G．研究発表

1．論文発表

なし

2．学会発表

1）低酸素性虚血性脳症における興奮毒性の影響 第1回 JPM, 名古屋.

2）The Effects of Excitotoxicity with Hypoxic-Ischemic Encephalopathy Piglet Model. The 4th World Congress on Controversies in Neurology (CONy), Barcelona, Spain.

H．知的財産権の出願・登録状況

なし

1．特許取得

なし

2．実用新案登録

なし

3．その他

なし

11. 原因不明の運動障害症例の病因解析、特に神経伝達物質病に関する検討

研究要旨

当院ならびに、鳥取・島根両県の重症心身障害児施設に通院・入所している原因不明の重度運動障害症例を対象に、神経伝達物質病の可能性を神経所見から調査した。神経伝達物質病に比較的特徴的な眼球運動発作と症状の日内変動を呈する例は少なかった。同意の得られた症例に対して尿有機酸分析と髄液プテリジン分析を行ったが診断例はなかった。

研究分担者

前垣　義弘（鳥取大学医学部　脳神経小児科、準教授）

A．研究目的

神経伝達物質病は稀な疾患である上に、症状の特異性に乏しいため、臨床症状から疾患を疑うことは困難である。小児期早期より重度の運動障害を生じる疾患は、周産期脳障害や先天異常、染色体異常など多岐にわたる。臨床症状や画像検査、通常の血液・尿・髄液検査で確定診断されない重度の運動障害の症例は少なくない。本研究は、このような診断未確定の重度運動障害症例において、神経伝達物質病の可能性のある例に対して、診断目的に精査を行う。

B．研究方法

当院通院中の症例ならびに、鳥取・島根の重症心身障害児施設入所児（者）で、原因不明の運動障害を呈する症例を対象に、症例調査票に主要症状を記載し、同意の得られた場合には、血液アミノ酸分析、尿有機酸分析、髄液HVA、5HIAA、DOPAおよびプテリジン分析を実施する。

C．研究結果

対象症例を表1に示す。対象症例のうち初期症状の記録のある15例の症状を表2に示す。

尿有機酸分析を9例に実施したが4-ヒドロキシイソ酪酸上昇例はなかった。アミノ酸分析は7例に実施されており異常を認めなかった。髄液HVA、5HIAA、プテリジン分析（3例）にて診断に至った例はなかった。

表1．対象症例

	入所患者数	候補患者数
鳥取大学医学部附属病院		7
鳥取県立総合療育センター	25+α	
国立病院機構　鳥取医療センター	160	3（<30歳）
国立病院機構　松江医療センター	80	13
西部島根医療福祉センター	100	9

表2．対象症例の初期症状（n=15）

発症年齢	乳児11、1歳2
筋緊張低下	12
筋緊張亢進	4
寡運動	4
ジストニア	3
ミオクローヌス、振戦	5
舞踏運動・アテトーゼ	9
尖足、対麻痺	6
眼球運動発作	3（一過性1）
言語発達遅滞	13
哺乳不良	2
運動の退行	5
日内変動	0
けいれん発作	12

D．考　察

原因不明の重度運動障害症例において、神経伝達物質病に比較的特徴的な神経徴候である眼球運動発作や症状の日内変動を認める例は少なかった。同意の得られた症例について精査を行ったが、確定診断のついた症例はなかった。類似の状態を呈する未知の疾患が多数存在するものと思われる。

E．結　　論

重症心身障害施設に入所している原因不明の重度運動障害症例において、神経伝達物質病の精査を行ったが診断例はなかった。

F．健康危険情報

なし

G．研究発表

1．論文発表

なし

2．学会発表

なし

H．知的財産権の出願・登録状況

1．特許取得

なし

2．実用新案登録

なし

3．その他

なし

平成22年度厚生労働科学研究費補助金（難治性疾患克服研究事業）研究報告書
研究課題：「小児神経伝達物質病の診断基準の作成と新しい治療法の開発に関する研究」

12. 九州における新規小児神経伝達物質病患者の発見に関する研究

研究要旨

平成21年に施行された小児神経伝達病の全国調査では、九州地区は全体の疾患の報告が少なかった。その要因として、発症が本当に少ないのか、疾患の認識不足、理解不足、および診断の困難さによるものかを検討する。その後、平成21年は新生児発症の非ケトーシス型高グリシン血症の1例が発見され、平成22年は瀬川病の1家系が発見された。今後、各種の医療機関および、重症心身障害施設でも引き続き調査を行うことが重要と考えられる。

研究分担者

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研究協力者

山下　裕史朗（久留米大学　小児科・准教授）
渡邊　順子（久留米大学　小児科・講師）
小篠　史郎（熊本大学　発達小児科・助手）
木村　重美（熊本大学　発達小児科・准教授）
高柳　俊光（国立佐賀病院機構佐賀病院・小児科部長）

A．研究目的

稀少疾患である、小児伝達物質病の疾患概念の啓蒙をはかり、理解をすすめ、未診断の患児・者の発見および治療的介入を検討する。

B．研究方法

班会議で既に作成した調査用紙、パンフレットを用いて、九州地区の小児神経地方会、神経内科地方会、その他で紹介する。対象は疾患に関与する可能性のある、新生児科医、小児神経科専門医、神経内科医、先天代謝異常症専門医、先天代謝異常症スクリーニング機関、重症心身障害施設等の医師である。

C．研究結果

初年度の全国調査の結果、九州地区で把握された患者の内訳は瀬川病6例、芳香アミノ酸脱炭酸酵素（AADC）欠損症0例、コハク酸セミアルデヒド脱水素酵素（SSADH）欠損症1例、非ケトーシス型高グリシン血症（NKH）0例であった。その後新生児発症のNKH患者1例が発見され、昨年度はその症例の詳細な報告と代謝マップ、診断基準を中心に報告した。今回は熊本で診断された瀬川病の1例を中心に報告する。また、母親、母の姉が類似の症状があるため、今後の遺伝子検索を含めた診断、治療を含めて検討する予定である。

症例は6歳女児で、3歳頃から平らな場所でもよく転ぶようになり、バス停から家までの距離でも数回休憩を取らないと歩けなくなり、年単位で症状が目立ち6歳より左下肢優位の姿勢ジストニアを呈するようになった。症状は、午前中は軽度だが午後は歩けなくなり著明な日内変動を呈した。血液生化学、血清アミノ酸、Cu、セルロプラスミン、頭部MRI異常なし、脳脊髄液ではNeopterin 4.18 nM（8.0－25）、Biopterin 2.6 nM（10－20）、BH4 40.65 nMであった。遺伝学的検査は検討予定である。その後、確認された家族歴で、母親が44歳で健康だが20年以上前から夕方になると足が重くなるとの事である。また母親の姉が現在48歳、約20年前から、午後から夕方にかけて足が疲れて動かなくなり、徐々に進行し、48歳から神経内科受診しアマンタジン・ビペリデン内服中であり、瀬川病が強く疑われている。

D．考　察

昨年報告した新生児発症の、非ケトーシス型高グリシリン血症も、本疾患を意識して検査しないと見逃される可能性があると考えられた。瀬川病は神経内科領域でも認識が不十分で、緊張性筋無力症と診断され治療されていた。瀬川病は一般には治療に反応がよく、予後良好な病気であるため、更なる啓蒙が必要と考えられた。AADC欠損症は九州では発見されていないが、間歇的な眼球回転発作（oculogyric crisis）、四肢のジストニアに注目し診断できる可能

性がある。またSSADHは乳児期からの精神遅滞、筋緊張低下、睡眠障害等の症状に着目し、重症心身障害施設などでも注意して調査していく必要性が考えられる。

E．結　論

瀬川病は九州でも新たに1人の患児と母、母の姉が同じ病気が疑われ、潜在的にもっと存在している可能性が示唆された。

F．健康危険情報

無し。

G．研究発表

1．論文発表

Kawano G, Iwata O, Iwata S, Kawano K, Obu K, Kuki I , Rinka H, Shiomi H, Yamanouchi H, Kakuma T, Takashima S, Matsuishi T. Determination of outcomes following acute child encephalopathy and encephalitis : pivotal effect of early and delayed cooling. ADC 2010 in press.

2．学会発表

Haruo Shintaku, Masaya Segawa, Mitsuhiro Kato, Shinji Saitoh, Shin-ichiro Hamano, Masaya Kubota, Jun Tohyama, Jun Natsume, Yoshihiro Maegaki, Toyojiro Matsuishi, Hideji Hattori, Shuhei Ide, Yasusi Itoh, Hiroki Fujioka, Yoshiko Nomura. Nationwide epidemiological study of pediatric neurotransmitter disease in Japan (1st report) March26-28, Taipei, Taiwan, 2010.

H．知的財産権の出願・登録状況

1．特許取得

無し。

2．実用新案登録

無し。

3．その他

無し。

平成22年度厚生労働科学研究費補助金（難治性疾患克服研究事業）研究報告書
研究課題：「小児神経伝達物質病の診断基準の作成と新しい治療法の開発に関する研究」

13. 唾液中メラトニン濃度測定によるAADC欠損症のスクリーニング検査の可能性について

研究要旨

重症心身障害児（者）（以下重症児（者））の中には、眼球上転発作やジストニアを示す例がありAADC欠損症が疑われる症例が存在する。しかし診断に必要な髄液採取は実施困難な場合が多く、重症児（者）でも抵抗なく実施可能な簡便なスクリーニング検査が望まれる。我々は、重症児（者）8例とAADC欠損症患者1例に対して唾液中メラトニン濃度測定を行った。夜間唾液中メラトニン濃度は重症児（者）群では40.0 ± 13.4pg/mlと一般の脳障害では障害が重度な場合でもメラトニン分泌は低下しないことが示された。AADC欠損症例では夜間唾液中メラトニン濃度は2.6pg/mlであり、メラトニン分泌が重度に障害されていた。重症児（者）からAADC欠損症をスクリーニングする方法として夜間唾液中メラトニン濃度測定は有効であると考えた。

研究分担者

井手　秀平（東京都立東部療育センター）

A．研究目的

AADC欠損症は、乳児期前半に異常眼球運動やジストニアで発症し、多くは寝たきりで発語のない状態のまま発達が停滞する。成長とともに嚥下機能や排痰機能の低下があり下気道感染を繰り返す症例も多い。このような経過は脳性麻痺に精神遅滞を合併した重症児（者）においてもしばしば認められる臨床経過である。AADC欠損症に比較的特異な症状とされる、眼球上転発作やジストニアでも重症児に随伴する場合がまれではない。本邦でのAADC欠損症患者の報告は3例のみであるが、より多くのAADC欠損症患者が重症心身障害児（者）の中に潜在していることが予想される。

AADC欠損症の診断には髄液検査（HVA、5HIAAなどモノアミンやセロトニンの代謝産物の濃度測定）が必須である。しかし未診断のまま学齢期や成人となった重症児（者）では診断に対するニーズが低く、痛みを伴う髄液採取を行うことは難しい。より簡便な尿中VMA、HVA測定では少なからず正常値を示す症例があることが知られておりスクリーニング検査としては不適である。頭部MRI検査や脳波検査も特異的所見がなくスクリーニングには使用できない。

AADCはメラトニンの前駆体であるセロトニンの合成酵素でありAADC欠損症患者ではメラトニン合成も障害されていることが予想される。メラトニンはより簡便に採取可能な唾液でも測定可能であり、その濃度は血漿と相関することが知られている。本研究の目的は、唾液中メラトニン濃度測定が重症児（者）の中からAADC欠損症をスクリーニングする検査として使用可能かどうかの検討を行うことである。

B．研究方法

対象は、東京都立東部療育センターに長期入所中の重症児（者）8名（以下SMID群）およびAADC欠損症の1名（14歳男児）である。SMID群は全例が寝たきりで寝返り不可の運動障害と言語理解や発語のない最重度精神遅滞の状態の合併がある。年齢は8歳～30歳（平均17歳）、男性5名女性3名である。また全例が経管栄養と気管切開をしており、6名が人工呼吸器を使用している。

唾液検体は、日中の11時から13時と夜間23時から1時に、直接吸引をして採取した。採取後は速やかに冷凍し2日以内に－80℃の冷凍庫に移して測定まで保存した。唾液中メラトニンはDirect MELATONI ELISA キット（BUHLMAN社）を用いて測定した。SMID群とAADC欠損症患者の日中と夜間のメラトニン値を比較したほか、SMID

群の中では追視の有無で2群にわけて唾液中メラトニン値を比較した。

本研究は、東京都立東部療育センターの倫理委員会にて承認を受けた。対象者の家族からは文書による承諾を得た。

C．研究結果

SMID 群の結果

重症児（者）8例では（図1）、夜間唾液中メラトニン濃度は 40.0 ± 13.4pg/ml であった。この値は健常者の既報告値と同程度の値であった。日中唾液中メラトニン濃度は 20.0 ± 15.6pg/ml であった。日中は健常者ではメラトニン分泌は抑制されているため、それと比較すると重症児者では日中のメラトニン分泌は高い傾向があった。次に重症度の指標として追視のある症例（4例）と追視の無い症例（4例）で結果を比較した（図2）。神経障害のより重度と考えられる追視なし群でも夜間のメラトニン分泌は保たれていた。一方で日中のメラトニン分泌は追視なし群のほうが追視あり群よりも高濃度の傾向があった（p=0.06）。夜間唾液中メラトニン濃度と日中唾液中メラトニン濃度の比は、追視あり群のほうが追視なし群よりも高値の傾向があり（p=0.16）、障害の重い症例のほうが日中のメラトニン分泌抑制機能が障害されていると考えた。

SMID 群と AADC 欠損症例の比較（図3）

AADC 欠損症例では、夜間唾液中メラトニン濃度が 2.6pg/ml、日中唾液中メラトニン濃度は 8.9pg/

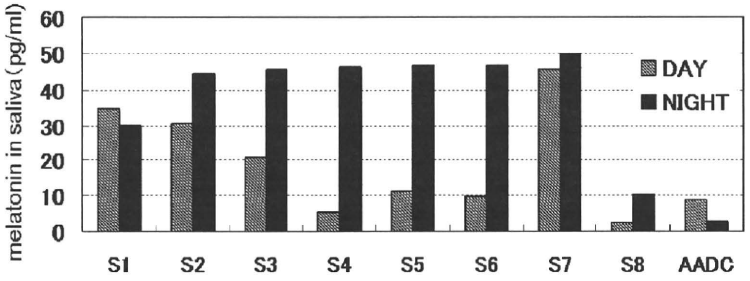

図1．重症心身障害児（者）症例（S1 ～ S8）と AADC 患者の日中と夜間の唾液中メラトニン濃度.

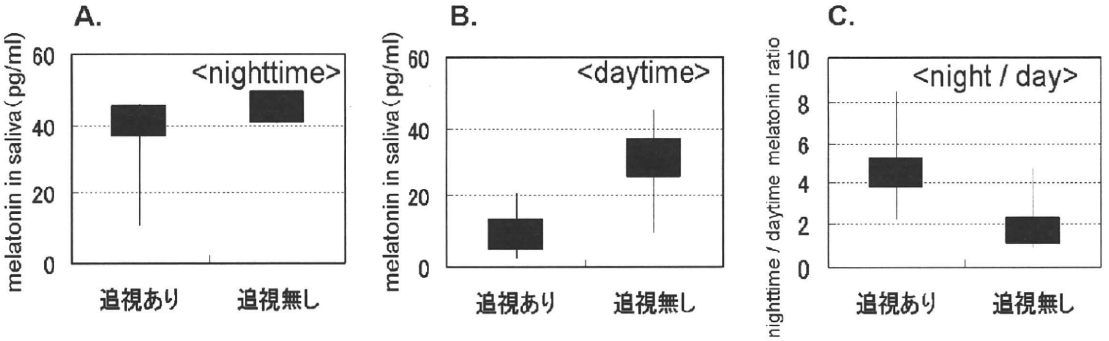

図2．重症心身障害児（者）8例の唾液中メラトニン濃度．夜間の唾液中メラトニン分泌は追視あり（4例）なし（4例）に関係なく保たれていた（A）．日中の唾液中メラトニン分泌は追視なしの群では高値の傾向があり（B）夜間と日中の唾液中メラトニン濃度比は追視なし群で低い傾向があった（C）.

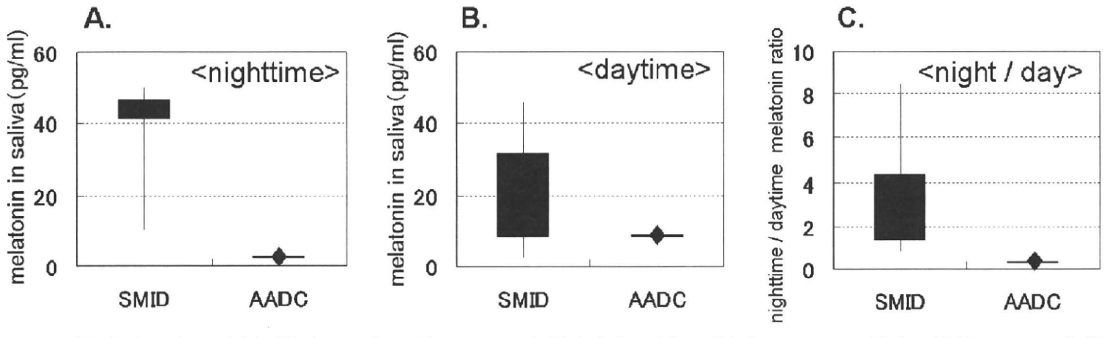

図3．重症心身障害児（者）群（SMID）8例と AADC 欠損症患者1例の唾液中メラトニン濃度の比較。AADC 欠損症患者では夜間の唾液中メラトニン濃度が SMID 群と比較して低値であり（A）、日中唾液中メラトニン濃度は SMID 群と有意な差はなかった（B）．AADC 欠損症患者では日中のメラトニン濃度のほうが高値であったため、夜間と日中の唾液中メラトニン濃度比は AADC 欠損症患者で低値であるという特徴を示した（C）.

ml であり、夜間と日中の唾液中メラトニン濃度の比は 0.29 であった。夜間唾液中メラトニン濃度は SMID 群の最低値 10.5 と比較しても十分低く、平均値から－ 2.8SD 離れていた。日中唾液中メラトニン濃度は SMID 群の最低値 2.5 よりも高値だった。夜間と日中の唾液中メラトニン濃度の比は、SMID 群での最低値は 0.86 であり、AADC 欠損症例ほどあきらかな夜間と日中のメラトニン濃度の逆転を示した例はなかった。

D．考　察

SMID 群には、非常に広汎は大脳障害をしめし松果体の同定すら困難な症例も含まれており、それでもメラトニン分泌が障害されていないことは驚くべきことであった。AADC 欠損症例の夜間メラトニン分泌は SMID 群と比べて十分に低値でありスクリーニング検査として使用可能と考えた。

日中のメラトニン分泌は SMID 群の中でも障害がより重い症例でむしろ高値となる傾向があった。松果体の主な入力系として、網膜からの光情報の入力系がある。この経路は網膜から視交叉上核、視床下部室傍核を経由し最終的に上頚神経節を経て交感神経系からの入力として松果体へ至るとされている。SMID 群での日中のメラトニン分泌抑制障害は、この経路の障害である可能性が考えられた。AADC 欠損症ではノルエピネフリン産生が障害されており交感神経系は重度に障害されることが知られている。ADC 欠損症例で、日中のメラトニン分泌が夜間よりも高値と逆転していた背景には交感神経の障害が関係している可能性があると考えた。

E．結　論

重症心身障害児（者）の中から AADC 欠損症の可能性のある症例をスクリーニングする方法として、夜間唾液中メラトニン濃度測定検査は有効である。

F．健康危険情報

なし

G．研究発表

1．論文発表

1）Ide S, Sasaki M, Kato M, Shiihara T, Kinoshita S, Takahashi JY, Goto Y. Abnormal glucose metabolism in aromatic L-amino acid decarboxylase deficiency. Brain Dev. 2010 Jun ; 32(6) : 506-10.

2．学会発表

なし

H．知的財産権の出願・登録状況

1．特許取得

なし

2．実用新案登録

なし

3．その他

なし

Ⅳ. 研究成果の刊行に関する一覧表

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
Segawa M, Nomura Y, Nishiyama N	Dopa-Responsive Dystonia	Stacy MA	Handbook of Dystonia, 2nd edition. Series : Neurological Disease and Therapy	Informa Healthcare		未定	
久保田雅也	ヒト随意運動の発達	五十嵐隆, 久保田雅也	「ここまでわかった小児の発達」	中山書店	東京	2010	Pp2-6
久保田雅也	MEG を用いた小児の発達の解析	五十嵐隆, 久保田雅也	「ここまでわかった小児の発達」	中山書店	東京	2010	Pp114-119

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
Chieko Fujii, Yuki Sato, Shohei Hrada, Naoko Kakee, Yan-Hong Gu, Tadaaki Kato, Haruo Shintaku, Misao Owada, Fumiki Hirahra, Hozo Umehashi, Makoto Yoshino	Attitude to extended use and long-term storage of newborn screening blood spots in Japan.	Pediatr Int	52	393-397	2010
Haruka Hirakawa, Hiroshi Sawada, Yumi Yamahama, Shin-Ichiro Takikawa, Haruo Shintaku, Akira Hara, Keisuke Mase, Tomoyoshi Kondo, Teruhiko Iino	Expression Analysis of the Aldo-Keto Reductases Involeved in the Novel Biosynthetic Pathway od Tetrahydrobiopterin in Human and Mouse Tissues.	J. Biochem.	146(1)	51-60	2009
Tomiko Kuhara, Morimasa Ohse, Yoshito Inoue, Toshihiko Shinka, Yoshiyuki Okano, Haruo Shintaku, Kazuhisa Hongou, Toshio Miyawaki, Wakaba Morinobu, Hirosi Tamai, Kenji Omrura	Urinary metabolic profile of phenylketonuria in patients receiving total parenteral nutrition and medication.	Rapid Mommun. Mass Spectrom.	23	3167-3172	2009
Hiroki Fujioka, Haruo Shintaku, Satoshi Kudo, Tsunekazu Yamano	Plasma Phenylalanine Level in Dopa-Responsive Dystonia.	Movement Disorders	24(15)	2289-2308	2009

発表者氏名	論文タイトル名	発表誌名	巻 号	ページ	出版年
Segawa M	Hereditary progressive dystonia with marked diurnal fluctuation.	Brain Dev	33(3)	195-201	2011
瀬川 昌也	小児の歩行機能異常	BRAIN and NERVE	62(11)	1211-1220	2010
Nomura Y, Nagao Y, Kimura K, Hachimori K, Segawa M	Epilepsy in autism : A pathophysiological consideration.	Brain Dev	32(10)	799-804	2010
Ide S, Sasaki M, Kato M, Shiihara T, Kinoshita S, Takahashi JY, Goto Y.	Abnormal glucose metabolism in aromatic L-amino acid decarboxylase deficiency.	Brain Dev	32	506-510	2010
Brun L, Ngu LH, Keng WT, Ch'ng GS, Choy YS, Hwu WL, Lee WT, Willemsen MA, Verbeek MM, Wassenberg T, Regal L, Orcesi S, Tonduti D, Accorsi P, Testard H, Abdenur JE, Tay S, Allen GF, Heales S, Kern I, Kato M, Burlina A, Manegold C, Hoffmann GF, Blau N.	Clinical and biochemical features of aromatic L-amino acid decarboxylase deficiency	Neurology	75	64-71	2010
Sueda K, et al.	MEG time-frequency analyses for pre and post surgical evaluation of patients with epileptic rhythmic fast activity.	Epilepsy Res	88	100-107	2010
Asahina N, et al.	Vaccine-associated paralytic poliomyelitis in a non-immunocompromised infant.	Pediatr Int	52	838-841	2010
Nakamura M, et al.	MERRF/MELAS overlap syndrome : A double pathogenic mutation in mitochondrial tRNA genes.	J Med Genet	47	659-664	2010
Yagyu K, et al.	The applications of timeh-uichifrequency analyses to ictal magnetoencephalography in neocortical epilepsy.	Epilepsy Res	90	199-206	2010
Saitsu H, et al.	Dominant-negative mutations in alpha-II spectrin cause West syndrome with severe cerebral hypomyelination, spastic quadriplegia, and developmental delay.	Am J Hum Genet	86	881-891	2010
Yamazawa K, et al.	Parthenogenetic chimaerism/mosaicism with a Silver-Russell syndrome-like phenotype.	J Med Genet	47	782-785	2010

発表者氏名	論文タイトル名	発表誌名	巻 号	ページ	出版年
Hamano S, Higurashi N, Koichihara R, Oritsu T, Kikuchi K, Yoshinari S, Tanaka M, Minamitani M.	Interictal cerebral blood flow abnormality in cryptogenic West syndrome.	Epilepsia	51, 7	1259-1265	2010
Tajima A, Ohashi T, Hamano S, Higurashi N, Ida H.	Gaucher Disease Patient With Myoclonus Epilepsy and a Novel Mutation.	Pediatric Neurology	42, 1	65-68	2010
Kikuchi K, Hamano S, Goto F, Takahashi A, Ida H.	Epileptic focus in a case of subcortical band heterotopia : SISCOM and ictal EEG findings.	Epilepsy & Seizure	3, 1	192-198	2010
日暮憲道, 浜野晋一郎, 田中学, 大場温子, 黒田直宏, 吉成聡, 南谷幹之	脊髄疾患, 心因反応が疑われていた瀬川病の女児例－鑑別点とその病態.	埼玉小児医療センター医学誌	25, 1	76-80	2009
Kubota M, Chida J, Hoshino H, Kashii H, Ozawa H, Koide A, Hoshino A, Koyama A, Mizuno Y, Yamaguchi M, Yao D, Yao M, Kido H	Thermolabile CPT II variants and low blood ATP levels are closely related to severity of acute encephalopathy in Japanese children.	Brain Dev	doi : 10. 1016/ j.braindev. 2010. 12. 012		2011
Kawano G, Iwata O, Iwata S, Kawano K, Obu K, Kuki I, Rinka H, Shiomi H, Yamanouchi H, Kakuma T, Takashima S, Matsuishi T.	Determination of outcomes following acute child encephalopathy and encephalitis : pivotal effect of early and delayed cooling.	ADC	in press		2010

研究成果の刊行物・別刷

PEDIATRICS INTERNATIONAL Official Journal of the Japan Pediatric Society

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Original Article

Attitude to extended use and long-term storage of newborn screening blood spots in Japan

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Abstract ***Background***: Residual dried blood spots (DBS) remaining after routine newborn screening (NBS) tests are candidate specimens for extended uses such as quality assurance and the development of new technology. A trial of NBS using tandem mass-spectrometry was launched in 2004 in Japan. The aim of the present study was to analyze the attitudes of the public, patient families, and medical professionals toward the extended use and long-term storage of residual DBS, and to construct a standardized informational brochure.

Methods: A questionnaire was sent to randomly selected members of the public, members of the Japanese Phenylketonuria (PKU) Association, medical staff of a general hospital, staff of a children's hospital, obstetricians and gynecologists, pediatricians and NBS personnel. Associated responses, which were given in a free comment format, were analyzed by text mining.

Results: The awareness ratio of NBS was low in the public (26.6%), but despite this, when a brief explanatory note on NBS was provided, 71.7% of them recognized the necessity of NBS. They were less positive than medical professionals and PKU patient families regarding the extended use of DBS for forensic investigation, for the study of health problems, or long-term storage of residual DBS, regardless of whether these factors affected them personally or not. Among the medical professionals, obstetricians and pediatricians exhibited a higher ratio of negative responses toward the extended use and long-term storage of DBS than others.

Conclusion: The general public is more conservative than PKU patients and their families or medical professionals about the extended use or long-term storage of residual DBS. Presentation to the public, particularly to couples of childbearing age, of appropriate explanatory information on NBS itself, or the extended use or long-term storage of residual DBS, is recommended.

Key words biobank, dried blood spots, long-term storage, newborn screening, text mining.

The newborn screening (NBS) program commenced in 1977 in Japan.[1] Dried blood spots (DBS) remaining after regular NBS tests are the best candidates for use not only as quality control specimens for NBS and the development of new screening technology, but also for scientific research and forensic investigation. Systematic, large-scale collection and storage of biological specimens in facilities generally termed "Biobanks", is now in operation in some countries, following the first implementation in Iceland and the UK in 1998.[2] Residual DBS are a potential source for biobanking owing to the high coverage rate among populations.[3]

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The rationale for extended use and long-term storage of residual DBS is currently under debate. It is controlled by regulations or statutes specific to the NBS program, or as part of governmental policy, in certain European countries,[4,5] Australia, New Zealand,[6] and in some States of the USA.[7,8] Guidelines for the handling of DBS, including the general rules for extended use, were presented in Japan in 1998.[9] These guidelines require that prior parental consent be given for the extended use of DBS for each specific purpose. A trial of screening on tandem mass-spectrometry was launched in 2004 in Japan. With implementation of this pilot study, substantiation of the requirement outlined in the guidelines has become mandatory. The rationale for long-term storage of residual DBS is also another issue to be discussed. The aim of the present research was to analyze the public's comments on the extended use and long-term storage of residual DBS and to offer proposals for socially acceptable policy thereof in Japan.

Methods

The questionnaire consisted of brief explanatory notes and seven questions (Table 1). This questionnaire was based on a poll questionnaire developed in the UK.[5] The questionnaire was sent to the following: (i) members of the public; (ii) members of the Japanese Phenylketonuria (PKU) Association (JPA); (iii) medical staff (excluding clerical employees) of Kurume University Hospital (KUH); (iv) personnel (including clerical employees) of the National Center for Child Health and Development (NCCHD); (v) members of the Japan Pediatric Society (JPS); (vi) members of the Japanese Society of Obstetrics and Gynecology (JSOG); and (vii) members of the Japanese Society for Mass-screening (JSM). Selection of addressees was randomized using SPSS version 14 (SPSS Japan, Tokyo, Japan). The questionnaire was sent to members of the public and members of the JPS (parents of patients) and JSOG via email, and to the other groups via regular mail. The period of survey was 1 April 2006–30 November 2007.

Analysis of free comments by text mining

Free comments were analyzed by text mining using the True Teller program (Nomura Research Institute, Tokyo, Japan), as follows.

Free comments written in Japanese were first subjected to word segmentation. Nouns were then extracted and ranked in the order of their usage frequency in the texts. Clusters of semantically close words were constructed first from the top 10 nouns, and then groups of words with lower frequency were added to the parent cluster. Semantic similarity was determined at each step by dependency parsing and by directly examining the original text.

Results

Question 1

The demographic background of the participants is summarized in Table 2. The recovery rate ranged from 26.9% (JSOG members) to 60.4% (JSM members). There was a predominance of female responders from the JPA, and a predominance of male

Table 1 Content of questionnaire

QUESTIONNAIRE ON THE USE OF BLOOD SPOTS REMAINING AFTER NEWBORN SCREENING TESTS

Question 1. Please provide your gender, birth year and month, and occupation (please specify).
Question 2a. Did you know that newborn screening tests are conducted in our country?
1. Yes, I knew.
2. No, I have never heard of it /I only came to know of it through this questionnaire.
Question 2b. If you answered "1. Yes, I knew." to question 2a:
How did you know of newborn screening tests? Please mark every option that applies.
1. Because I have undergone the test
2. Because my child(ren) has (have) undergone the test
3. Because I am engaged in medical service
4. Because I am a member of a newborn screening test-associated organization.
5. Because I am a member of a society of patients' or their families.
6. Other (please specify)
Question 3. Do you think newborn screening tests is necessary?
1. Yes 2. No 3. Unsure
Question 4. How do you feel about the use of residual blood spots by screening laboratories or research laboratories in order to maintain and improve newborn screening technology?
1. Agree 2. Neither agree nor disagree 3. Disagree 4. Unsure
Please give your free comments.
Question 5. Stored residual blood spots can be used to identify victims of a fire or natural disaster with the aid of DNA analysis. How do you feel about residual blood spots being used for such purposes?
1. Agree 2. Neither agree nor disagree 3. Disagree 4. Unsure
Please give your free comments.
Question 6a. How do you feel about your own or your family's residual blood spots being used to study health problems or medical research that directly involve you or your family?
1. Agree 2. Neither agree nor disagree 3. Disagree 4. Unsure
Please give your free comments.
Question 6b. How do you feel about your own or your family's residual blood spots being used to study health problems or medical research that do not directly involve you or your family?
1. Agree 2. Neither agree nor disagree 3. Disagree 4. Unsure
Please give your free comments.
Question 7a. How do you feel about the storage of residual blood spots for a long period (several decades), if they may be used in the future for the benefit of you or your family?
1. Agree 2. Neither agree nor disagree 3. Disagree 4. Unsure
Please give your free comments.
Question 7b. How do you feel about the storage of residual blood spots for a long period (several decades), if they may be used in the future for the benefit of society in general?
1. Agree 2. Neither agree nor disagree 3. Disagree 4. Unsure
Please give your free comments.
End of Questionnaire

Occupation was excluded from these questions when they were addressed by the members of the public and Japanese Phenylketonuria Association.

Table 2 Participant demographic data

	Members of the public	JPA members	KUH medical staff	NCCHD personnel	JPS members	JSOG members	JSM members
No. addressees	2127	351	1177	751	1012	992	396
No. respondents	1030	182	651	355	390	267	263
Recovery rate (%)	48.8	36.2	55.3	47.3	38.5	26.9	66.4
Median age (years) (range)	41.5 (21–61)	43.0 (19–80)	36.0 (19–62)	35.0 (22–76)	49.0 (25–81)	49.0 (23–80)	48.5 (26–93)
Male/Female ratio (%)	50.7/49.2	20.9/79.1	37.0/62.7	33.0/67.0	63.0/37.0	87.1/12.9	58.2/41.8

JPA, Japan Phenylketonuria Association; JPS, Japan Pediatric Society; JSM, Japanese Society for Mass-screening; JSOG, Japan Society of Obstetrics and Gynecology; KUH, Kurume University Hospital; NCCHD, National Center for Child Health and Development.

responders from the JSOG. Medical personnel respondents consisted of physicians (n = 1106), nurses (n = 324), laboratory technicians (n = 36), pharmacists (n = 15), radiologists (n = 12), midwives (n = 3), dieticians (n = 3) and others (n = 24). Occupation was not requested for members of the public or the JPA (Table 1).

The responses to questions in each group are given in Table 3. Data on differences between male and female responses are not presented unless described specifically. In Table 3, data are presented for "yes" or "no," and "agree" or "disagree" responses alone. The remaining answers were classified as "ambiguous" collectively, and are not given in the table.

Question 2

The ratio of members of the public who answered that they knew that NBS was conducted (26.6%) was lower than that of any of the other groups of subjects (question 2a). This ratio was lower in male (13.2%) than in female (40.4%) members of the public.

The leading reasons whereby members of the public had become aware that NBS was conducted (question 2b) were as follows: "because I am engaged in medical service" (56.2%), followed by "because my child(ren) underwent the test" (28.8%). Among 507 female members of the public who responded, 324 (63.9%) answered that they had ever given birth. Of the 324, 183 (56.5%) replied that they had known of NBS. Among these 183, 164 (89.6%) knew of NBS because their babies had undergone the test.

Question 3

The ratio of those who answered that NBS was necessary was 71.7% in members of the public, while it was ≥85.7% in the other groups. The ratio of respondents who were opposed to NBS was <0.8% in every group.

Question 4

The ratio of respondents who responded positively towards the use of residual DBS to maintain and improve NBS technology was lower in members of the public and medical staff of a general hospital (KUH) than in the groups that were involved in conducting NBS or had benefited from it (patients and their families).

Question 5

The ratio of positive responses toward the use of residual DBS for forensic purpose in members of the public was closer to the ratios in the other groups, when compared to other questions.

Question 6

The ratio of positive responses toward the use of residual DBS to study health problems or medical problems that directly involved themselves or their family (Question 6a) varied from 63.1% (members of the public) to 81.4% (JSM members). In the event that the purpose of DBS use was unrelated to themselves or their family, the ratio was lower in every group, except JSOG members, who gave identical ratios of affirmative answers for both questions (Question 6b).

Question 7

The ratio of responses that were positive towards storage of residual DBS in the event that they might be used for themselves or their family was relatively uniform among all the groups, except members of the public, who had a lower ratio (Question 7a). This tendency was reproduced in responses regarding the event that residual DBS might be used for the benefit of society in general, although the positive response ratio was generally lower in every group, with the exception of JMS members.

In every group the ratio of negative responses was generally higher in questions 6b and 7b than in all the other questions.

Text mining analysis

Three concepts were generated from the clusters of words that were constructed as described in the previous section. The clustered words (shown in parentheses) and concepts deduced from them (shown in brackets following an arrowhead) were as follows: (i) (privacy, administration, information, identity, etc.) > [personal data]; (ii) (agreement, approval, written consent etc.) > [availability of consent]; and (iii) (progress, research, medical science, therapy etc.) > [progress in medicine]. The frequency of usage of words that are relevant to each concept is illustrated in Figure 1.

In brief, words related to the "personal data" concept appeared at approximately the same frequency in each group (Fig. 1a). The usage frequency of words related to the "availability of consent" concept, however, was lower in members of the public and JPA members than in the other groups (Fig. 1b). In contrast, words related to the "progress in medicine" concept were more frequently used in members of the public and JPA members than in the other groups (Fig. 1c).

Discussion

The ratio of members of the public who were aware that NBS was being conducted in Japan was <27% (Question 2a). Nearly half

Table 3 Responses

Groups		Members of the public	JPA members	KUH medical staff	NCCHD personnel	JPS members	JSOG members	JSM members	Total
No. respondents		1030	182	651	355	390	267	263	3138
Questions[†]	Answers (%)[†]								
Question 2a (Awareness of NBS)	Yes	26.6	93.4	84.3	82.0	99.0	99.3	99.2	69.9
	No	73.4	6.0	14.7	17.7	0.8	0.4	0.8	29.7
Question 3 (Necessity of NBS)	Yes	71.7	99.5	85.7	88.4	97.9	97.4	98.9	85.8
	No	0.8	0	0.8	0.6	0.3	0.4	0.4	0.6
Question 4 (Extended use for NBS itself)	Agree	54.4	81.3	66.8	73.2	86.2	87.4	90.1	69.8
	Disagree	2.8	0.5	1.5	2.0	2.6	5.6	3.8	2.6
Question 5 (Forensic use)	Agree	74.0	78.6	82.3	80.2	85.9	87.9	83.3	79.5
	Disagree	1.8	1.1	1.8	2.8	2.3	5.2	2.3	2.3
Question 6a (Use for own health problems)	Agree	63.1	78.6	76.5	76.3	80.8	72.3	81.4	73.0
	Disagree	3.6	2.2	2.9	2.5	4.1	7.9	0.4	3.8
Question 6b (Use for health problems of others)	Agree	53.4	69.8	70.5	73.2	74.6	72.3	79.1	66.5
	Disagree	8.2	4.4	3.8	3.9	7.4	10.5	0.4	6.5
Question 7a (Long-term storage for own benefit)	Agree	67.8	79.7	79.1	81.7	80.8	80.1	79.8	76.1
	Disagree	3.6	2.7	2.9	1.4	4.4	6.4	6.1	3.7
Question 7b (Long-term storage for benefit of others)	Agree	59.9	70.9	76.0	75.5	77.5	77.2	80.6	71
	Disagree	4.5	2.7	3.2	2.5	5.0	8.2	5.3	4.4

[†]Synopses of questions are shown in brackets. Please refer to Table 1 for full text of questions. [‡]Answers "yes" or "no" and "agree" or "disagree" alone are indicated. The remaining answers were classified as "ambiguous" collectively, and are not given in the Table.

JPA, Japan Phenylketonuria Association; JPS, Japan Pediatric Society; JSM, Japanese Society for Mass-screening; JSOG, Japan Society of Obstetrics and Gynecology; KUH, Kurume University Hospital; NBS, newborn screening; NCCHD, National Center for Child Health and Development.

of the female members of the public who had ever given birth replied that they did not know that NBS was being implemented. This result would imply that briefing on NBS was not provided in enough detail to be remembered by mothers, or was not given at all. This low awareness ratio appears to be closely linked with the lower usage frequency of words related to the "availability of consent" concept in members of the public than in other groups (Fig. 1b).

Approximately 70% of members of the public agreed with the significance and necessity of NBS, when some information on NBS was provided (Question 3). In addition, members of the public used words related to the "progress in medicine" concept most frequently among all the seven groups, when their questionnaire responses were analyzed by text mining (Fig. 1c). All these results indicate that the public is potentially interested in NBS and anticipates benefit from it. Therefore, intensive publicity regarding the purpose and benefit of NBS among the general public, and in particular couples who intend to have children, is expected to be useful.

Approximately 66.8–90.1% of respondents in each group, excluding members of the public, had positive responses toward the extended use of residual DBS to evaluate and improve current NBS technology and to develop new technologies (Question 4). The extended use of DBS, and the duration of storage of residual DBS and associated personal data, are governed by legislation or by recommendations proposed by academic societies in Denmark,[4] UK,[5] Australia, New Zealand,[6] France,[10] The Netherlands[10] and 16 States in the USA.[8] In Japan, the general guidelines that control the extended use of DBS were set out in 1998.[9] For routine screening tests, oral or written information is provided to parents in various ways in accordance with this guideline in individual areas. Notification on the storage policy, however, for residual DBS is generally not included in such information. Explicit documentation and publicity regarding storage policy is essential to ensure transparency about the outcomes of DBS and to avoid public confusion about their storage, as was encountered in the Enschede disaster.[10] The articulation of policies regarding storage of human materials and data is strongly recommended in the operation of human biobanks and genetic databases in general, according to a recent OECD recommendation draft.[11] Residual DBS are systematically registered and stored nationwide and used for research in Denmark[4] and the UK.[5] Purposes of storage are specified in these countries. They are, in brief, later retesting, quality assurance, improvement and development of new NBS programs, and research. Residual DBS are used for research purposes and the system is termed "biobank" in these countries.[4,5] In laboratories in Japan, the purposes, duration and conditions of storage of residual DBS are diverse.[12] A common purpose of storage is to provide for possible retesting. It is crucial to make the purpose of storage of residual DBS clear in order to first determine the duration and conditions of storage, because the stability of analytes on DBS is largely unknown.[8] All of these results indicate that presentation to the public, particularly couples of child-bearing age, of appropriate explanatory information on the extended use and long-term storage of residual DBS is necessary. Presentation of information on the NBS

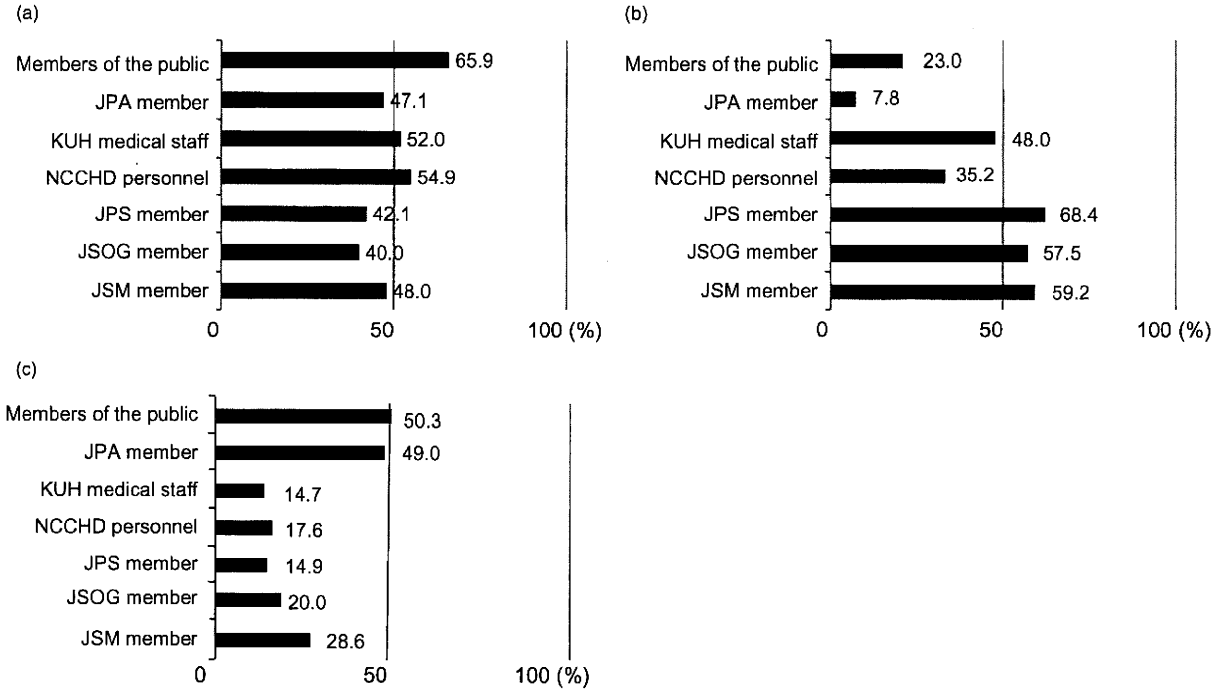

Fig. 1 Usage frequency of words (%) related to the concepts (a) personal data; (b) availability of consent; and (c) progress in medicine. The usage frequency number is defined as the sum of every word that is related to each concept divided by number of comments made for every question. JPA, Japanese Phenylketonuria Association; JPS, Japan Pediatric Society; JSM, Japanese Society for Mass-screening; JSOG, Japanese Society of Obstetrics and Gynecology; KUH, Kurume University Hospital; NCCHD, National Center for Child Health and Development.

program to couples during pregnancy may allow better understanding of the program.[13]

Attitudes toward the use of DBS for forensic purposes were similar among all the groups (Question 5). It remains open whether or not consent for extended use for such a purpose should be included in routine explanatory brochures. Legislation or regulation regarding whether or not a screening laboratory can release stored DBS upon judicial order should also be discussed.

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References

1 Matsuda I. Bioethical issues in newborn mass screening; Experiences in Japan. *Acta Paediatr.* 1999; **432**(Suppl.): 104–5.
2 Austin MA, Harding S, McElroy C. Genebanks: A comparison of eight proposed international genetic databases. *Community Genet.* 2003; **6**: 37–45.
3 Nørdgaard-Pedersen B, Simonsen H. Biological specimen banks in neonatal screening. *Acta Paediatr.* 1999; **88**(Suppl.): 106–9.
4 Nørgaard-Pederson B, Hougaard DM. Storage policies and use of the Danish Newborn Screening Biobank. *J. Inherit. Metab. Dis.* 2007; **30**: 530–36.
5 Oliver S, Stewart R, Hargreaves K, Dezateux C. *The Storage and Use of Newborn Babies' Blood Cards: A Public Consultation.* Social Science Research Unit, Institute of Education, University of London, London, 2005.
6 Webster D. Storage and use of residual dried blood spots. *Southeast Asian J. Trop. Med. Public Health* 2003; **34**(Suppl. 3): 49–51.
7 Olney RS, Moore CA, Ojodu JA, Lindegren ML, Hannon WH. Storage and use of residual dried blood spots from state newborn screening programs. *J. Pediatr.* 2006; **148**: 618–22.
8 Therrell BL, Johnson A, Williams D. Status of newborn screening programs in the United States. *Pediatrics* 2006; **117**: S212–S252.
9 Matsuda I. The implementation guideline for newborn screening. *J. Jpn. Soc. Massscreen.* 1998; **8**(Suppl. 2): 1–5 (in Japanese).
10 Couzin-Frankel J. Science gold mine, ethical minefield. *Science* 2009; **324**: 166–8.
11 Directorate for Science, Technology and Industry, Organization for Economic Cooperation and Development (OECD). *Guidelines for Human Biobanks and Genetic Research Databases (HBGRDs).* DST1/STP/BIO(2008)31/REV1. OECD, Paris, 2009.
12 Umehashi H, Tazaki R, Kuroda Y, Fukushi M, Umehashi M, Sumiyoshi Y. *A study on the management and use of residual blood card with attention to ethical issues.* The Ministry of Health, Labor and Welfare of Japan, Term 2001 (Research on Children and Families), Tokyo, 2002 (in Japanese)
13 Detmar S, Hosli E, Dijkstra N, Nijsingh N, Rijnders M, Verweij M. Information and informed consent for neonatal screening: Options and preference of parents. *Birth* 2007; **34**: 238–44.

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Expression Analysis of the Aldo-Keto Reductases Involved in the Novel Biosynthetic Pathway of Tetrahydrobiopterin in Human and Mouse Tissues

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Tetrahydrobiopterin (BH_4) acts as a cofactor of the aromatic amino-acid hydroxylases, and its deficiency may result in hyperphenylalaninemia (HPA) and decreased production of the neurotransmitters. BH_4 is synthesized by sepiapterin reductase (SPR) from 6-pyruvoyl-tetrahydropterin (PPH_4). A patient with SPR deficiency shows no HPA; however, an SPR knockout mouse exhibits HPA. We have reported on the SPR-unrelated novel biosynthetic pathway from PPH_4 to BH_4 (salvage pathway II) in which 3α-hydroxysteroid dehydrogenase type 2 and aldose reductase work in concert. In this study, we performed the expression analysis of both proteins in humans and wild-type mice. The results of expression analysis indicated that salvage pathway II worked in human liver; however, it did not act in human brain or in mouse liver and brain. For this reason, a patient with SPR deficiency may show progressive neurological deterioration without HPA, and SPR knockout mice may exhibit HPA and abnormal locomotion activity.

Key words: AKR1B1, AKR1C3, Aldo-keto reductase, BH_4 deficiency, SPR deficiency.

Tetrahydrobiopterin (BH_4) is a cofactor for aromatic amino-acid hydroxylases (*1*, *2*), which catalyses the initial steps in phenylalanine degradation in the liver and the rate-limiting steps in the biosynthesis of catecholamine and indoleamine neurotransmitters in the brain. BH_4 is also required by nitric oxide synthase, which generates nitric oxide, a messenger molecule involved in various processes in many tissues (*3*, *4*).

The pathway of the *de novo* biosynthesis of BH_4 from GTP involves GTP cyclohydrolase I (GTPCH-I, EC 3.5.4.16), 6-pyruvoyl-tetrahydropterin synthase (PTPS, EC 4.6.1.10) and sepiapterin reductase (SPR, EC 1.1.1.153). SPR catalyses the last step of the biosynthesis, in which the diketo group on the side chain of PPH_4 is converted into the corresponding diol form in BH_4 (*5–7*). A deficiency of BH_4 causes hyperphenylalaninemia (HPA), which leads to the abnormal development of mammalian neonates.

In 2001, SPR deficiency was first discovered in a patient with progressive psychomotor retardation and dystonia. However, the patient showed normal urinary pterins without HPA (*8–10*). These findings suggest that an enzyme or enzymes other than SPR may be involved in the formation of BH_4 from PPH_4.

Park *et al.* (*11*) previously reported that human monomeric carbonyl reductase (CBR) reduces PPH_4 to both 1′-oxo-2′-hydroxypropyl-tetrahydropterin (1′-$OXPH_4$) and 1′-hydroxy-2′-oxopropyl-tetrahydropterin (2′-$OXPH_4$) and that aldose reductase (AKR1B1, EC 1.1.1.21) catalyses the reduction of 2′-$OXPH_4$ to BH_4. Therefore, if both AKR and CBR proteins exist in the tissue, BH_4 can be synthesized from PPH_4 without SPR. However, the 2′-$OXPH_4$-forming activity of CBR is quite low compared to its 1′-$OXPH_4$-forming activity. Therefore, the BH_4-forming activity, which involves CBR and AKR1B1, functions with difficulty in humans.

Blau *et al.* (*9*) proposed that BH_4 is synthesized through salvage pathway I in the case of SPR deficiency (Fig. 1). In salvage pathway I, sepiapterin, which is generated non-enzymatically from 1′-$OXPH_4$, is converted to dihydrobiopterin (BH_2) by CBR. The final reduction to BH_4 in the liver is catalysed by the enzyme dihydrofolate reductase (DHFR, EC 1.5.1.3). The activity of DHFR is ~10× lower in the brain than in the liver. Thus, sepiapterin is reduced to dihydrobiopterin by CBR but cannot be further reduced to BH_4 owing to low DHFR activity in the brain. Therefore, they concluded that a patient with SPR deficiency shows progressive psychomotor retardation without HPA.

We previously discovered two carbonyl reductases (CRI and CRII) that are involved in the formation of BH_4 from PPH_4 in the fat body of the *lemon* mutant and the

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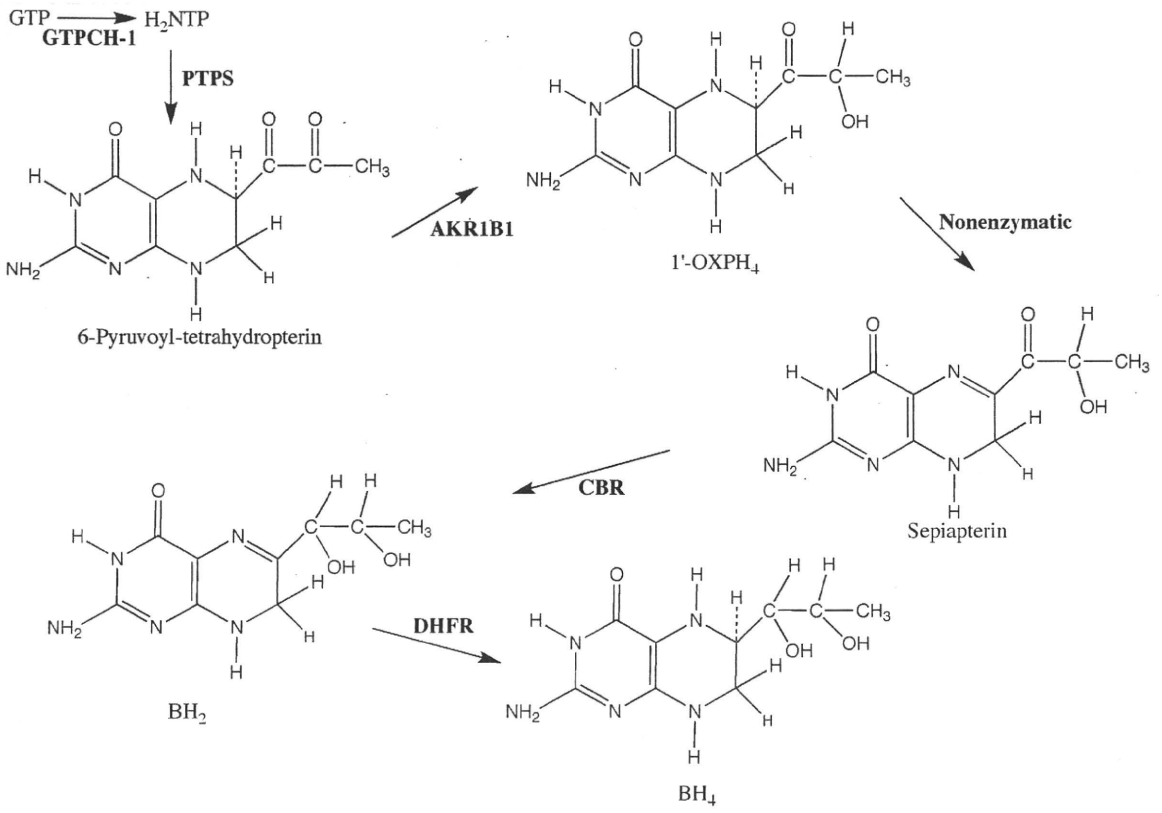

Fig. 1. **The SPR-unrelated BH_4 biosynthetic pathway (salvage pathway I).** In the absence of SPR, 1′-$OXPH_4$ is oxidized non-enzymatically to sepiapterin, which is reduced to BH_4 by CBR and DHFR.

normal strain of the silkworm, *Bombyx mori* (*12–14*). Furthermore, we have reported on a novel alternative biosynthetic pathway (salvage pathway II) from PPH_4 to BH_4, in which 3α-hydroxysteroid dehydrogenase type 2 (AKR1C3, EC 1.1.1.213) and AKR1B1 work in concert (Fig. 2) (*15*). Salvage pathway II shows that AKR1C3 efficiently catalyses the reduction of PPH_4 to the intermediate metabolite, 2′-$OXPH_4$, which is reduced to BH_4 by AKR1B1.

Recently, Yang *et al.* (*16*) indicated that the SPR knockout mouse exhibited HPA, dwarfism and impaired body movement. Furthermore, $Spr^{-/-}$ mice, as reported by Takazawa *et al.* (*17*), also showed HPA. In spite of adequate activity of CBR and DHFR in the mouse liver, SPR knockout mice show HPA. Thus, salvage pathway I, which is proposed by Blau *et al.* (*9*), may not function in mouse liver. We believe that salvage pathway II works in human liver but not in wild-type mouse liver. Consequently, humans who lack *SPR* may show no HPA, and SPR knockout mice show HPA. In order to verify this hypothesis, we examined the expression analysis of AKR1B1 and AKR1C3 proteins in human and wild-type mouse tissues using anti-AKR1B1 or anti-AKR1C3 antibodies. In the case of mice, western blot and immunohistochemical analyses showed that the AKR1C3 protein was expressed in the liver but not in the brain. In contrast, the AKR1B1 protein was detectable in the brain but not in the liver.

In the case of humans, western blot analysis showed that the AKR1B1 and AKR1C3 proteins were both expressed in the liver; however, AKR1B1 was only expressed in the brain, and AKR1C3 could not be detected in the brain.

These results of the expression analysis of AKR1B1 and AKR1C3 by means of immunohistochemistry and western blot analysis could be explained by the relationship between HPA in SPR knockout mouse and the absence of HPA in patients with SPR deficiency. Moreover, they suggest that the SPR-unrelated BH_4 formation pathway from PPH_4, which is involved in the AKR enzymes, functions in the human liver. In this report, for the first time, we explain the reasons that SPR knockout mice show abnormal locomotion activity with HPA and a patient with SPR deficiency exhibits progressive neurological deterioration without HPA.

MATERIALS AND METHODS

Chemicals and Enzymes—BH_4 and sepiapterin were purchased from Dr Schircks (Jona, Switzerland). Dihydroneopterin triphosphate (NH_2TP) was synthesized enzymatically from GTP by the method of Yoshioka *et al.* (*18*) using purified GTP cyclohydrolase I from chicken liver (*19*). 1′-$OXPH_4$ and 2′-$OXPH_4$ standards were prepared as described previously (*12*). Other chemicals were of analytical grade and obtained from

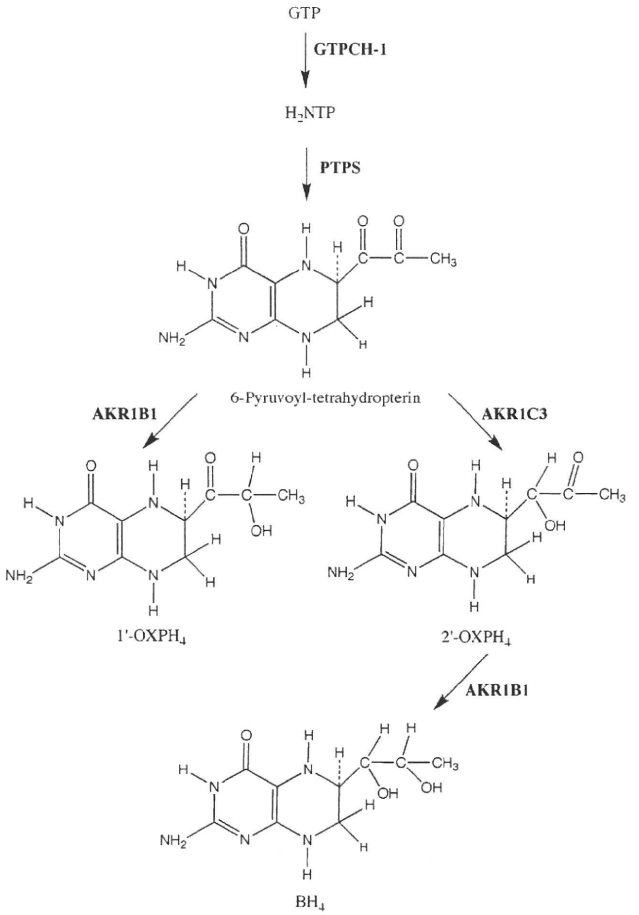

Fig. 2. **The SPR-unrelated BH_4 biosynthetic pathway (salvage pathway II).** BH_4 is synthesized from PPH_4 by AKR1C3 and AKR1B1.

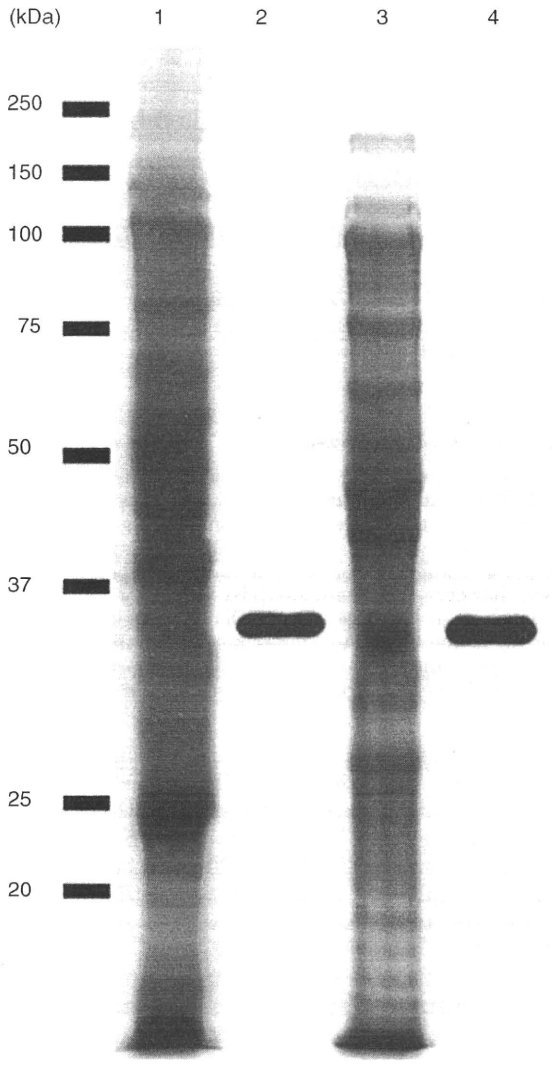

Fig. 3. **Specificity of anti-AKR1B1 and anti-AKR1C3 antibodies.** Lanes 1 and 3 show the SDS/PAGE analysis of the protein from the *E. coli* lysate, which expressed human AKR1B1 and AKR1C3, respectively. The gel was stained with Coomassie Brilliant Blue R-250. Lanes 2 and 4 are immunoblot analyses using the anti-AKR1B1 antibody and the anti-AKR1C3 antibody, respectively. Immunoblot analysis shows that both antibodies can only react with one species of ~36 kDa. Ten micrograms of crude *E. coli* lysate was used.

commercial sources. PPH_4 synthase was purified from chicken liver by the method of Takikawa *et al.* (*20*).

Human Tissues and Animals—Human tissues were obtained in compliance with the Ethical Committee of Wakayama Medical University and Osaka City University Medical School's Ethics Committee. Three human brains were obtained following autopsies: Patient 01 (P01) (female) was 68 years old with Alzheimer's disease; Patient 02 (P02) (female) was 78 years old with oophoroma; and Patient 03 (P03) (female) was 48 years old with bacterial meningitis. One human liver was obtained following an autopsy: Patient 04 (P04) (male) was 1 year old with galactosialidosis. Another was obtained as a result of a biopsy: Patient 05 (P05) (male) was 5 years old with amylopectinosis. The autopsy samples from brain and liver were obtained between 5 and 7 h post mortem. The autopsy samples and biopsy liver sample were stored at −70°C until use. Male mice of BALB/c (3 weeks old), an example of wild-type mouse, were obtained from a breeder.

Production of an Anti-AKR1B1 and an Anti-AKR1C3 Antibody—Rabbit polyclonal antibodies against the purified recombinant human AKR1B1 and AKR1C3 proteins (*21*, *22*) were raised. For immunization, a solution containing the purified proteins (1 mg/ml) was emulsified with Freund's complete adjuvant having twice the volume of the antigen solution. Rabbits received a dose of 0.5 mg of the proteins (AKR1B1, AKR1C3) intradermally at multiple sites on their backs. Doses of 0.5 mg of the proteins in Freund's incomplete adjuvant were then given as booster injections at 2 weeks intervals by subcutaneous injections. More than six booster injections were necessary to obtain a satisfactory antibody titer.

Western Blot Analysis—Western blot analyses of mouse liver, brain, kidney, heart and lung lysates and of human