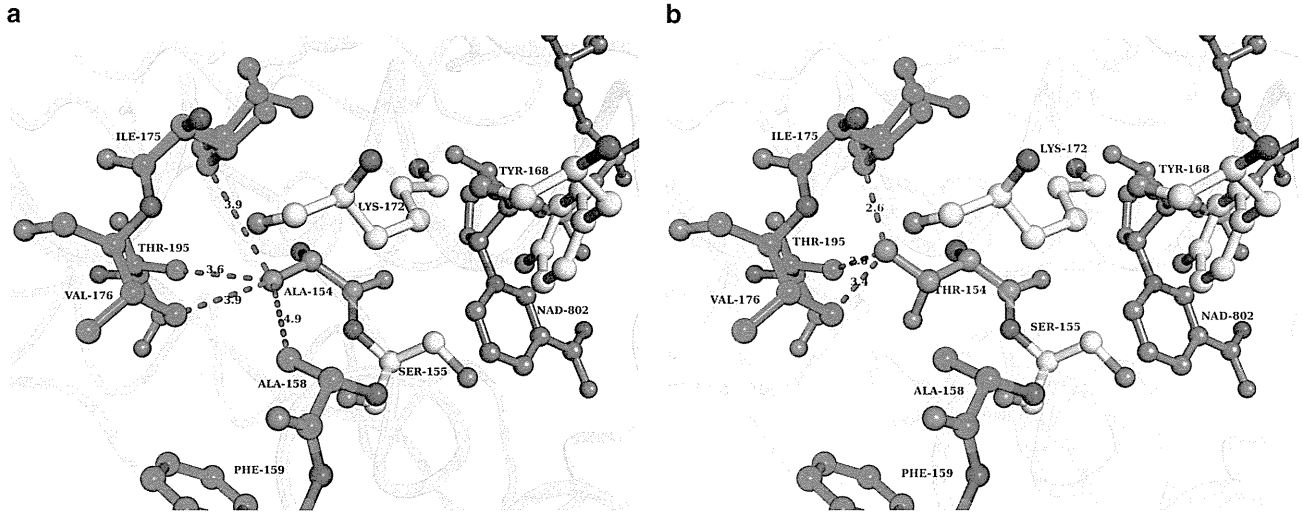


**Figure 3** Structural analysis. (**a**) Environment of residue Ala154 as seen in PDB ID 2023. Oxygen atoms are shown in red, nitrogen in blue and carbon is color coded as follows: Ala154 in magenta, the catalytic triad comprising residues Ser155, Tyr168 and Lys172 in yellow, and NAD in blue. Ala158, Phe159, Ile175, Val176 and Thr195 (Cγ) are some of the residues pointing toward the side chain of Ala154, creating a hydrophobic pocket. These are highlighted in green. Ile175 has a double conformation. The relevant distances are shown with red dashes. (**b**) Possible steric clashes in HSD10 disease due to mutation of Ala154 into Thr154. Thr154 is shown in magenta. Ala154 was mutated to Thr154 using PDB-entry 2023 by the program COOT. The expected steric clashes of the Thr154 side chain with Ile175, Val176 and Thr195 are highlighted by red dashes.

and fatty acyl-CoA substrates such as 2-methyl-3-hydoxybutyryl-CoA are predicted to be equally affected.

## DISCUSSION

This is believed to be the first report of HSD10 disease in Asia. Since the discovery of the first patient in 2000,[1] fewer than 30 patients have been described.[1,2,5,9–21] Typically, this disorder is suspected when patients with neurological degeneration or psychomotor retardation show similar urinary organic acid or blood acylcarnitine profiles with T2 deficiency. However, our patient experienced a severe ketoacidotic episode with blood pH 7.01 and blood total ketone level of 14 mM after a 5-day history of gastroenteritis. This clinical picture is similar to T2 deficiency, although the onset of the first severe ketoacidotic episode at the age of 6 years is late compared with that in typical T2-deficient patients who develop such crises around the age of 6 months to 2 years.[7,8] The first patient described by Zschocke *et al.*[1] had metabolic decompensation with ketonuria on day 2 of life. Disturbance in isoleucine catabolism may be attributed to such reversible metabolic decompensation in HSD10 disease, and appears to be independent from pathophysiology of neurodegeneration in HSD10 disease.

In the patients with HSD10 disease described thus far, broad clinical heterogeneity has been found.[5,30] The classical presentation that is observed in most patients, which was called the infantile form by

Zschocke,[5] is characterized by a period of more or less normal development in the first 6–18 months of life. This is followed by a progressive neurodegenerative disease course in conjunction with progressive cardiomyopathy, leading to death at the age of 2–4 years or older. Patients with a common mutation c.388C>T (p.R130C) present with the infantile form. Some patients with other mutations have more severe neonatal forms. Atypical presentation was reported in three families. (1) Only one patient with c.745G>C (p.E249Q) mutation developed normally in the first 5 years of life and then showed neurological deterioration.[14] This was classified as the juvenile form by Zschocke.[5] (2) The proband of a family with c.495A>C (p.Q165H) mutation showed growth retardation, feeding difficulty and microcephaly but his neurological status remained normal at up to age 5 years. Moreover, his male cousin with the same mutation achieved normal neurodevelopment until his current age of 8 years, with a height and weight in the 25th percentile.[17] (3) Four boys in a large family showed X-linked intellectual disability, choreoathetosis and abnormal behavior with a normal urinary organic acid profile, and they had an apparent synonymous mutation that affected splicing efficiency in the HSD17B10 gene.[13] Our patient with a novel c.460G>A (p.A154T) mutation showed no neurological degeneration, at least until age 6.5 years, and normal growth. Hence, our patient had a milder phenotype than in patients with juvenile HSD10 disease.

There is evidence that the neurological degeneration observed in HSD10 disease is not caused by a deficiency in the isoleucine metabolism-related 2M3HBD activities of HSD17B10.[17,21] Instead, defects in neuroactive steroid metabolism[21] and/or the non-enzymatic function of the protein required for mitochondrial integrity and cell survival[17] may be responsible for the neurological manifestations. The HSD17B10 protein is one of three component proteins of mitochondrial RNase P, which is essential for mitochondrial translation.[6] Reduced function as a component of RNase P may contribute to clinical severity. The p.R130C mutation common for infantile form reduced not only its mutant HSD10 level but also that of another RNase P component, MRPP-1, suggesting that HSD10 is important for the maintenance of the MRPP1–HSD10 subcomplex of RNase P.[31] Analysis of the consequences of the A154T mutation on the tertiary structure suggests that A154T mutation affects enzyme activity of both 2-methyl-3-hydroxybutyryl-CoA and neurosteroids. The enzymological characterization of the expressed HSD17B10 A154T variant is required to confirm this observation. Mitochondrial morphological changes using MitoTracker staining have been reported,[17] and we also observed punctate and fragmented mitochondrial organization in our patient. Mitochondrial respiratory chain complex IV activity was decreased in both fibroblasts with A154T and those with L122V, although the decreased level did not fulfill the minor diagnostic criteria of Bernier *et al.*[27] Mitochondrial respiratory chain enzyme assay was reported to be normal in fibroblasts with V65A mutation. Further investigation in other fibroblasts with HSD10 disease is necessary to confirm that reduced complex IV activity is one of the characteristics in HSD10 disease.

We have described a patient with mild phenotype HSD10 disease with a novel A154T mutation, who is believed to be the first patient with HSD10 disease in Asia. Accumulation of more data on phenotype–genotype correlation of HSD10 disease is important to understand the molecular basis of the disease.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Journal of Human Genetics website (http://www.nature.com/jhg)

Supplemental Table S1. Genomic PCR primers

| | | |
|---|---|---|
| exon 1 | sense | 5'-g.470ATCCCCATCCCGTGGAGTGG |
| | antisense | 5'-g.683AGTGCTGACTTTCACCTCTTGA |
| exon 2 | sense | 5'-g.810GGAGAAGCAGCACACCTAGT |
| | antisense | 5'-1279TCCCACAGTGCTTGAAGGCT |
| exon 3,4, 5 | sense | 5'-g.2309CCTCTCCCTTCTCACAAATCT |
| | antisense | 5'-g.3139TGCTGCTGCTTAGGTGGTGGAT |
| exon 6 | sense | 5'-g.3130AGCAGCAGCAGCCTTTTATCT |
| | antisense | 5'-g.3565ATTAGGCACAGAGGGCGACT |

Nucleotides are numbered according to NG_008153 RefSeqGene.

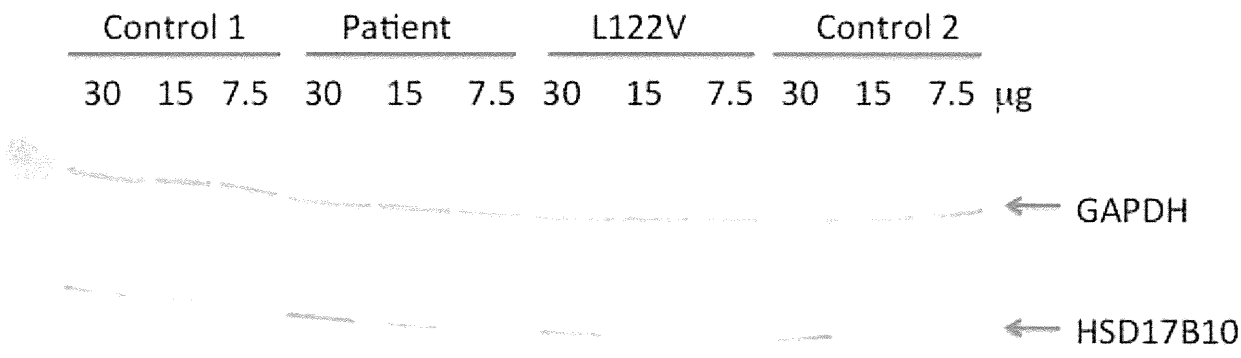
Supplemental Table S2. Enzyme assay using blood mononuclear cells.

| | acetoacetyl-CoA thiolase | | | SCOT |
|---|---|---|---|---|
| | -K+ | +K+ | +K/-K | |
| After 1st episode | | | | |
| Control | 6,4 | 16.1 | 2.5 | 12.1 |
| Patient | 10.8 | 21.2 | 2.0 | 13.7 |
| After 2nd episode | | | | |
| Control | 6.9 | 12.7 | 1.8 | 14.3 |
| Patient | 5.8 | 13.1 | 2.3 | 17.0 |

Enzyme activities were expressed as nmol/min/mg protein.

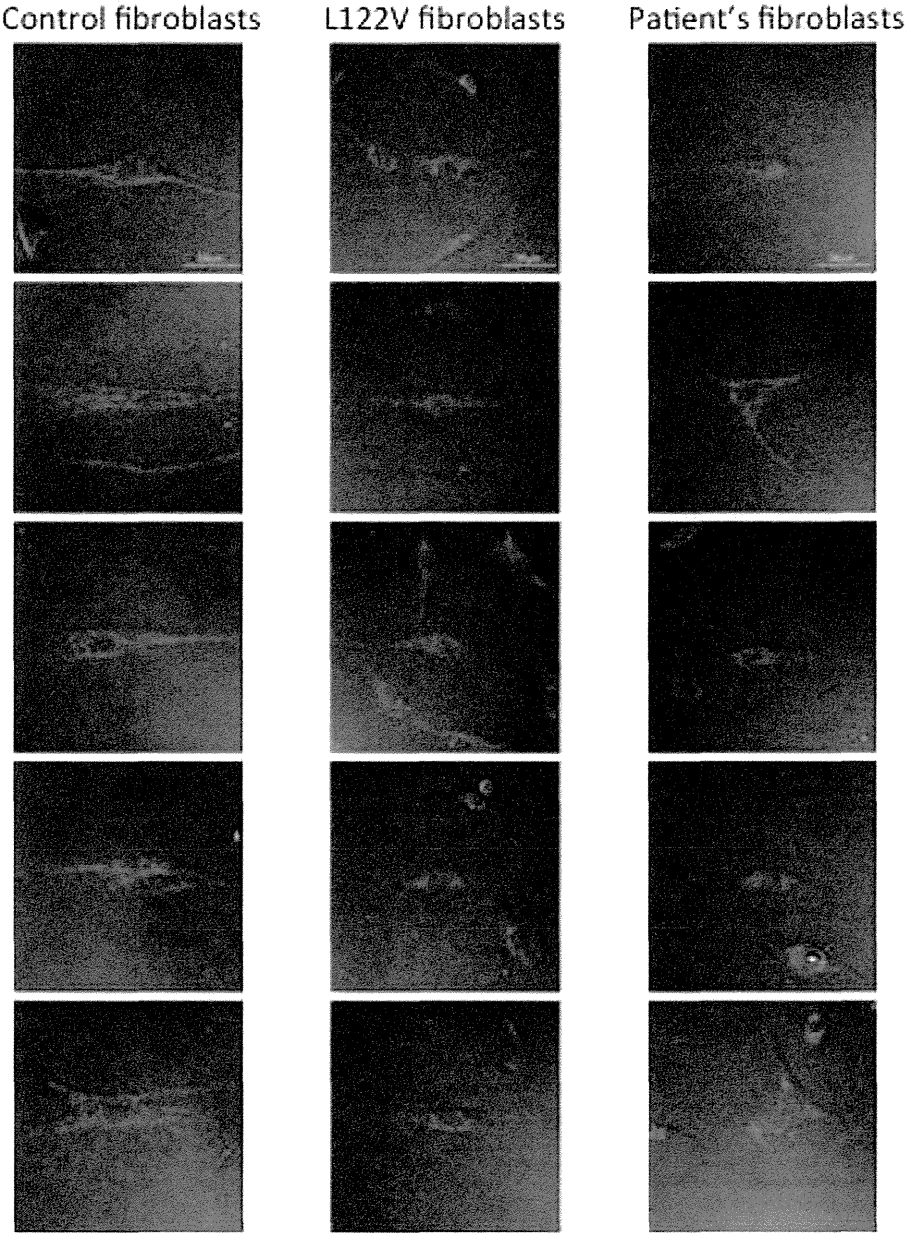
## Supplemental Figure 1

Immunoblot analysis. Serially diluted samples 30, 15, and 7.5 micrograms of fibroblast protein extracts were applied. The lane L122V indicates fibroblasts with HSD10 disease in which the mutation was p. L122V. A mixture of anti-rat HSD17B10 antibody and anti-human GAPDH antibody was used as the first antibody.



# Supplemental Figure 2

Fig. 2 only shows representative photos for mitochondrial staining. This supplemental figure shows that similar findings were observed in other fibroblasts.



Supplemental Table S3. Respiratory chain enzyme activities

| | COI | COII | COII+III | COIII | COIV | CS |
|---|---|---|---|---|---|---|
| Control fibroblasts | | | | | | |
| Crude activity (%) | **176.1** | **134.4** | **207.2** | **185.6** | **123.2** | **186.4** |
| CS ratio (%) | **91.7** | **69.5** | **105.2** | **93.7** | **64.9** | |
| COII ratio (%) | **130.3** | | **149.3** | **137.5** | **93.3** | |
| L122V fibroblasts | | | | | | |
| Crude activity (%) | **117.9** | **152.1** | **161.2** | **143.7** | **55.5** | **111.2** |
| CS ratio (%) | **103.0** | **131.9** | **137.2** | **121.7** | **49.0** | |
| COII ratio (%) | **77.0** | | **102.6** | **94.0** | **37.1** | |
| Patient's fibroblasts | | | | | | |
| Crude activity (%) | **118.0** | **117.4** | **101.6** | **166.0** | **51.5** | **98.0** |
| CS ratio (%) | **117.0** | **115.5** | **98.2** | **159.5** | **51.6** | |
| COII ratio (%) | **99.9** | | **83.8** | **140.8** | **44.6** | |

Supplemental Figure 3

Blue native polyacrylamide gel electrophoresis analysis of skin fibroblasts. Twenty μg proteins of isolated mitochondria from fibroblasts were solubilized in dodecyl maltoside and subjected to blue native polyacrylamide gel electrophoresis and Western blotting. The amount of fully assembled complex IV was shown to be slightly decreased both in this patient and another one with L122V.