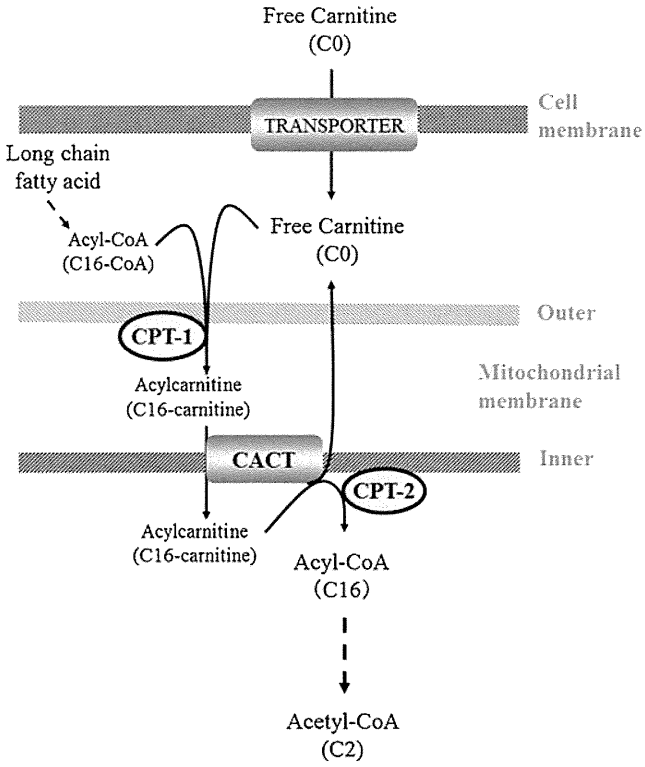

Fig. 1 Pathway for mitochondrial fatty acid beta-oxidation. Transporter: carnitine uptake transporter; *CPT-1*: carnitine palmitoyltransferase-1, *CACT*: carnitine acylcarnitine translocase, *CPT-2*: carnitine palmitoyltransferase-2. *Solid arrows* indicate single reactions; *dashed arrows* indicate multiple reactions or steps

encephalopathy, hepatomegaly, myopathy, hypoglycemia, and hyperammonemia, mainly result from low carnitine concentration in the tissues. On the other hand, secondary carnitine deficiency occurs in some conditions such as organic acidemias, renal dialysis, long-term medication (antiepileptic drugs or some antibiotics), and alimentary deficiency of L-carnitine [7–9].

It is necessary to make a differential diagnosis of PCD from the secondary carnitine deficiency or other false-positive cases, and diagnosis is confirmed by demonstrating reduced transport in skin fibroblasts from the patients. Until now, cluster-tray method using radioisotope-labeled substrate was used for the diagnosis of PCD [4, 10–12]. However, such a diagnostic method requires handling of radioactive substrates and focused only on diagnosis of PCD. Gene sequencing in SLC22A5 is one diagnostic method for PCD. However, it is molecularly heterogenous, and around 50 different mutations have been identified [6]. After acylcarnitine analysis using tandem MS analysis became available in the worldwide, blood acylcarnitine analysis was used as an initial method for diagnosis of FAO disorders and a detection of FAO disorders has been increased. However, it is necessary to confirm the diagnosis of the diseases with detailed analysis. The in vitro probe acylcarnitine (IVP) assay using cultured fibroblasts and tandem mass spectrometry (MS/MS) has been used to evaluate FAO capacity in the cultured cells and make a diagnosis of FAO disorders [13–15]. However, conventional IVP assay is not feasible to diagnose PCD or CPT1 deficiency, because excess amount of free carnitine is added to the experimental medium at the beginning. Estimation of free carnitine, which is the key marker for the above diseases, in experimental medium was nonsense for diagnosis of these disorders. We developed a novel functional assay for PCD and CPT1 deficiency using the IVP assay, with some modifications. This method uses different concentrations of exogenous free carnitine and measures intracellular as well as extracellular acylcarnitine (AC) levels, which overcomes the disadvantage of the conventional IVP assay in the diagnosis of carnitine cycle disorders.

Materials and methods

Materials

Hexanoylcarnitine (C6), octanoylcarnitine (C8), decanoylcarnitine (C10), and palmitoylcarnitine (C16) were purchased from Sigma–Aldrich (St Louis, MO, USA). Methanol, acetonitrile, and formic acid were purchased from Wako (Osaka, Japan). As an internal standard, a labeled carnitine standard kit (NSK-B), which contains $^{2}[H]_{9}$-carnitine, $^{2}[H]_{3}$-acetylcarnitine, $^{2}[H]_{3}$-propionylcarnitine, $^{2}[H]_{3}$-butyrylcarnitine, $^{2}[H]_{9}$-isovalerylcarnitine, $^{2}[H]_{3}$-octanoylcarnitine, $^{2}[H]_{9}$-myristoylcarnitine, and $^{2}[H]_{3}$-palmitoylcarnitine, was purchased from Cambridge Isotope Laboratories (Andover, MA, USA).

Preparation of standard solutions of ACs

Standard solutions containing 1, 10, 25, and 50 μmol/L each of C6, C8, C10, and C16 were used to validate the recovery and determine linear concentration range of ACs after extraction by the Folch method [16]. The ACs were dissolved in methanol (99.8 %), and the prepared standard solution was analyzed directly and after extraction by the Folch method.

Subjects

Human skin fibroblasts from six healthy controls (volunteers) and seven patients with various carnitine cycle disorders—three each with PCD and CPT2 deficiency and one with CPT1 deficiency—were analyzed. In all cases, diagnoses were confirmed by mass spectrometric analyses (gas chromatography-mass spectrometry and MS/MS), enzyme assay, and protein or mutational analyses. Informed consent was obtained from the patients or their families. This study was approved by the Ethical Committee of the Shimane University School of Medicine.

In vitro probe acylcarnitine (IVP) assay using MS/MS

An IVP assay was performed, as described, with some modifications [13, 15, 17], and principle of IVP assay was shown Fig. 2. Briefly, 3×10^6 cells were seeded in triplicate onto a six-well microplate (35 mm i.d.; Iwaki) and cultured until confluent. After washing twice with Dulbecco's phosphate buffered saline (DPBS; Invitrogen, Carlsbad, CA, USA), the cells were subsequently cultured for 96 h in 1 ml of a special experimental minimal essential medium (MEM) containing bovine serum albumin (0.4 % essential fatty acid-free BSA; Sigma), two different concentrations of C0 (Sigma)—10 μmol/L (reduced level, lower compared with physiological level) and 400 μmol/L (excess level)—and unlabelled palmitic acid (0.2 mmol/L; Nacalai Tesque). C0 and AC levels in the culture medium (extracellular fraction) and in the intracellular extract were analyzed after a 96-h incubation period using MS/MS (API 3000; Applied Biosystems, Foster City, CA, USA), as described [18].

Intracellular acylcarnitine extraction

Intracellular C0 and ACs were extracted using the Folch method, with some modification [16]. Briefly, harvested cells were washed twice with DPBS buffer. The cell pellet was resuspended in 100 μl volume of DPBS buffer and immediately frozen in liquid N_2. In order to separate phospholipids and cell debris, 250 μl of Folch reagent (chloroform/methanol, 2:1) was added to the resuspended cell pellet. After vigorous mixing using a vortex mixer, the solution was centrifuged for 10 min at 15,000 rpm at 4 °C. The debris layer around the interface between the aqueous and lipid phases was removed, and the extracted aqueous and lipid phases were mixed and thereafter dried under a nitrogen stream at 50 °C. ACs in culture medium supernatants and extracted intracellular ACs lysate were analyzed using MS/MS (API 3000; Applied Biosystems, Foster City, CA, USA). Briefly, methanol (200 μl) including an isotopically labeled internal standard (Cambridge Isotope Laboratories, Kit NSK-A/B, Cambridge, UK) was added to 10 μL of supernatant from culture medium and extracted intracellular ACs, for 30 min. Portions were centrifuged at 1,000×*g* for 10 min, and then 150 μL of supernatant was dried under a nitrogen stream and butylated with 50 μL of 3 N *n*-butanol-HCl at 65 °C for 15 min. The dried butylated sample was dissolved in 100 μL of 80 % acetonitrile/water (4:1 *v/v*), and then the ACs in 10 μL of the aliquots were determined using MS/MS [18] and quantified using Chemo-View™ software (Applied Biosystems/MDS SCIEX, Toronto, Canada).

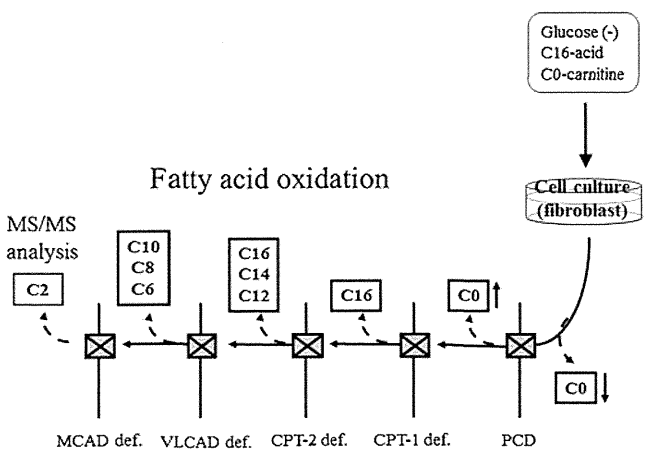

Fig. 2 Principle of in vitro probe acylcarnitine assay. *C2, C4, C6, C8, C10, C12, C14*, and *C16* represent acylcarnitines

Protein concentration and cell viability

Protein concentrations were measured by a modification of the Bradford method using the Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA) [19]. The percentage of viable cells was determined at 24, 48, 72, and 96 h of incubation using the modified 3-(4,5-dimethyl-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) assay [20].

Data and statistical analysis

The results are expressed as mean±SD from at least three independent experiments for IVP assay in each cultured cell and three intra-assays and three inter-assays for recovery of standard AC solutions, and statistical significance was evaluated using Student's *t*test in Microsoft Excel. The AC concentrations were expressed as nanomoles per milligram protein.

Results

Recovery of ACs during Folch extraction

The AC standards in the aqueous or lipid fraction were analyzed separately using MS/MS, after extraction by the Folch procedure, and compared with direct analysis of the total mixed standard solutions using three inter-assays and three intra-assays of analysis of standard AC solution. As shown in Fig. 3, most of the C6 and C8-carnitines fractionated to the aqueous phase, while almost all C16-carnitine was exclusively retained in the lipid phase. The amount of C10-carnitine was comparable in both aqueous and lipid phases.

To determine the loss of C0 and ACs during Folch extraction, the standard AC solution was analyzed directly after routine sample preparation for MS/MS and compared with that after Folch extraction. The recovery of ACs in the

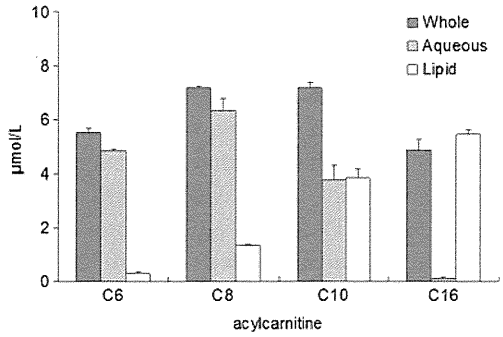

Fig. 3 Recovery of ACs during extraction using the Folch method. Standard solutions of 10 μmol/L each of C6-, C8-, C10-, and C16-carnitine were used to determine the recovery of ACs in the aqueous and lipid fractions during extraction using the Folch method. *Grey column*: ACs in the whole extract after Folch method; *striped column*: ACs in the aqueous fraction of Folch extraction; *open column*: ACs in lipid fraction of Folch extraction. Data are expressed as mean±SD (micromoles per liter) from three intra-assays and three inter-assays, and statistical significance was evaluated using Student's *t*test in Microsoft Excel

standard solutions after direct analysis and Folch extraction procedure was analyzed three times by inter-assay. The inter-assay CV of acylcarnitines ranged from 3.21 to 8.33 %. No statistical difference was seen between direct analysis and after Folch extraction.

Acylcarnitine profile in extracellular medium of cultured fibroblasts with excess and reduced concentrations of free carnitine

Using fibroblasts from various carnitine cycle disorders, AC profiles were determined in the extracellular medium with reduced or excess concentration of C0. Reported conventional IVP assay used excess levels of C0 (400 μmol/L) [14, 15, 17, 21]. With excess amount of C0 (Table 1, "Medium (C0-excess, 400 μM)"), a selective increase in C16 and a decrease in acetylcarnitine (C2) was observed in cases of CPT2-deficient fibroblasts. AC profiles in media from PCD- and CPT1-deficient fibroblasts were similar to that of healthy controls. In PCD fibroblasts, C2 was 53.1 % of the normal control while C2 in CPT1-deficient fibroblasts was 140 % of the normal control. No statistical difference in C0 level was observed among CPT2-, PCD-, and CPT1-deficient fibroblasts and a healthy control.

In the extracellular medium containing reduced C0, C16 remains higher in cells with CPT2 deficiency, while AC profiles were similar to those observed in C0-excess for PCD- and CPT1-deficient cells and the healthy controls (Table 1, "Medium (C0-reduced, 10 μM)").

Acylcarnitine profile in intracellular lysate with various concentrations of free carnitine

The intracellular C0 and ACs were measured after AC extraction using the Folch method. C16 in the intracellular lysate from CPT2-deficient fibroblasts was significantly elevated in both reduced and excess C0 conditions similar to those in extracellular medium, and diagnostic significant was kept. In the excess C0 condition, CPT1- and PCD-deficient fibroblasts could not be distinguished clearly; based on the C0 levels, even C16 level was relatively low (Fig. 4a). On the other hand, the intracellular C0 under conditions with reduced C0 was 41.78±1.47 and 6.31±2.88 nmol/mg protein/96 h in the normal controls (n=6) and patients with PCD (n=3), respectively, and the C0 levels of PCD cells were significantly lower (p<0.001) as shown in Fig. 4b. This indicated that the C0 uptake was significantly decreased in PCD compared with control in

Table 1 Acylcarnitine profiles of in vitro probe acylcarnitine assay

	Acylcarnitines, nmol/mg protein/96 h						
	C0	C2	C6	C8	C12	C14	C16
Medium (C0 excess, 400 μM)							
Control (n=6)	411.74±23.08	11.80±1.54	2.60±0.09	1.70±0.47	0.79±0.22	0.34±0.19	2.06±0.77
PCD (n=3)	432.18±18.76	6.25±0.96	2.09±0.40	0.94±0.54	0.41±0.33	0.20±0.10	1.72±0.57
CPT-1 (n=1)	357.69±34.16	16.52±5.60	1.73±0.87	0.54±0.94	0.18±0.14	0.17±0.16	1.36±0.98
CPT-2 (n=3)	376.56±42.71	6.88±0.72	0.94±0.65	0.41±0.22	1.70±0.35	0.80±0.05	18.73±1.07
Medium (C0 reduced, 10 μM)							
Control (n=6)	9.85±0.30	1.70±0.74	0.78±0.30	0.18±0.09	0.10±0.08	0.03±0.01	0.51±0.11
PCD (n=3)	10.03±0.71	0.74 ±0.33	0.75±0.31	0.06±0.04	0.03±0.01	0.01±0.01	0.20±0.08
CPT-1 (n=1)	11.06±0.75	7.56±3.10	0.98±0.30	0.55±0.62	0.09±0.09	0.08 ±0.07	0.01 ±0.02
CPT-2 (n=3)	9.73±1.94	0.64±0.23	0.54±0.20	0.11±0.03	0.22±0.06	0.04±0.01	2.79±0.38

The results are expressed as mean±SD from three independent experiments with triplication in each cell line. The AC concentration was expressed as nanomoles per milligram protein. *C0* free carnitine, *C2* acetylcarnitine, *C6* hexanoylcarnitine, *C8* octanoylcarnitine, *C12* dodecanoylcarnitine, *C14* myristoylcarnitine, *C16* palmitoylcarnitine

C0-reduced condition. Concentration of C16 was also significantly low in PCD in C0-reduced condition. Under the C0-reduced condition, intracellular C0 was much higher, but C16 was much lower in CPT1-deficient fibroblasts, compared with the levels in controls (Fig. 4b).

The ratio of intracellular C0 to extracellular C0 in PCD was significantly lower than that of the controls ($p<0.001$) in the C0-reduced condition, while that in C0-excessive condition was not significantly different (Fig. 5). Cell viability was measured using the MTT assay under reduced or excess concentrations of C0. The percentage of viable cells cultured in C0-reduced medium was equivalent to that in C0-excess media (data not shown).

Discussion

The present study developed a novel IVP assay for the accurate diagnosis of PCD and CPT1 deficiency. Although previous studies reported that IVP assay was a powerful method for the diagnosis of most FAO disorders [13, 14, 21], this assay turned out to be unable to identify PCD and CPT1 deficiencies. At first, we used a C0-excess experimental medium, which contained 400 μmol/L of C0, according to previous reports [13, 14, 21]. Extracellular

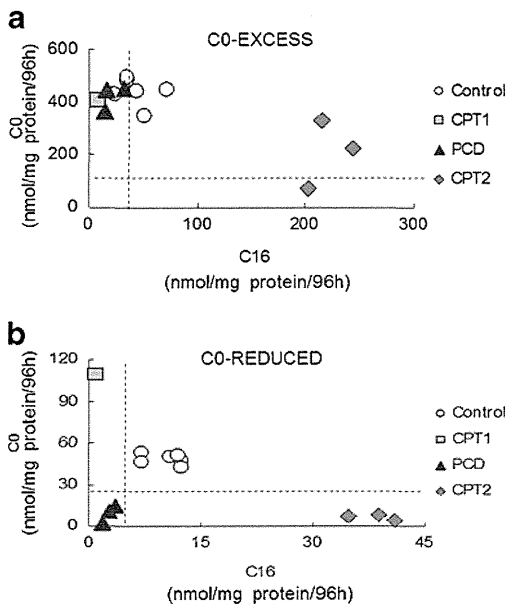

Fig. 4 Intracellular C0 and C16 correlation in patients with carnitine cycle disorders. **a** C0-excessive condition (−*E*); **b** C0-reduced condition (−*R*). *open circle*: healthy control ($n=6$); *closed triangle*: PCD ($n=3$); *closed square*: CPT1 deficiency ($n=1$); *closed diamond*: CPT2 deficiency ($n=3$). Cells were incubated in experimental medium with 400 or 10 μmol/L of free carnitine and 200 μmol/L of palmitic acid. After 96-h incubation, cells were harvested, and intracellular free carnitine (C0) and palmitoylcarnitine (C16) were extracted using Folch method and measured using MS/MS. Data of mean values of triplicates are presented (nanomoles per milligram protein per 96 h)

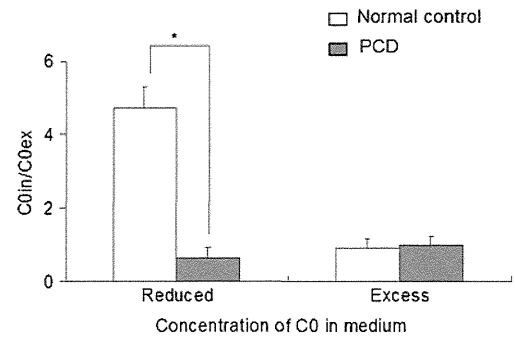

Fig. 5 Ratio of intracellular C0 to extracellular C0. *Open square*: normal control ($n=6$); *closed square*: PCD ($n=3$). Extra- and intracellular C0 of cells with normal control and PCD were measured in C0-reduced (10 μmol/L) and C0-excess (400 μmol/L) conditions using MS/MS. Data are expressed as mean±SD of six normal controls and three patients with PCD. Experiment in each cell line was repeated twice with triplications. Significant differences between normal control and PCD are shown as $*p<0.001$

AC profiles of patients with PCD and CPT1 deficiency showed a pattern similar to that of normal controls by the conventional assay that contains excessive C0 (400 μmol/L) in the culture medium, since C0 moves across the cell membrane down its concentration gradient by passive diffusion. Long-chain fatty acids are transferred across the inner mitochondrial membrane with the assistance of carnitine and carnitine cycle enzymes. The subsequent FAO functions normally even in PCD, and AC profile in PCD is similar to that in normal FAO. Next, we used 50 μmol/L of C0 because the normal range of free carnitine in human plasma was approximately 25 to 50 μmol/L [6]. However, there was no diagnostic difference compare with C0-excess condition, and data are not shown. We analyzed IVP assay in C0-deficient condition (10 μmol/L of C0).

It is known that fibroblasts and muscle and cardiac cells have a high-affinity, low-capacity transporter system [22], and carnitine concentrations in the tissues are much higher than those in serum [23]. Analysis of intracellular C0 and ACs is more relevant for the diagnosis of PCD and CPT1 deficiency because it was shown that C0 was decreased in PCD and increased in CPT1 deficiency in those tissues. When we analyzed cell lysates with MS/MS after direct sonication, artificial peaks of ACs were detected, and the background peaks of mass spectrum were high and hampered the subsequent analyses (data not shown). Hence, we extracted intracellular ACs using a modified Folch method and analyzed both the intracellular lysate and the extracellular medium. This allowed visualization of clear peaks of C0 and ACs in the intracellular lysate, validating that the Folch extraction can be used for simultaneous quantitation of intracellular C0 and a wide range of ACs (short- to long-chain AC).

Uptake of C0 and abnormalities in ACs were associated with the concentration of C0 in culture medium. In the C0-excess condition, it was hard to differentiate PCD from control

cells. Levels of C0 and C16 were overlapped with those of normal control. On the other hand, in the C0-reduced condition, intracellular C0 was significantly decreased in PCD while being increased in CPT1 deficiency, compared with that in normal control. C0-reduced medium was changed after fibroblasts equilibrated in MEM, and normal control could force to uptake free carnitine in C0-deficient condition while cells with PCD could not uptake sufficiently in that condition. Furthermore, the following fatty acid oxidation cycle interrupted, and C16 also decreased in PCD. This correlation of C0 and C16 in the C0-reduced condition is more informative for the diagnosis of carnitine cycle disorders (Fig. 4b). Since cells with PCD cannot uptake C0 via the cell membrane, the finding of reduction of both C0 and C16 is specific for PCD. In case of CPT1 deficiency, C0 uptake is normal, but it cannot bind acyl-CoA ester, resulting in reduced long-chain acylcarnitine production, and FAO is disturbed. Therefore, the stored intracellular ACs were consumed by FAO, and intracellular C16 as well as total ACs were decreased, and C0 was accumulated in intracellular lysate. In contrast, the AC profile of low level of C0 and high level of C16 is diagnostic for CPT2 deficiency. In this disease, normally transferred long-chain AC cannot be converted back from ACs to acyl-CoA esters and C0, the substrate for FAO. Additionally, the ratio of intracellular and extracellular C0s can sensitively distinguish PCD from control in the C0-reduced medium because carnitine transporter of normal cells was forced to uptake C0 up to physiological level in C0-reduced condition while cells with PCD failed for it. In excessive C0 condition, ratio of intracellular and extracellular C0 was similar to that in normal control and PCD since C0 transfer by passive diffusion across the cell membrane.

In conclusion, the simultaneous analysis of intracellular and extracellular C0 and ACs under the various concentrations of free carnitine in the culture medium is useful for diagnosis of FAO, especially carnitine cycle disorders. This study confirms that the newly modified IVP assay is an easy and safe method to diagnose PCD and CPT1 deficiency.

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Expanded newborn mass screening with MS/MS and medium-chain acyl-CoA dehydrogenase (MCAD) deficiency in Japan

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Abstract

Expanded newborn mass screening (NBS) with tandem mass spectrometry (MS/MS) and medium-chain acyl-CoA dehydrogenase (MCAD) deficiency in Japan are described. Prognosis of patients with inborn metabolic disease was compared between groups detected in the symptomatic and pre-symptomatic stages. Furthermore, clinical, biochemical and genetic findings of Japanese children with MCAD deficiency, which is a most important target of MS/MS screening, was investigated. Our study concluded as follows: 1) the detection incidence in MS/MS screening in Japan is totally about 1 in 9,000, which might be smaller than that of the other countries; 2) Outcomes of patients detected by NBS (pre-symptomatic stage) is more favorable than that of cases detected after symptomatic onset; 3) the incidence of MCAD deficiency in the Japanese population is 1 in 110 thousands, which is approximately 10 times smaller than that in Caucasian; 4) Japanese patients with MCAD deficiency have a common mutation, c.449-452delCTGA, which covers about 45% of alleles of MCAD gene, but not have 985A>G, which is a common mutation of Caucasians patients; 5) genotype/phenotype correlation was not observed in MCAD deficiency; 6) prognosis of the non-symptomatic group is much more favorable than that of the symptomatic group. In conclusion, it is indicated that detection of inborn metabolic disease in the pre-symptomatic stage by NBS is essential to prevent children affected with target diseases of NBS including MCAD deficiency from neurological impairments or infant death.

Key words

expanded newborn mass screening, MS/MS, MCAD deficiency, genotype/phenotype correlation, prevention of neurological impairment

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1. Introduction

The expanded newborn screening (NBS) using tandem mass spectrometry (MS/MS) is becoming popular worldwide. In Japan, a pilot screening was initiated at Fukui University in 1997 (1), and the national

project of MS/MS screening (Principle Investigator, Dr. Seiji Yamaguchi, Shimane University) funded by Grant-in-Aid for Scientific Research from the Ministry of Health, Labour, and Welfare was started in 2004. For the pilot screening, about 10 laboratories in Japan joined up to 2012. The Japanese government issued notice urging implementation of MS/MS to NBS in 2011. Eventually, the MS/MS screening is becoming spread from 2012 to 2013 (2), and will initiate officially nationwide in next year (2014).

In the expanded NBS, it is considered that medium-chain acyl-CoA dehydrogenase (MCAD) deficiency is a most important screening target, particularly in Caucasian people, because the incidence is high (1 in 10,000), and MCAD deficiency is a causative disease of sudden infant death, and preventable by detection in NBS (3). In this paper, clinical and genetic aspects of MCAD deficiency as well as the results of the pilot MS/MS screening in Japan are described.

2. Pilot MS/MS Screening In Japan

a) Results of pilot MS/MS screening in Japan

A total number of babies screened by MS/MS during the period between 1997 and 2012 was 1,949,987 (about 1.95 million), and 217 cases affected with disease were found as shown in Table 1. The detection incidence of Japanese babies was calculated to be 1 in 8,986, while that in USA was estimated to be 1 in 4,353 (4). The prevalence of Japanese is likely smaller than that of the other countries.

b) The detection prevalence of each disease group

The incidence of amino acidemias was totally 1 in 27 thousands; that of organic acid disorders, 1 in 22 thousands; and that of fatty acid disorders, 1 in 34 thousands. The most common disease in Japan was propionic acidemia, which was found in 1 in 45,349 (about 45 thousands); and phenylketonuria, 1 in 52,702 (53 thousands); followed by methylmalonic acidemia and MCAD deficiency, each of which in 1 in 108,333 (110 thousands). A considerable number of Japanese patients with propionic acidemia show a mild phenotype with a common mutation, 1304T>C (Y435C) in *PCCB* gene (5).

c) Comparison of outcomes between cases detected by NBS and by tests after symptomatic onset

Outcomes of cases with organic and fatty acid disorders detected in NBS were compared with those detected after symptomatic onset by MS/MS, GC/MS and/or molecular investigation in Shimane University. As shown in Table 2, in organic acid disorder, normal development was achieved in 58 of 70 cases (83%) of the NBS group, as of at least 1 year of age. In contrast, normal development was gotten in only 28 of 114 cases (19%) in the "symptomatic" group.

In fatty acid disorder, normal development was achieved in 40 of 45 cases (89%) in the NBS group, although that was in 25 of 52 cases (48%) in the symptomatic group. Hence, beneficial effect of NBS using MS/MS was indicated. Through the results of the pilot study, we proposed 16 kinds of disease which should be screened as primary targets as marked with black circle in Table 1, in consideration of the false negative rate, complexity of diagnostic approaches, or the benefit of detection by NBS.

Table 1. Results of the pilot screening using MS/MS in Japan (1997 to 2012)

Disease		No. of Cases	Incidence (Japan)	Estimated in USA*
AMINO ACIDEMIA		72	(1:27 K)	(1:15 K)
• Phenylketonuria		37	1: 53 K	1: 19 K
• MSUD		1	1: 1,950 K	1: 159 K
• Homocystinuria		3	1: 650 K	1: 38 K
• Citrulinemia type I		6	1: 330 K	1: 173 K
• Argininosuccinic A		2	1: 980 K	1: 591 K
Citrin deficiency		23	1: 85 K	n.a.
ORGANIC ACIDEMIA		87	(1: 22 K)	(1:16 K)
• Methylmalonic acidemia		18	1: 110 K	1: 67 K
• Propionic acidemia		43	1: 45 K	1: 276 K
• Isovaleric acidemia		3	1: 650 K	1: 129 K
• MCD		3	1: 650 K	1: 1,380K
• Methylcrotonylglycinuria		13	1: 150 K	1: 44 K
• HMGL deficiency		—	—	1: 1,380K
• Glutaric acidemia type1		7	1: 280 K	1: 109 K
β-ketothiolase deficiency		—	—	1: 591 K
FATTY ACID DISORDER		58	(1: 34 K)	(1:10 K)
• CPT1 deficiency		5	1: 390 K	n.a.
• VLCAD deficiency		12	1: 160 K	1: 69 K
• MCAD deficiency		18	1: 110 K	1: 17 K
• TFP/LCHAD deficiency		2	1: 980 K	1: 276 K
CPT2 deficiency		7	1: 280 K	n.a.
CACT deficiency		—	—	n.a.
Glutaric acidemia type 2		6	1: 330 K	n.a.
Carnitine uptake defect		7	1: 280 K	1: 49 K
SCHAD deficiency		1	1: 1,950 K	n.a.
TOTAL	Affected cases	217	1: 8,986	1: 4,353
	Screened babies	1,949,987		

•, primary target disease in the MS/MS screening in Japan. "K" means thousands. * Estimated incidence in USA, based on live birth for 2006 (n=4,138,349) (4). Abbreviation: −, not detected; n.a., not applicable; MCD, multiple carboxylase deficiency; HMGL, 3-hydroxy-3- methyl-glutaryl-CoA lyase; CPT1 and CTP2, carnitine palmitoyl transferase-I and -II, respectively; VLCAD and MCAD, very-long-, and medium-chain acyl-CoA dehydrogenase, respectively; TFP, mitochondrial trifunctional protein; CACT, carnitine acylcarnitine translocase; LCHAD and SCHAD, long- and short-chain 3-hydroxyacyl-CoA dehydrogenase, respectively.

3. Clinical, Biochemical and Genetic Investigation of Japanese MCAD Deficiency

a) Outline of MCAD deficiency

MCAD deficiency was first discovered in children with sudden infant death syndrome-like illness in 1982, and has been found at an incidence of 1 in 10,000. A common mutation, 985A>G, covering about 90% of the alleles in this disease was seen among Caucasian population. Acute symptoms of MCAD deficiency include acute

Table 2. Comparison of outcomes between presymptomatic and symptomatic detection cases in Japanese children with organic academia and fatty acid disorder.

Disease	NBS (MS/MS screening)	Symptomatic (Shimane)
No. of cases	115	196
ORGANIC ACID DISORDER	70	144
Normal development	58 (83%)	28 (19%)
Handicaps or death	12 (17%)	116 (81%)
FATTY ACID DISORDER	45	52
Normal devlopment	40 (89%)	25 (48%)
Handicaps or death	5 (11%)	27 (52%)

NBS, newborn mass screening, MS/MS screening, cases detected by the pilot study between 2004 and 2011. Symptomatic, cases after symptomatic onset and detected by MS/MS, GC/MS or molecular tests in Shimane University from 2001 to 2011.

encephalopathy-like symptoms, or even sudden death (6), following after long fasting or infection, although the patients have no symptoms in the stable condition. Concerning the prognosis, it has been claimed that as many as 35% of MCAD deficiency patients are asymptomatic lifelong, but that over 25% of the symptomatic cases die suddenly during the first episodic attack (3).

Abnormal laboratory tests in the acute condition include hypoglycemia or hyperammonemia. Biochemical markers in MS/MS analysis of blood acylcarnitines are elevation of C8, C6 or C10, or C8/C10. Elevation of hexanoylglycine (HG) and suberylglycine (SG) as well as dicarboxylic acids is often observed by GC/MS analysis of urinary organic acids.

b) Prevalence of MCAD deficiency in Japan

According to the results of pilot screening using MS/MS, MCAD deficiency was found at the incidence of 1 in 110 thousands, and was most common among fatty acid disorders in the pilot study of Japan, as shown in Table 1. However, the incidence is about 10 times smaller than that of Caucasian whose incidence is 1 in 10 thousands (3).

c) Subjects of MCAD deficiency

Ages at onset, and diagnosis, clinical, biochemical and genetic findings of a total 20 Japanese cases whose blood C8 acylcarnitine, a diagnostic marker of MCAD deficiency, was over the cut off value (0.3 nmol/mL) in MS/MS analysis were investigated (Table 3). Cases 1 through 9 were identified after symptomatic onset (symptomatic group), while cases 10 through 18 were detected in the non-symptomatic or pre-symptomatic stage (non-symptomatic group). Cases 10 through 17 were detected by NBS while Case 18 was by the sibling screening using MS/MS. Acylcarnitine analysis was performed at Shimane University or the other laboratories. GC/MS analysis (7) and gene analysis (8) were performed at Shimane University. The final diagnosis was confirmed by gene analysis, which revealed that cases 19 and 20 were heterozygotes (carrier group). Clinical information was surveyed using questionnaire.

Table 3. Clinical, biochemical and genetic profiles of Japanese cases of MCAD deficiency

No.	Age at onset	Age at diag.	NBS	MS/MS	GC/MS		Genotype		Outcome
				C8 (<0.3)	HG	SG	Allele 1	Allele 2	
Symtpomatic group									
1	8m	8m	−	5.97	11.1	44.5	c.449-452del	c.157C>T	impair
2	1y0m	1y0m	−	4.52	n.a	n.a	IVS4+1G>A	c.422 A>T	SID
3[a]	1y0m	8y10m	−	1.57	45.4	29.6	c.449-452del	c.449-452del	impair
4	1y1m	1y1m	−	7.00	14.7	112.2	del. ex 11-12	del. ex 11-12	impair
5	1y3m	1y3m	−	high*	n.a	n.a	del. ex 11-12	del. ex 11-12	impair
6[b]	1y4m	1y4m	−	3.33	9.9	15.3	c.449-452del	c.449-452del	impair
7	1y7m	1y7m	−	4.12	6.1	6.4	c.275C>T	c.157C>T	impair
8[a]	1y8m	1y8m	−	4.75	69.3	1.2	c.449-452del	c.449-452del	SID
9	2y2m	2y2m	−	1.71	n.a	n.a	c.449-452del	c.449-452del	normal
Non-symptomatic group									
10	−	5d	+	5.92	12.9	14.8	c.1085G>A	c.843A>T	normal
11	−	5d	+	5.37	6.3	39.9	c.449-452del	c.154A>G	normal
12	−	5d	+	4.82	15.3	3.8	IVS3+2T>C	c.843 A>T	normal
13	−	5d	+	4.04	n.a	n.a	c.449-452del	c.212 G>A	normal
14	−	5d	+	2.78	11.5	5.9	c.449-452del	c.134 A>G	normal
15	−	5d	+	2.59	3.1	(-)	c.1085G>A	c.1184A>G	normal
16	−	5d	+	2.58	(-)	3.2	c.449-452del	IVS3+5G>A	normal
17	−	5d	+	0.49	9.7	1.5	c.449-452del	c.820 A>C	normal
18[b]	−	5y5m	−	1.37	n.a	n.a	c.449-452del	c.449-452del	normal
Carrier group									
19	−	5d	+	0.44	(-)	(-)	c.845C>T	(-)	normal
20	−	4m	−	0.51	(-)	(-)	c.843A>T	(-)	normal

Cases 3 and 8 (a-a), and cases 6 and 18 (b-b) are sibling cases, respectively. Abbreviation: diag, diagnosis; NBS, newborn mass screening; −, none; +, NBS received; MS/MS, blood acylcarnitine analysis; GC/MS, urinary organic acid analysis; HG and SG, hexanoylglycine and suberylglycine, respectively; high*, elevated but detailed value not available. n.a, data not available in Shimane University; (-), not detected; SID, sudden infant death; c.449-452del, 4 base deletion of CTGA. impair, neurological impairments as sequallae. Unit: C8, nmol/mL (cut off, <0.3); HG and SG, peak area ratio to IS (%) (7) on GC/MS (normal, both undetectable).

d) Comparison between Symptomatic and non-symptomatic groups of MCAD deficiency

1) Ages at onset and diagnosis: In the symptomatic group, the ages at onset was 8 mo to 2 yr 2 mo. Cases 3 and 8, and cases 6 and 18, were siblings. In 9 cases of the non-symptomatic group, 8 cases were detected on 5 day after birth by NBS, and the other one (case 18) was identified at the age of 5 yr 5 mo by sibling screening.

2) Clinical findings of symptomatic case: In the symptomatic group, all 9 cases had acute encephalopathy- or sudden death-like illness in the acute stage. Hypoglycemia was observed in all 7 cases tested, while hyperammonemia was seen in 4 of the 9 cases.

3) Biochemical findings: As shown in Table 2, C8 (cut off, <0.3 nmol/mL) ranged between 1.57 and 7.00 in the symptomatic group, while C8 did between 0.49 and 5.92

in the non-symptomatic group. No significant difference between these two groups was seen in the level of C8. The C8 value of the carrier group (cases 19 and 20) was 0.44 and 0.51, respectively, which was lower compared to those of the 18 affected cases. No significant difference was seen in the urinary excretion amounts of HG or SG between these two groups (Table 3).

4) Gene mutation: c.449-452delCTGA (c.449del4) was identified in 16 of 36 alleles (44%) in 18 Japanese patients with MCAD deficiency. The homozygote of c.449del4 mutants were observed in 4 and 1 cases in the symptomatic and non-symptomatic groups, respectively. A common mutation, 985A>G, found in Caucasian population was never identified in Japanese cases (8). On the other hand, it was reported that the c.449del4 mutation was in 3 of 5 alleles of 3 Korean MCAD deficiency cases (9). It would be interesting to investigate and compare the genotypes of Japanese MCAD deficiency with those of the other Asian countries and the other ethnic groups.

5) Outcomes: With respect to the outcomes, 8 of 9 cases of the symptomatic group resulted in severe handicaps or sudden death, whereas all 9 cases of the non-symptomatic group showed normal development and growth (Table 3). It was likely that there were no genotype/phenotype correlation, although existence of the correlation is not clear enough in the present point (10). These findings indicate that pre-symptomatic detection is important for the favorable outcome in MCAD deficiency. Namely, NBS is essential (11, 12). Furthermore, our data suggests no clear genotype/phenotype in MCAD deficiency.

4. Conclusion

Our study indicated that: 1) the detection incidence in MS/MS screening is totally about 1 in 9,000 in Japan, which might be 2 times smaller than that of other countries; 2) the outcomes of patients detected by NBS is more favorable compared with that of cases detected after symptomatic onset; 3) the incidence of MCAD deficiency is 1 in 110 thousands in Japanese population. This is approximately 10 times smaller than that in Caucasian population; 4) 45% of alleles of *MCAD* gene in Japanese patients have a common mutation, c.449-452delCTGA. The genetic background of Japanese cases is likely the same with Korean patients, but different from those in Caucasians with MCAD; 5) clinical severity of MCAD deficiency may be similar despite the different genetic mutations, suggesting that genotype does not necessarily predict phenotype in MCAD deficiency; 6) prognosis of the symptomatic cases with MCAD deficiency was poor, whereas that of the non-symptomatic group was excellent, indicating "pre-symptomatic detection" is essential to prevent children affected with MCAD deficiency from impairments or sudden death.

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G protein-linked signaling pathways in bipolar and major depressive disorders

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The G-protein linked signaling system (GPLS) comprises a large number of G-proteins, G protein-coupled receptors (GPCRs), GPCR ligands, and downstream effector molecules. G-proteins interact with both GPCRs and downstream effectors such as cyclic adenosine monophosphate (cAMP), phosphatidylinositols, and ion channels. The GPLS is implicated in the pathophysiology and pharmacology of both major depressive disorder (MDD) and bipolar disorder (BPD). This study evaluated whether GPLS is altered at the transcript level. The gene expression in the dorsolateral prefrontal (DLPFC) and anterior cingulate (ACC) were compared from MDD, BPD, and control subjects using Affymetrix Gene Chips and real time quantitative PCR. High quality brain tissue was used in the study to control for confounding effects of agonal events, tissue pH, RNA integrity, gender, and age. GPLS signaling transcripts were altered especially in the ACC of BPD and MDD subjects. Transcript levels of molecules which repress cAMP activity were increased in BPD and decreased in MDD. Two orphan GPCRs, GPRC5B and GPR37, showed significantly decreased expression levels in MDD, and significantly increased expression levels in BPD. Our results suggest opposite changes in BPD and MDD in the GPLS, "activated" cAMP signaling activity in BPD and "blunted" cAMP signaling activity in MDD. GPRC5B and GPR37 both appear to have behavioral effects, and are also candidate genes for neurodegenerative disorders. In the context of the opposite changes observed in BPD and MDD, these GPCRs warrant further study of their brain effects.

Keywords: G-protein coupled receptor (GPCR), transcriptome, bipolar disorder, major depressive disorder, GPR37, GPRC5B, cyclic AMP, phosphatidylinositol

INTRODUCTION

The G-protein linked signaling system (GPLS) comprises G-proteins, G protein-coupled receptors (GPCRs), GPCR ligands, and downstream effector molecules. G-proteins interact with both GPCRs and downstream effectors such as cyclic adenosine monophosphate (cAMP), phosphatidylinositols, and ion channels. The GPLS is implicated in the pathophysiology and pharmacology of both major depressive disorder (MDD) and bipolar disorder (BPD). GPCRs are integral membrane proteins with key roles in numerous physiological cellular processes. GPCRs can signal ligand binding events by conformational changes that activate intracellular G proteins. Active G proteins in turn trigger intracellular downstream signaling pathways. Evidence suggests that altered GPCRs and their two major downstream signaling pathways, mediated by effectors such as cAMP and phosphatidylinositol, may be involved in the pathophysiology of BPD and MDD as well as in the mechanism of drug treatment for these disorders. Altered immunoreactivities and functional activities of proteins related to cAMP signaling pathways have been reported in the brain and peripheral blood cells of BPD patients (Jope et al., 1996; Karege et al., 1996; Gould and Manji, 2002; Chang et al., 2003). Also, mood stabilizers and antidepressants have been shown to affect cAMP and phosphatidylinositol signaling (Donati and Rasenick, 2003; Harwood, 2005; Xu et al., 2007; Racagni and Popoli, 2008). There are at least 210 GPCRs for which a natural ligand has been identified. There are 150 orphan GPCRs, with no known ligand or function (Wise et al., 2004), that might be relevant to downstream signaling dysregulation observed in

BPD and MDD. Among the signal transmission systems associated with GPCRs, monoaminergic, and neuropeptidergic systems are believed to be dysregulated in BPD and MDD (Lieb et al., 2002; Elhwuegi, 2004; Carvajal et al., 2006; Ruhe et al., 2007).

Messenger RNA expression levels of a number of GPCRs, their ligands, and effector molecules involved in the GPLS pathways have been measured in post-mortem brains of BPD and MDD patients (Young et al., 1996; Spleiss et al., 1998; Caberlotto and Hurd, 2001; Kuromitsu et al., 2001; Zhou et al., 2001; Gurevich et al., 2002; Pandey et al., 2002; Agam et al., 2003; Chang et al., 2003; Dwivedi et al., 2004; Escriba et al., 2004; Lopez-Figueroa et al., 2004; Sherrin et al., 2004). Brain imaging studies have implicated anterior cingulate cortex (ACC) and the dorsolateral prefrontal cortex (DLPFC) in the pathophysiology of mood disorders (Harrison, 2002; Rogers et al., 2004; Frazier et al., 2005). In this study, the DLPFC and ACC were selected for microarray gene expression profiling of BPD and MDD patients focusing on the involvement of genes involved in GPLS pathways in these disorders and the effect of drug treatments. These cortical regions were selected because of the integral role of the ACC and DLPFC in the control of impulses, memory, learning, and hedonic evaluation, and depression. This study compares an a priori set of genes, in both mood disorders, using two cortical regions. There have been a number of previous studies comparing MDD and BPD by microarray analysis to controls, e.g., studies involving samples from the Stanley Foundation Neuropathology Brain collection (Kim and Webster, 2010). However, few studies have analyzed gene expression by microarray analysis of the Anterior Cingulate in both mood disorder groups, aside from studies involving the Pritzker Neuropsychiatric Disorders papers which involved selected pathways of glutamate, mitochondria, and growth factors (Evans et al., 2004; Choudary et al., 2005; Vawter et al., 2006). We hypothesized that there would be dysregulation of the GPLS in both mood disorders compared to controls, and that some overlap between the signature of each mood disorder would be found, given that most of our subjects were in a depressive state near the time of death.

MATERIALS AND METHODS

SUBJECTS

The primary cohort consisted of 22 subjects, including seven healthy controls, six patients with bipolar disorder type I (BPD), and nine patients with MDD (Cohort A). The MDD results were validated with an additional independent cohort, including seven controls and five MDD patients (Cohort B). Toxicology data and/or recent clinical data were examined for the BPD and MDD subjects and detailed clinical information is summarized in **Table 1**. To minimize potential confounding effects of agonal events, we used three criteria in subject selection: brain tissue pH of >6.6, ribosomal RNA 28S/18S ratio of >1.5, and sudden death with the agonal period lasting less than 1 h. We have previously found large differences in gene expression values when using longer agonal periods, >1 h and as long as 1–2 days, and would have found large effects due only to mixing samples with long and short agonal duration (Li et al., 2004; Tomita et al., 2004; Vawter et al., 2006; Atz et al., 2007).

Brain tissue and clinical information were obtained by the UCI Brain Bank staff with the informed consent of decedents' next-of-kin, and were processed through a standard protocol approved by the local UCI Institutional Review Board (Tomita et al., 2004). None of the subjects included in this study had specific agonal conditions including cancer, hypoxia, coma, pyrexia, seizure, dehydration, hypoglycemia, multi-organ failure, skull fracture, ingestion of neurotoxic substances or prolonged agonal duration, which are known to affect tissue pH, RNA integrity and gene expression profiles in post-mortem brain (Li et al., 2004; Tomita et al., 2004). The variables for gender, age, post-mortem interval (PMI), tissue pH, and 28S/18S ratio were balanced between diagnostic and control groups. Patients who were prescribed lithium and selective serotonin reuptake inhibitor (SSRI) at the time of death are also indicated (**Table 1**).

MICROARRAY GENE EXPRESSION

The experimental procedures are described elsewhere (Evans et al., 2004; Tomita et al., 2004). Total RNA was extracted from ACC and DLPFC of Cohort A (**Table 1**), and purified with silica-based mini-spin columns (Qiagen, Valencia, California). The oligonucleotide microarray experiments were conducted utilizing Affymetrix U133A Gene Chips following the manufacturer's protocol (Affymetrix, Santa Clara, CA). To detect gene expression differences between diagnostic groups reliably, we replicated the experiments as follows: Cohort A used 22 subjects' total RNA from ACC which were processed at two laboratories (University of California, Irvine and University of California, Davis) and DLPFC that were processed independently (University of California, Davis and University of Michigan, Ann Arbor). For Cohort B 12 total RNA samples from ACC and DLPFC (additional 12 subjects) were processed at the same independent laboratories as cohort A. In summary, for each BPD subject there were duplicate arrays run, and for MDD subjects there were duplicate arrays run, plus an additional cohort of MDD subjects were run also with duplicate arrays.

DATA ANALYSES

Since a considerable number of probe sequences in the Affymetrix original chip definition file (CDF) were found to incorrectly BLAST to other transcripts, a refined CDF based on an UniGene cluster was developed (Evans et al., 2004; Dai et al., 2005), and was used for data analyses presented in this paper. The refined CDF file is available on http://brainarray.mhri.med.umich.edu/brainarray/.

The focus of this study was 445 GPLS genes found on the Affymetrix arrays used in this study (Table S1). Signal intensities were extracted with Robust Multi-array Average (RMA) for each probe set and each subject. Gene-wise Pearson's correlation coefficients between experimental duplicates were calculated. Only the genes that were significantly correlated ($p < 0.05$) between experimental duplicates were considered to be reliably measured genes, and were subjected to downstream analyses. These genes were analyzed in a mixed-model ANOVA utilizing Partek Pro 6.0 (Partek, MO) for the main effect of the diagnostic classification (BPD, MDD, control) and for sex (Evans et al., 2004). We calculated the false discovery rate (FDR) in the microarray

Table 1 | Demographics of two cohorts with mood disorder and matched controls, Cohorts A and B.

Gender (L)[a]	Age	Diagnosis	PMI	pH	28S/18S	SSRI[b]	Manner of death	Medications, time of death
COHORT A (BPD, MDD, CONTROL)								
Male	9	BPD	11.25	6.91	1.55	NP	Exsanguination rupture of aorta	
Male (L)	3	BPD	9	7.12	2.1	NP	Hanging	Mood Stabilizer, Antipsychotic, Anticholinergic
Male (L)	26	BPD	19	6.92	1.81	NP	Carbon monoxide poisoning	Antipsychotic, Mood Stabilizer, Mood Stabilizer, Antianxiety Agent, Anticholinergic
Female	56	BPD	29	6.83	2.17	SSRI+	Acute strychnine intoxication	Antidepressant-Atyp[#], Antidepressant-HCA[#], Antianxiety Agent, Hypnotic, Antidepressant-SSRI
Male	52	BPD	28	7.05	2.36	NP	Acute hemorrhage slash wound to neck	
Male (L)	59	BPD	15.5	6.99	1.58	NP	Myocardial infarction	Mood Stabilizer
Female	72	MDD	21	7.13	2.28	NP	Pulmonary edema due to acute intoxication	Hypnotic, Antianxiety Agent, Antianxiety Agent, Antidepressant-HCA, Antidepressant-HCA
Male	19	MDD	18	7.11	2.17	NP	Asphyxiation due to hanging	
Male	58	MDD	24	6.93	2.26	SSRI+	Hanging	Antidepressant-SSRI, Antidepressant-Atyp
Male	49	MDD	31	7	1.96	SSRI+	Accidental overdose of propoxyphene, norpropoxyphene, amitriptyline, nortriptyline, sertaline, norsertaline and zolpidem	Antidepressant-SSRI, Antianxiety Agent, Hypnotic, Antidepressant-HCA
Male	46	MDD	27	6.91	2.07	NP	Occlusion of left anterior descending coronary artery	Hypnotic, Antianxiety Agent, Antianxiety Agent
Male	49	MDD	27	7.19	2.24	NP	Hanging	
Male	52	MDD	16	6.82	1.82	SSRI+	Acute myocardial infarction	Antidepressant-SSRI, Antidepressant-Atyp, Antipsychotic, Hypnotic
Female	48	MDD	37	6.95	1.86	SSRI+	Overdose morphine	Anticonvulsant, Antidepressant-SSRI, Antianxiety Agent
Male	39	MDD	27.5	6.79	1.77	SSRI+	Hanging	Anticonvulsant, Antidepressant-SSRI
Female	60	Control	24	6.99	1.77	NA	Myocardial infarction	
Male	70	Control	27	7.03	1.89	NA	Myocardial infarction	
Male	18	Control	22	6.97	2.53	NA	Freshwater drowning	
Male	58	Control	26	7.02	1.99	NA	Ventricular fibrillation	
Male	55	Control	18	6.89	2.2	NA	Myocardial infarction	
Male	45	Control	21	6.86	1.92	NA	Congestive heart failure	
Male	44	Control	23	6.87	1.9	NA	Myocardial infarction	
COHORT B (MDD, CONTROL)								
Male	35	MDD	24.75	7.04	2.14	NP	Hanging	Antidepressant-Atyp[#]
Male	47	MDD	29	7.25	2.08	NP	Hanging	
Female	80	MDD	15	6.68	2.21	NP	Hypertrophic cardiomyopathy	
Male	63	MDD	28.5	7.17	1.57	NP	Ruptured aortic abdominal aneurysm	
Male	66	MDD	32	7.05	2.07	SSRI+	Cardiac event	Antidepressant-SSRI
Male	77	Control	7.25	6.62	2.16	NA	Cardiac event	
Male	39	Control	18	6.81	1.67	NA	Cardiac arrhythmia	
Male	39	Control	30	7.02	1.88	NA	Electrocution	

(Continued)

Table 1 | Continued

Gender (L)[a]	Age	Diagnosis	PMI	pH	28S/18S	SSRI[b]	Manner of death	Medications, time of death
Male	41	Control	22.5	7.01	2.08	NA	Severe coronary arteriosclerosis	
Male	65	Control	13.5	6.88	2.05	NA	Hemorrhagic pericarditis	
Male	49	Control	27.5	6.68	1.68	NA	Coronary Insufficiency	
Female	45	Control	32	6.6	1.71	NA	Bilateral extensive pulmonary embolism	

[a] *L, prescribed lithium at time of death.*

[#] *Antidepressant-Atypical, Antidepressant-heterocyclic.*

Mean + standard deviation of age: BPD, 47.5 ± 18.7; MDD, 48.0 ± 14.2; control, 50 ± 16.7.

Mean + standard deviation of PMI: BPD, 18.6 ± 8.4; MDD, 25.4 ± 6.5; control, 23.0 ± 3.1.

Mean + standard deviation of pH: BPD, 7.0 ± 0.1; MDD, 7.0 ± 0.1; control, 6.9 ± 0.1.

Mean + standard deviation of 28S/18S: BPD, 1.9 ± 0.3; MDD, 2.0 ± 0.2; control, 2.0 ± 0.3.

Mean ± standard deviation of age: MDD, 58.2 ± 17.5; control, 50.7 ± 14.7.

Mean ± standard deviation of PMI: MDD, 25.9 ± 6.6; control, 21.5 ± 9.1.

Mean ± standard deviation of pH: MDD, 7.0 ± 0.2; control, 6.8 ± 0.2.

Mean ± standard deviation of 28S/18S: MDD, 2.0 ± 0.3; control, 1.9 ± 0.2.

[b] *NP, not prescribed SSRI; NA, not applicable for SSRI.*

study by Benjamini-Hochberg step-down procedure using Partek Genomics setting the FDR to 0.05. Since the three diagnostic groups were well matched on age and pH (**Table 1**), the gene expression values were not tested by ANCOVA. However, in *post-hoc* analysis, we determined whether the tissue pH was correlated with any differentially expressed GPLS genes. The *post-hoc* analysis showed that there were no significant correlations found with tissue pH and differentially expressed GPLS genes. A *post-hoc* group comparison of subjects with SSRI treatment and without treatment was conducted in MDD subjects, and similar analysis for lithium was conducted for BPD subjects.

The hypergeometric distribution was used for calculating probabilities that the observed number of genes in each gene category for cAMP or PI signaling pathways (listed in Table S2) were detected as differentially expressed genes in the comparisons between patients and control. False discovery rate were evaluated by QVALUE (Storey and Tibshirani, 2003). Calculations for hypergeometric probabilities were performed using SISA online statistics package (http://www.quantitativeskills.com/sisa/distributions/hypergeo.htm).

QUANTITATIVE PCR

For further technical evaluation of the microarray data, we evaluated mRNA expression levels by quantitative real-time reverse transcriptase PCR (qPCR) for the seven genes listed in **Table 3**. We followed up on results primarily that met FDR in cohort A for BPD, and both Cohorts A and B for MDD, except for RGS20, which we followed up based upon Cohort A only. These genes selected for qPCR analyses met the criteria of FDR at the level of accepting 5% false positives and percentage change greater than 20% in the ACC microarray results. The threshold cycle (C_t) values for the target transcripts were normalized to averaged C_t values for two reference transcripts, Jagged 1 (JAG1) and solute carrier family 9 isoform 1 (SLC9A1), which showed equivalent expression levels among the three diagnostic groups (BPD, MDD, and control) throughout the 3 brain regions on our normalized microarray data. After DNase digestion and purification, total RNA (1 μg) samples from each of the 34 subjects in Cohorts A and B were used as a template for first-strand cDNA synthesis using poly-dT primer. The mRNA for each transcript was measured using the SybrGreen system with Prism model 7000-sequence detection instrument (Applied Biosystems, Foster City, California) and primer sequences listed in Table S1. The C_t, which correlatedinversely with the target mRNA concentration, was measured as the cycle number at which the SybrGreen fluorescent signal increased abovea pre-set threshold level. The qPCR experiments were performed three times for each transcript, and the C_t triplicate values were averaged.

In situ HYBRIDIZATION

The gene expression fold changes for GPRC5B and GPR37 showed an increase in BPD and decrease in MDD. We selected those genes for *in situ* hybridization (ISH) using the ACC regions from six representative subjects to evaluate the cellular localization of GPRC5B and GPR37 mRNA expression. A formal statistical analysis of the ISH data was not applied due to low subject number. ISH was performed following a protocol as described elsewhere (Neal et al., 2001; Lopez-Figueroa et al., 2004). Briefly, frozen ACC tissue blocks were cut into 12-μm sections on a cryostat. After fixation with 4% paraformaldehyde for 1 h, sections were hybridized overnight with a ^{35}S-labeled antisense cRNA probes for GPRC5B and GPR37. Following hybridization, wash, RNase A treatment, dehydration and air-dry, sections were exposed to Kodak XAR-5 X-ray film for 21 days. Technical control studies were performed by (1) using ^{35}S-labeled sense RNA probes for GPRC5B and GPR37, and (2) pre-treating tissue with RNase A before hybridizing with the antisense probes.

RESULTS

Significantly dysregulated GPLS genes that passed FDR ($p < 0.05$) in either Cohort A or B and either mood disorder, BPD

or MDD, are listed in **Table 2**. For completeness, the uncorrected p-values are also shown, as some genes only met FDR for a single comparison. In general, equal numbers of GPLS genes passed FDR in BPD and MDD in the ACC (10 genes for BPD and 10 genes for MDD that passed FDR), compared with DLPFC (1 gene for BPD and 5 genes for MDD). Thus, about three fold more gene expression differences in mood disorder were found in the ACC showed compared to the DLPFC. The genes in **Table 2** also showed non-significant correlations between gene expression and tissue pH (all p-values > 0.1) suggesting that residual pH-agonal effects on the expression levels of GPCR genes did not remain in the data set, even though complete elimination of these factors may not be possible with matching of subject's age and pH, and inclusion of subjects that had rapid deaths.

CYCLIC AMP SIGNALING PATHWAY

The genes involved in cAMP signaling pathways, which were differentially expressed in ACC of BPD patients compared to controls, are listed in **Table 2**. The cAMP signaling pathway is shown in **Figure 1A**. The data show that six significant transcripts out of 39 that repress cAMP signaling activity (**Table 1**) were increased in ACC from BPD patients at the transcriptional level. The group of molecules that act negatively on cAMP signaling activity was significantly over-represented in BPD based on a hypergeometric distribution ($p = 0.037$, $q = 0.36$). Thus, there was a nominal enrichment of genes that act negatively on cAMP signaling activity (G_i proteins, phosphodiesterase, protein kinase A inhibitors, and protein phosphatase) in BPD in the ACC.

Table 2 | GPLS genes significantly dysregulated in anterior cingulate cortex by microarray.

Category	Anterior Cingulate Cortex								
	Symbol	BPD Cohort A			MDD Cohort A			MDD Cohort B	
		% change	Unadjusted *p*-value	FDR	% change	Unadjusted *p*-value	FDR	% change	Unadjusted *p*-value	FDR
A. Cyclic AMP signaling pathway	NPY1R	24.4	0.000889	$p < 0.05$	21.9	0.000855	$p < 0.05$	−17.7	0.02108	
	NPY	33.0	1.48E−06	$p < 0.05$	3.6	0.37116		3.6	0.54313	
	SST	22.1	5.4E−05	$p < 0.05$	−13.3	0.002288	$p < 0.05$	−6.5	0.10886	
	GRM3	34.4	0.000113	$p < 0.05$	−10.9	0.081528		−17.9	0.01528	
	EDG1	−4.1	0.194276		−21.2	5.44E−07	$p < 0.05$	−10.4	0.00180	
	EDG2	18.2	0.000195	$p < 0.05$	4.8	0.1806		−4.5	0.22364	
	GNAI1	40.4	0.000289	$p < 0.05$	−2.4	0.740184		−9.0	0.26898	
	RGS20	15.6	0.005655	$p < 0.05$	−29.2	5.85E−06	$p < 0.05$	−13.9	0.00992	
	PDE1A	24.1	0.000599	$p < 0.05$	14.4	0.01141	$p < 0.05$	−8.0	0.19030	
	PDE8A	12.5	0.004505	$p < 0.05$	−23.1	3.96E−06	$p < 0.05$	−17.8	0.00138	
	PKIA	16.6	0.001318	$p < 0.05$	9.4	0.025332		−1.2	0.82836	
	PPP1CA	16.8	3.61E−06	$p < 0.05$	1.1	0.627546		1.1	0.79955	
	PPP1R3C	−15.4	0.000544	$p < 0.05$	−63.2	6.98E−13	$p < 0.05$	−21.4	0.00006	$p < 0.05$
B. Phosphatidylinositol signaling pathway	NTSR2	−1.4	0.741755		−18.6	0.000195	$p < 0.05$	−24.7	0.00012	$p < 0.05$
	EDNRB	−1.0	0.765956		−31.2	1.9E−08	$p < 0.05$	−15.9	0.00070	$p < 0.05$
	EDG2	18.2	0.000195	$p < 0.05$	4.8	0.1806		−4.5	0.22364	
	INPP5F	16.2	0.002445	$p < 0.05$	21.8	6.25E−05	$p < 0.05$	8.0	0.30847	
	ITPKB	11.5	0.035537		−12.3	0.015197	$p < 0.05$	−34.0	0.00010	$p < 0.05$
	INPP1	24.9	9.4E−06	$p < 0.05$	−1.1	0.761718		−7.4	0.04372	
	PIK3C2A	22.3	0.020892	$p < 0.05$	−35.9	0.000393	$p < 0.05$	−13.1	0.15764	
	PIK3C2B	14.8	0.001516	$p < 0.05$	−11.1	0.005757	$p < 0.05$	−1.1	0.81543	
	ITPR1	5.5	0.123943		17.4	2.66E−05	$p < 0.05$	10.5	0.15830	
	PRKCB1	−0.5	0.886704		11.5	0.00341	$p < 0.05$	5.7	0.15413	
C. Orphan GPCRs	GPR37	38.3	9.46E−08	$p < 0.05$	−40.5	8.39E−09	$p < 0.05$	−27.0	0.00003	$p < 0.05$
	GPRC5B	24.6	6.13E−06	$P < 0.05$	−38.3	2.94E−09	$p < 0.05$	−30.3	0.00004	$p < 0.05$
	GPR56	7.2	0.161404		−15.9	0.00261	$p < 0.05$	−14.9	0.00298	
	GPR125	−8.1	0.088684		−11.5	0.011594	$p < 0.05$	−0.3	0.94843	

The GPLS expression percent change differences (% change) by cohort for genes that met FDR in Cohort A or B. Blank FDR cells indicated those genes did not pass FDR.

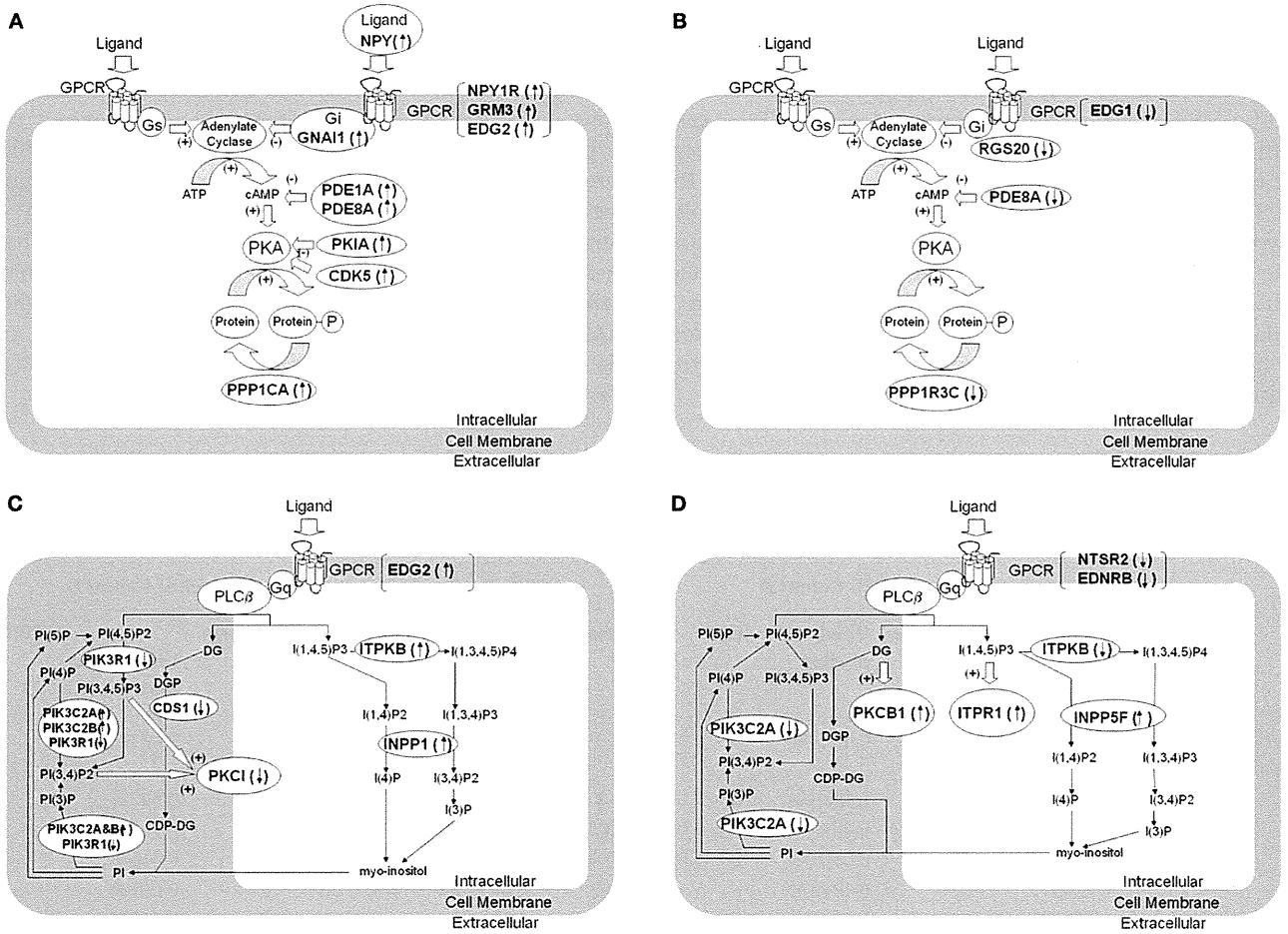

FIGURE 1 | Dysregulation of genes involved in cAMP- and phosphatidylinositol signaling pathways in brain tissue from patients with BPD and MDD. The figure summarizes differentially expressed genes regulating signaling pathways using second messengers of cAMP **(A,B)** and phosphatidylinositol **(C,D)** in brain tissue from BPD **(A,C)** and MDD **(B,D)** compared to control subjects. The differentially expressed genes are denoted in italic and bold. **(A)** shows increased expression of transcripts related to suppression of cAMP mediated signaling in BPD. **(B)** shows decreased expression of transcripts related to suppression of cAMP mediated signaling in MDD. **(C)** shows altered expression of transcripts related to phosphatidylinositol mediated signaling in BPD. **(D)** shows altered expression of transcripts related to phosphatidylinositol mediated signaling in MDD. GNAI1, G protein alpha inhibiting activity 1; RGS20, Regulator of G-protein signaling 20; PDE1A, Phosphodiesterase 1A; PDE8A, Phosphodiesterase 8A; PKIA, Protein kinase A inhibitor alpha; PPP1CA, Protein phosphatase 1, catalytic alpha; PPP1R3C, Protein phosphatase 1, regulatory 3C; INPP5F, Inositol polyphosphate-5-phosphatase F; ITPKB, Inositol 1,4,5-trisphosphate 3-kinase B; INPP1, Inositol polyphosphate-1-phosphatase; CDS1, CDP-diacylglycerol synthase 1; PIK3C2A, Phosphoinositide-3-kinase catalytic 2A; PIK3C2B, Phosphoinositide-3-kinase catalytic 2B; PIK3R1, Phosphoinositide-3-kinase regulatory 1; PRKCI, Protein kinase C iota; ITPR1, Inositol 1,4,5-triphosphate receptor 1; PRKCB1, Protein kinase C beta 1; NPY, Neuropeptide Y; NPY1R, Neuropeptide Y receptor Y1; NTSR2, Neurotensin receptor 2; EDNRB, Endothelin receptor type B; GRM3, Metabotropic Glutamate receptor 3; EDG1, Endothelial differentiation GPCR 1; EDG2, Endothelial differentiation GPCR 2.

In contrast, of those genes that repressed cAMP signaling activity in MDD (**Figure 1B** and **Table 2**) there was only one gene (PDE8A) differentially expressed, hence this signaling pathway was not over-represented ($p > 0.05$).

G_s/G_i-coupled GPCRs

Significant increases of NPY and NPY1R mRNA expression were found in BPD in ACC. GRM3 expression was also increased in ACC of BPD patients. G_i/G_{12}-linked EDG2 was increased in ACC of BPD, while G_i-linked EDG1 was decreased in ACC of MDD. EDG1 and EDG2 regulate neuronal and glial functions, including neurite retraction, neurogenesis, and axonal guidance (Takuwa et al., 2002).

G proteins

Our data showed an increase of GNAI1 in the ACC of BPD, but no change in GNAI1 expression in the DLPFC of BPD. Consistent with our findings in DLPFC, immunoreactivity and functional activity of G_i protein is not altered in the frontal cortex of BPD (Young et al., 1993; Friedman and Wang, 1996). RGS20 was significantly decreased in ACC of MDD cases.

Adenylate cyclase and protein kinase A

Expression levels of transcripts coding adenylate cyclases and cAMP-dependent protein kinases (PKAs) were not altered in BPD and MDD compared with controls in our study, which is consistent with a previous study (Chang et al., 2003). The reported

dysregulation in functional activities of adenylate cyclase and PKA (Gould and Manji, 2002) may not be due to alterations at the transcript level in the brain cortices.

Phosphatidylinositol signaling pathway

The phosphatidylinositol (PI) signaling pathway related genes which were differentially expressed in ACC of BPD patients are shown in **Figure 1C** and are summarized **Table 2**. The group of phosphatidylinositol 3-kinases was significantly over-represented in BPD based on a hypergeometric distribution ($p = 0.042$; $q = 0.36$).

The PI signaling pathway related genes which were differentially expressed in ACC of MDD patients compared with controls are summarized in **Figure 1D** and **Table 2**. Since antidepressants have been reported to affect PI signaling (Dwivedi et al., 2002; Quintero et al., 2005), the potential effect of drug treatment on the gene expression was considered. In cohort A, five MDD subjects were prescribed SSRI at the time of death, whereas remaining four MDD subjects were not prescribed and presumably not taking any SSRIs (**Table 1**). There were no significant differences in mRNA expressions of PKCB1, ITPR1 and INPP5F between SSRI-treated MDD and non-SSRI-treated MDD groups. However, ITPKB mRNA expression was significantly lower in the non-SSRI-treated MDD group than the SSRI-treated MDD group, which suggests that SSRIs may attenuate the altered expression in MDD toward levels observed in controls.

G_q-coupled GPCRs

NTSR2 was decreased in ACC of BPD. NTSR2 mRNA and NTSR2 binding was reported to be down regulated in transgenic mice over expressing corticotrophin releasing factor, which is linked to anxiety, stress and depression (Peeters et al., 2004). EDNRB was also decreased in ACC of MDD. Endothelin is known to be a vasoconstrictor peptide as well as a neuromodulator regulating neuronal excitability. The data did not show any significant differences in expression levels of G_q-linked monoamine GPCRs (serotonin-2 receptors and adrenergic alpha-1 receptors), which was consistent with previous microarray studies (Iwamoto et al., 2004; Konradi et al., 2004; Sibille et al., 2004; Aston et al., 2005).

Lithium and second messengers

The microarray data highlighted several target molecules of lithium treatment (**Figure 1C**). Lithium is known to block INPP1 (inositol polyphosphate-1-phosphatase) enzyme activity, and INPP1 mRNA expression was increased in ACC of BPD. Among the 6 BPD subjects, 3 BPD subjects treated with lithium at the time of death showed lower signal intensities for INPP1 and PIK3C2B compared with remaining non-lithium treated BPD subjects, although the difference did not reach significant changes. There is a modest genetic association between INPP1 and sensitivity to lithium treatment (Steen et al., 1998; Serretti, 2002). A second activity of lithium is activation of phosphatidylinositol 3-kinases and regulation of glycogen synthesis kinase 3 beta (GSK3B) activity (Sinha et al., 2005).

For MDD subjects, the mRNA expression of PKCB1, ITPR1, and INPP5F were increased in ACC, while ITPKB mRNA was decreased (**Figure 1D**). PKCB1 and ITPR1 are receptors for diacylglycerol (DG) and inositol 1, 4, 5-triphosphate (ITP), respectively, both of which are intracellular second messengers produced by PLC-beta through a G_q protein-dependent mechanism, while INPP5F and ITPKB metabolize ITP in a signal-terminating reaction. Previous reports suggest decreased PLC-beta activity in depressed patients (Pandey et al., 1997; Frey et al., 1998; Moore et al., 1999), and over expressed PKCB1 may down regulate PLC-beta activity (Pachter et al., 1992). The alterations in expression levels of the molecules interacting with DG and ITP may reflect functional impairments of G_q and PLC-beta in MDD.

ORPHAN G PROTEIN-COUPLED RECEPTORS

The two GPCRs that showed the most consistent differential expression patterns through both mood disorder analyses were GPCR family C, group 5, member B (GPRC5B) and G protein-coupled receptor 37 (GPR37)as shown in **Table 2** and validated by QPCR (**Table 3**). GPRC5B was significantly increased in ACC of BPD patients and in DLPFC of BPD. GPR37 was also significantly increased in ACC of BPD. GPRC5B was significantly decreased in ACC and DLPFC of MDD patients.

REAL TIME QUANTITATIVE PCR

We followed up on results primarily that met FDR in cohort A for BPD, and both Cohorts A and B for MDD, except for RGS20, which we followed up based upon Cohort A meeting FDR while Cohort B met uncorrected p-value significance only. Nine GPLS transcripts were selected for validation, five for BPD and four for MDD. The qPCR results showed 100% concordance between qPCR and microarray fold change for BPD in ACC (**Table 3**) and 4/5 transcripts were statistically significant. Four GPLS related transcripts showed 100% concordance between qPCR and microarray fold change for MDD in ACC (**Table 3**) and 4/4 transcripts were statistically significant. NPY, GPRC5B, GPR37, and INPP1 were significantly increased in ACC of BPD, whereas GPRC5B, GPR37, RGS20, and PPP1R3C were significantly decreased in the ACC of MDD.

In situ HYBRIDIZATION HISTOCHEMISTRY

GPR37 mRNA was preferentially expressed in subcortical white matter. The sense probe hybridization signal showed background signal. GPR37 mRNA expression in subcortical white matter was higher in ACC of both of the BPD subjects compared to both of the control subjects, whereas GPR37 mRNA expression in subcortical white matter was lower in ACC of both of the MDD subjects compared to both of the control subjects (**Figure 2**). This preliminary data suggests that the significant differential microarray and QPCR expressions of GPR37 between BPD/MDD and controls may reflect altered expression levels of GPR37 mRNA in subcortical white matter. mRNA expression of GPRC5B was not specific in ACC samples tested, most likely due to strong background hybridization issues.

DISCUSSION

The results of the study of GPLS transcriptional profiling showed differences in BPD and MDD in both the cAMP- and