（資料３：Th2ヘルパーT細胞マーカーの文献考察の要約）

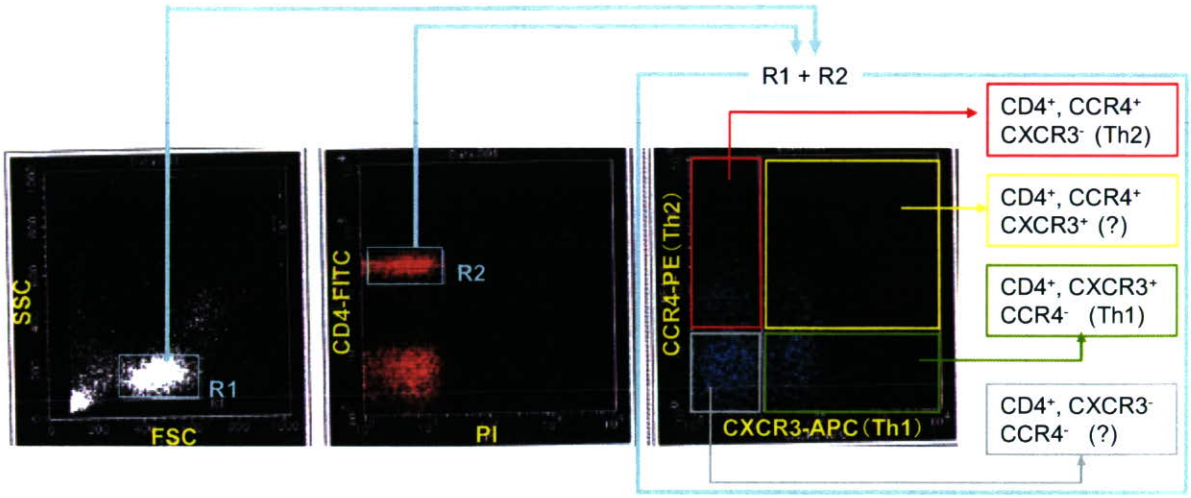
Th2

マーカー；CD4およびCCR4?

Ref No	マーカー	抗体	抗体メーカー	FCM機器	論文名	last	
H4	CRTH2	PE indirectly labeled CRTH2 (with biotinylation followed by PE-streptavidin)	分譲Dr. K. Nagata (BML,).	FACStar ; BD	J. Immunol	Seishi Kyoizumi *Department of Radiobiology, Radiation Effects Research Foundation*	2002(169)39-48.
H3*	CCR4	PE-antiCCR4	BD	FACSCalibur ;BD	Immunology Letters	Namit Ghildyal *Department of Immunotherapeutics*	102 (2006) 110-114
	*[When compared to the CRTh2 enrichment approach, the CCR4 approach gave 8- to 10-fold greater numbers of Th2 cells isolated directly]との記載あり						
H1	CD4	Cy5-antiCD4	EB	EPICS-XL ; Beckman	Cellular & Molecular	Qun Chen *Guangdong Medical College*	2005(2)5,387-392
	CRTH2	PE-antiCRTH2	Miltenyibiotec				
H5	CCR4	FITC-antiCCR4	PharMingen	FACScan ;BD	J. Leukoc. Biol.	Saburo Sone *University of Tokyo*	2001(70)749-755
	CD4	PE-antiCD4					
H6	CCR4	mouseIgG-antiCCR4	自作	FACSVantage /Calibar (BD)	Am J Pathol	James J. Campbell *Institute for Lung Health, Leicester University Medical School*	2002(160)347-355
H12	CCR3	FITC/NHS-LC-Biotin-antiCCR3	Boehringer Mannheim Corp/Pierce	FACScan;BD	Eur. J. Immunol.	Christina M. Parker*Brigham and Women's Hospital and Harvard Medical School, Harvard Medical School*	2000(30) 819-826
	CCR4	FITC/NHS-LC-Biotin-antiCCR4					
H28	CRTH2	biotin-antiCRTH2	自作	FACSCalibur	Clin Exp Immunol	S. SAITO *Toyama Medical and Pharmaceutical University,*	2001(123)105-111
H29	CRTH2	biotin-antiCRTH3	自作	FACSCalibur	FEBS Letters	Shoichi Takanoa *BioMedical Laboratories, Inc*	1999 (459) 195-199
H10	CD4	anti-CD4-APC	Beckman	FACSCalibur	Eur. J. Immunol.	Federica Sallusto *Institute for Research in Biomedicine,*	2003(33)474-482
	CCR4	anti-CCR4	PharMingen				
	CRTH2	anti-CRTh2	自作				
	CCR3	anti-CCR3	Millennium Pharmaceuticals				
	**検出は2次抗体を用いた。						
H11	CD4	PerCP-antiCD4	BD	FACScan;BD	Journal of Clinical Immunology	KAZUHIKO TAKEHARA *Kanazawa University Graduate School of Medical*	2003(23),4
	CCR4	anti-CCR4	自作				
H14	CD4	PerCP-antiCD4	BD	FACSCalibur; BD	Investigative Ophthalmology & Visual Science	Andrew D. Dick *University of Bristol*	2004(45)1,170-176
	CCR4	PE-antiCCR4	BD				
H16	CD4	PE- anti CD4	PharMingen	FACScan ; BD	Mod Rheumatol	Saburo Sone *Tokushima University*	2003 (13)114-120
	CCR4	FITC-antiCCR4					
H18	CD4	chlorophyll protein-antiCD4	BD	FACSCalibur machine	J ALLERGY CLIN IMMUNOL	Clare M. Lloyd, PhD *United Kingdom*	2003(112)6,1155-1161
	CCR3	R-PE-antiCCR3	R&D Systems),				
	CCR4	R-PE-antiCCR4	BD				
	CCR8	Anti-CCR8 (pAb)	AMS Biotechnology,				

（資料４：細胞ソーティング法によるTh1およびTh2ヘルパーT細胞の選別）

FACSによるヘルパーT細胞　Th1⁺細胞およびTh2⁺細胞の選別 (4 Way Sorting)



① FSC、SCCによりリンパ球分画（R1)を選別
② PI、CD4-FITCによりPI陰性のCD4⁺ヘルパーリンパ球分画（R2)を選別
③ R1、R2を展開し、CXCR3-APC、CCR4-PEにより、4 Way Sorting
　(1) PI陰性のCD4⁺, CXCR3⁺, CCR4⁻分画（Th1ヘルパーリンパ球細胞）
　(2) PI陰性のCD4⁺, CCR4⁺, CXCR3⁻分画（Th2ヘルパーリンパ球細胞）
　(3) PI陰性のCD4⁺, CCR4⁺, CXCR3⁺分画
　(4) PI陰性のCD4⁺, CCR4⁻, CXCR3⁻分画

（資料５：細胞数の再現性実験）

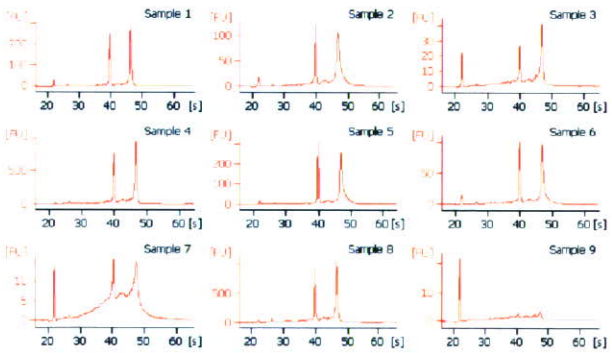
Sample	採血量	リンパ球	分画	%cell	Sorting cell
	[mL]	[個]			
健常者1-1	17	2.6×10^7	Th1	0.3	217300
			Th2	0.3	165073
			Th1/Th2		1.32
			WP	0.2	125522
			WN	0.9	521596
健常者1-2	13	3.4×10^7	Th1	0.9	267171
			Th2	0.6	207677
			Th1/Th2		1.29
			WP	0.4	131528
			WN	2.5	710538
健常者2-1	12	3.5×10^7	Th1	0.9	275834
			Th2	0.8	260448
			Th1/Th2		1.06
			WP	0.4	149658
			WN	4	1175080
健常者2-2	12.5	3.0×10^7	Th1	0.9	116011
			Th2	0.7	100212
			Th1/Th2		1.16
			WP	0.3	55079
			WN	3.7	609961
健常者3-1	10	3.5×10^7	Th1	0.3	89931
			Th2	0.3	90141
			Th1/Th2		1.00
			WP	0.1	44599
			WN	2.3	622728
健常者3-2	12	2.6×10^7	Th1	0.5	50104
			Th2	0.4	58497
			Th1/Th2		0.86
			WP	0.2	15160
			WN	2.8	277718

健常者３名から異なる採取日に採血した血液から細胞ソーティングを行い、Th1ヘルパーT細胞（Th1）、Th2ヘルパーT細胞（Th2）、Th1およびTh2マーカー共陽性細胞（Double Positive, WP）、Th1・Th2陰性ヘルパーT細胞（Double Negative, WN）の採取し、再現性を確認した。

（資料６：RNAの収量とクオリティーコントロール）

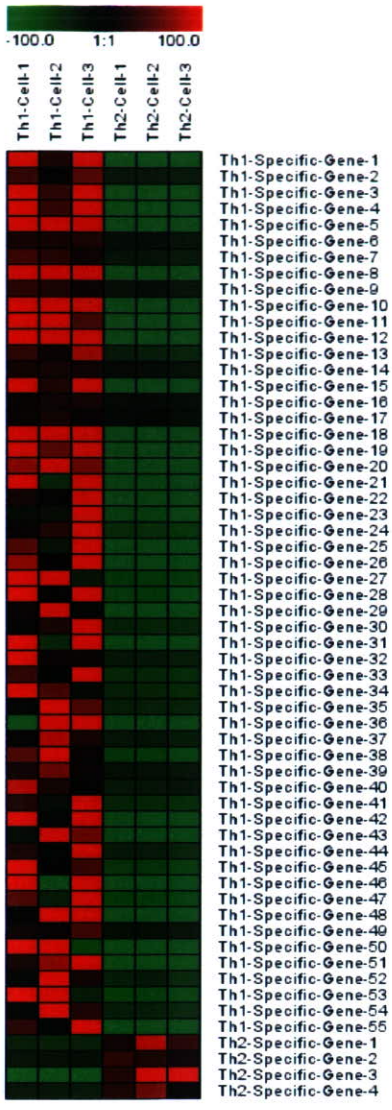
分画	Sample	採血量 [mL]	リンパ球 [個]	%cell	Sorting cell	totalRNA 溶出量[μL]	RNA Area	RNA濃度 (pg/μl)	rRNA比 [28S/18S]	RIN値
Th1	健常者1-1	17	2.6×10^7	0.3	217300	30	963.2	2559.6	1.5682	9.8 (B.02.02)
	健常者1-2	13	3.4×10^7	0.9	267171	30	1330.9	3536.5	1.8749	9.7 (B.02.02)
	健常者2-1	12	3.5×10^7	0.9	275834	30	742.8	2996.9	1.8654	9.6 (B.02.02)
	健常者2-2	12.5	3.0×10^7	0.9	116011	30	192.0	774.6	2.7857	8.1 (B.02.02)
	健常者3-1	10	3.5×10^7	0.3	89931	30	274.0	1674.8	1.0236	5.3 (B.02.02)
	健常者3-2	12	2.6×10^7	0.5	50104	30	274.0	1674.8	1.0236	5.3 (B.02.02)
Th2	健常者1-1	17	2.6×107	0.3	165073	30	737.8	1960.5	2.1725	9.3 (B.02.02)
	健常者1-2	13	3.4×107	0.6	207677	30	548.0	1456.2	1.6528	9.1 (B.02.02)
	健常者2-1	12	3.5×107	0.8	260448	30	688.9	2779.4	2.4421	10.0 (B.02.02)
	健常者2-2	12.5	3.0×107	0.7	100212	30	150.4	607.0	2.179	8.0 (B.02.02)
	健常者3-1	10	3.5×107	0.3	90141	30	227.4	1390.1	2.6666	9.2 (B.02.02)
	健常者3-2	12	2.6×107	0.4	58497	30	227.4	1390.1	2.6666	9.2 (B.02.02)
WN	健常者1-1	17	2.6×107	0.9	521596	30	3569.1	9484.1	1.518	9.4 (B.02.02)
	健常者1-2	13	3.4×107	2.5	710538	30	4408.8	11715.4	1.4489	9.2 (B.02.02)
	健常者2-1	12	3.5×107	4	1175080	30	7782.1	31398.2	1.991	9.8 (B.02.02)
	健常者2-2	12.5	3.0×107	3.7	609961	30	2327.7	9391.6	2.1019	9.8 (B.02.02)
	健常者3-1	10	3.5×107	2.3	622728	30	812.8	4969.0	0.2213	8.0 (B.02.02)
	健常者3-2	12	2.6×107	2.8	277718	30	812.8	4969.0	0.2213	8.0 (B.02.02)
WP	健常者1-1	17	2.6×107	0.2	125522	30	255.6	679.2	2.0105	8.0 (B.02.02)
	健常者1-2	13	3.4×107	0.4	131528	30	279.1	741.6	1.6428	7.3 (B.02.02)
	健常者2-1	12	3.5×107	0.4	149658	30	235.9	951.8	2.3477	10.0 (B.02.02)
	健常者2-2	12.5	3.0×107	0.3	55079	30	41.6	167.7	2.3923	6.6 (B.02.02)
	健常者3-1	10	3.5×107	0.1	44599	30	13.5	82.6	1.7426	7.5 (B.02.02)
	健常者3-2	12	2.6×107	0.2	15160	30	13.5	82.6	1.7426	7.5 (B.02.02)

Agilent BioAnalyzer 2100 泳動パターン（代表サンプル）



健常者３名から細胞ソーティングにより回収した、Th1ヘルパーT細胞（Th1）、Th2ヘルパーT細胞（Th2）、Th1およびTh2マーカー共陽性細胞（Double Positive, WP）、Th1・Th2陰性ヘルパーT細胞（Double Negative, WN）から総RNAを抽出し、総RNAのクオリティー・コントロールを行った。Agilent BioAnalyzer 2100にAgilent RNA Picoキットを用いて総RNAサンプルの泳動実験を行い、リボソームRNAの18Sと28Sの比（28S/18S比）やRIN(RNA Integrity Number)等の総RNAのクオリティー・コントロールの指標を確認した。総じて28S/18S比は1.5以上、RIN値は8以上あることが確認でき、総RNAの質は良好であることが確認された。28S/18S比1.5未満、RIN値7未満の検体は以降の実験には使用しない。

（資料７：マイクロアレイ解析による新規Th1細胞・Th2細胞特異的遺伝子群の特定）



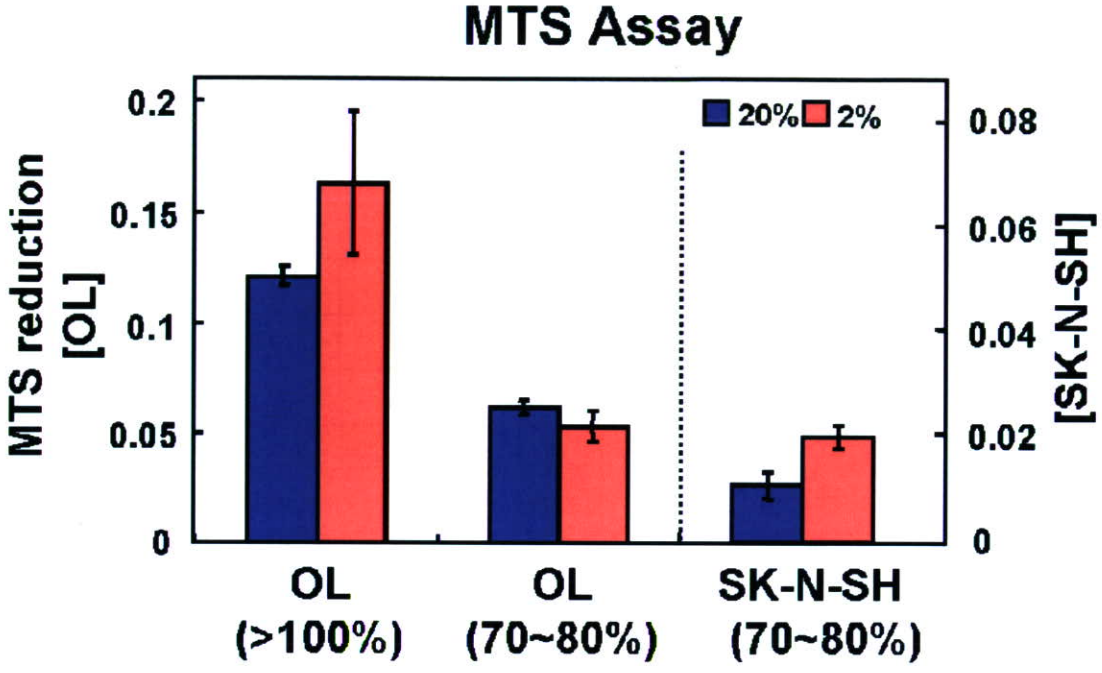
（資料８：Th1ヘルパーT細胞遺伝子発現プロファイル）

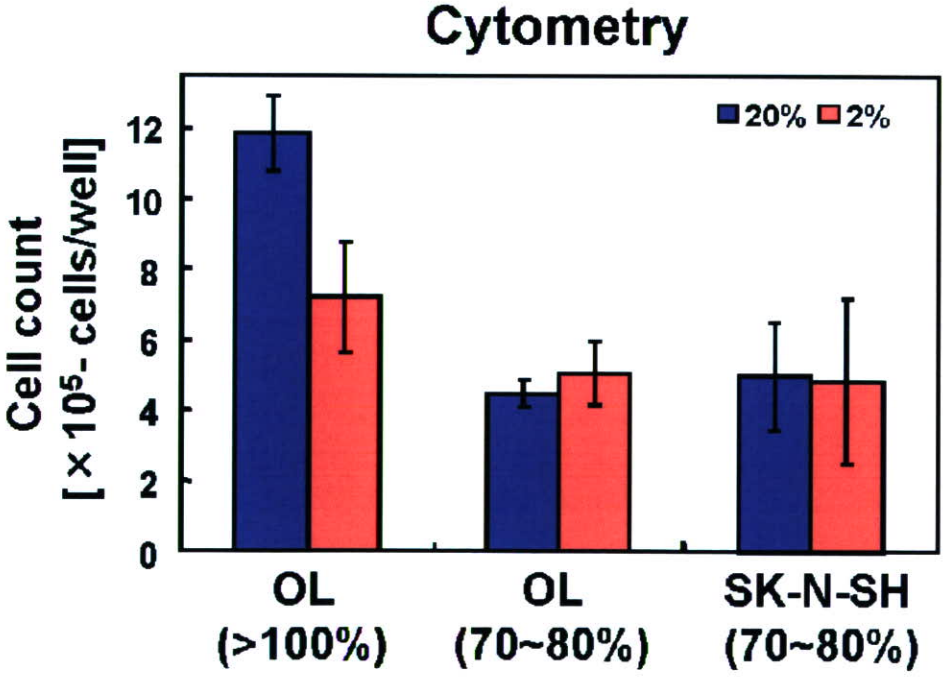
Gene Category	List Hits	List Total	Population Hits	Population Total	EASE score
response to biotic stimulus	35	182	755	9445	0.000002
defense response	31	182	698	9445	0.0000219
immune response	28	182	628	9445	0.0000593
response to pest/pathogen/parasite	21	182	393	9445	0.0000615
response to stress	26	182	689	9445	0.00144
antigen presentation	5	182	28	9445	0.00186
response to external stimulus	38	182	1209	9445	0.00236
antigen presentation, exogenous antigen	4	182	15	9445	0.00266
antigen processing, exogenous antigen via MHC class II	4	182	15	9445	0.00266
cytokine binding	6	187	53	9638	0.00344
MHC class II receptor activity	4	187	17	9638	0.00394
catabolism	25	182	734	9445	0.00676
cell proliferation	28	182	900	9445	0.0124
cytoplasm	84	180	3578	9342	0.0159
antigen processing	4	182	28	9445	0.0159
intracellular	124	180	5701	9342	0.019
cell death	14	182	360	9445	0.0209

（資料９：Th2ヘルパーT細胞遺伝子発現プロファイル）

Gene Category	List Hits	List Total	Population Hits	Population Total	EASE score
cell death	13	128	360	9445	0.00326
death	13	128	364	9445	0.00357
RNA binding	13	131	380	9638	0.00517
apoptosis	12	128	335	9445	0.00538
programmed cell death	12	128	336	9445	0.0055
regulation of apoptosis	8	128	185	9445	0.0123
isomerase activity	5	131	91	9638	0.0344
mRNA splicing	3	128	22	9445	0.0348
intramolecular isomerase activity	3	131	24	9638	0.0411

（資料１０：低酸素状態下での神経細胞由来・オリゴデンドロサイト由来培養細胞の細胞増殖とミトコンドリア活性）





（資料１１：低酸素状態下での神経細胞由来・オリゴデンドロサイト由来培養細胞の遺伝子発現変化）

低酸素により発現変化がみられた遺伝子数

	OL (100%)	OL (70-80%)	SK-N-SH (70-80%)
Probe Set On Array	54613	54613	54613
Induced Transcript	2882	877	267
Supressed Transcripts	4012	745	295

これまでに低酸素により発現誘導・抑制が報告されている遺伝子と一致したOL細胞の遺伝子

Gene Title	Gene Symbol	Expression
angiopoietin-like 4	ANGPTL4	Induced
BCL2/adenovirus E1B 19kDa interacting protein 3-like	BNIP3L	Induced
MAX interactor 1	MXI1	Induced
N-myc downstream regulated gene 1	NDRG1	Induced
ribonuclease, RNase A family, 4	RNASE4	Induced
solute carrier family 2 (facilitated glucose transporter), member 1	SLC2A1	Induced
solute carrier family 2 (facilitated glucose transporter), member 3	SLC2A3	Induced
cyclin D1	CCND1	Repressed
proliferating cell nuclear antigen	PCNA	Repressed

（資料１２：低酸素状態下での神経細胞由来培養細胞の遺伝子発現変化）

低酸素により発現変化がみられた遺伝子群

SK-N-SH細胞

Gene Category		List Hits	List Total	Population Hits	Population Total	EASE score
phosphatidylinositol binding	I	2	17	3	1467	3.24E-02
phosphoinositide binding	I	2	17	4	1467	4.30E-02
DNA metabolism	I	4	18	77	1427	5.95E-02
membrane fraction	I	3	16	38	1402	6.02E-02
cell communication	I	8	18	319	1427	6.33E-02
plasma membrane	I	5	16	155	1402	7.43E-02
response to abiotic stimulus	I	3	18	44	1427	9.43E-02
extracellular space	R	6	47	35	1402	4.73E-03
extracellular	R	8	47	88	1402	2.17E-02
transition metal ion homeostasis	R	3	50	7	1427	2.17E-02
di-, tri-valent inorganic cation homeostasis	R	3	50	10	1427	4.36E-02
hemostasis	R	3	50	10	1427	4.36E-02
metal ion homeostasis	R	3	50	11	1427	5.21E-02
cell adhesion	R	5	50	43	1427	5.66E-02
homeostasis	R	3	50	12	1427	6.12E-02
cell homeostasis	R	3	50	12	1427	6.12E-02
cation homeostasis	R	3	50	12	1427	6.12E-02
ion homeostasis	R	3	50	12	1427	6.12E-02
cell ion homeostasis	R	3	50	12	1427	6.12E-02
enzyme activator activity	R	4	46	33	1467	7.75E-02
Rho GTPase activator activity	R	2	46	3	1467	8.93E-02
copper/cadmium binding	R	2	46	3	1467	8.93E-02
copper ion binding	R	2	46	3	1467	8.93E-02
copper ion homeostasis	R	2	50	3	1427	9.96E-02

I; Induced　R; Repressed

（資料１３：低酸素状態下でのオリゴデンドロサイト由来培養細胞の遺伝子発現変化）

低酸素により発現変化がみられた遺伝子群

OL細胞

Gene Category		List Hits	List Total	Population Hits	Population Total	EASE score
glucose metabolism	I	12	195	24	1427	1.11E-04
glucose catabolism	I	10	195	18	1427	2.14E-04
alcohol catabolism	I	10	195	18	1427	2.14E-04
hexose catabolism	I	10	195	18	1427	2.14E-04
monosaccharide catabolism	I	10	195	18	1427	2.14E-04
hexose metabolism	I	13	195	30	1427	2.64E-04
monosaccharide metabolism	I	13	195	30	1427	2.64E-04
glycolysis	I	9	195	16	1427	4.93E-04
carbohydrate catabolism	I	10	195	20	1427	5.76E-04
alcohol metabolism	I	14	195	37	1427	6.52E-04
energy derivation by oxidation of organic compounds	I	13	195	33	1427	7.42E-04
energy pathways	I	14	195	38	1427	8.74E-04
structural constituent of ribosome	R	42	111	68	1467	4.46E-32
ribosome	R	44	110	82	1402	1.40E-29
cytosolic ribosome (sensu Eukarya)	R	35	110	48	1402	1.99E-29
ribonucleoprotein complex	R	46	110	98	1402	2.66E-28
protein biosynthesis	R	49	111	125	1427	1.28E-25
structural molecule activity	R	45	111	119	1467	6.33E-23
RNA binding	R	42	111	107	1467	6.30E-22
macromolecule biosynthesis	R	49	111	159	1427	3.21E-20
small ribosomal subunit	R	19	110	20	1402	4.55E-19
cytosol	R	38	110	100	1402	1.62E-18
cytosolic small ribosomal subunit (sensu Eukarya)	R	18	110	19	1402	6.15E-18
eukaryotic 48S initiation complex	R	18	110	19	1402	6.15E-18
biosynthesis	R	49	111	185	1427	4.39E-17

I; Induced　R; Repressed

II. 研究成果の刊行に関する一覧

研究成果の刊行に関する一覧表

論文発表

（1）原著

1. Atz M, Walsh D, Cartagena P, Li J, Evans S, Choudary P, Overman K, Stein R, Tomita H, Potkin S, Myers R, Watson SJ, Jones EG, Akil H, Bunney WE Jr, Vawter MP. Methodological considerations for gene expression profiling of human brain. J Neurosci Methods, 2007;163(2): 295-309.

2. Li JZ, Meng F, Tsavaler L, Evans SJ, Choudary PV, Tomita H, Vawter MP, Walsh D, Shokoohi V, Chung T, Bunney WE Jr, Jones EG, Akil H, Watson SJ, Myers RM. Sample matching by inferred agonal stress in gene expression analyses of the brain.
BMC Genomics. 2007; 8(1): 336 [Epub ahead of print]

（2）総説

1. 富田博秋、田中千晶：精神疾患研究におけるミトコンドリア関連遺伝子の発現変化～病態か、アーチファクトか？. 脳と精神の医学, 19(2):印刷中

III. 研究成果の刊行物・別冊

ELSEVIER

Journal of Neuroscience Methods 163 (2007) 295–309

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Methodological considerations for gene expression profiling of human brain

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Abstract

Gene expression profiles of postmortem brain tissue represent important resources for understanding neuropsychiatric illnesses. The impact(s) of quality covariables on the analysis and results of gene expression studies are important questions. This paper addressed critical variables which might affect gene expression in two brain regions. Four broad groups of quality indicators in gene expression profiling studies (clinical, tissue, RNA, and microarray quality) were identified. These quality control indicators were significantly correlated, however one quality variable did not account for the total variance in microarray gene expression. The data showed that agonal factors and low pH correlated with decreased integrity of extracted RNA in two brain regions. These three parameters also modulated the significance of alterations in mitochondrial-related genes. The average *F*-ratio summaries across all transcripts showed that RNA degradation from the AffyRNAdeg program accounted for higher variation than all other quality factors. Taken together, these findings confirmed prior studies, which indicated that quality parameters including RNA integrity, agonal factors, and pH are related to differences in gene expression profiles in postmortem brain. Individual candidate genes can be evaluated with these quality parameters in post hoc analysis to help strengthen the relevance to psychiatric disorders. We find that clinical, tissue, RNA, and microarray quality are all useful variables for collection and consideration in study design, analysis, and interpretation of gene expression results in human postmortem studies.

Keywords: Microarray; Gene expression; Postmortem brain tissue; pH; RNA quality; Agonal factors

1. Introduction

It has been a challenge to locate precise candidate genes for complex psychiatric disorders using methods that were successful for simple Mendelian disorders. Complex psychiatric disorders are not caused by one gene, but rather by multiple genes (Mitchell et al., 1993; Craddock and Jones, 1999; Shastry, 2005). Complex disorders have been difficult to map for reasons of disease heterogeneity, misclassification and environmental influences. An accurate, yet comprehensive gene expression profile of brain tissue may result in better understanding of the genotype and phenotype relationships (Nestler et al., 2002; Bunney et al., 2003; Mirnics and Pevsner, 2004; Altar et al., 2005; Erraji-Benchekroun et al., 2005; Newton et al., 2005).

One highly used technique of gene expression profiling in psychiatric disorders has been microarray studies that use postmortem brain tissue (Barrett et al., 2001; Luo and Geschwind, 2001; Mirnics and Pevsner, 2004) followed by quantitative real time PCR to confirm candidate genes (Jurata et al., 2004; Mimmack et al., 2004). Microarray is a high-throughput method used to screen thousands of genes for alterations in expression between groups. The resulting data has suggested novel pathways linked to psychiatric disorders (Bunney et al., 2003; Hosack et al., 2003; Mootha et al., 2003a,b).

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The reliability and reproducibility of microarray results (Auer et al., 2003; Buesa et al., 2004; Shergill et al., 2004) must be constantly evaluated (Konradi et al., 2004; Ryan et al., 2004) and is an important question. Previously reports have shown that standard factors such as age, postmortem interval, and medical and family history from proxy respondents increased data reliability (Deep-Soboslay et al., 2005). It has been reported that the most critical aspect of postmortem research is the integrity of the sample (Mirnics and Pevsner, 2004; Tomita et al., 2004) and the pH (Li et al., 2004; Mexal et al., 2006; Vawter et al., 2006; Lipska et al., 2006). It has been suggested that samples used for gene expression studies must be of the highest quality (or matched quality) to represent underlying molecular pathophysiology (Dumur et al., 2004; Mirnics and Pevsner, 2004) and most investigators will attempt to avoid samples with highly degraded RNA. However, inherent in many comorbid psychiatric disorders (e.g. drug overdose and suicide), potential subjects are likely to have varying degrees of quality, and thus elimination of subjects with less than ideal quality would severely restrict research avenues in psychiatric disorders. Therefore, many studies match samples based on several quality parameters. Human postmortem brain tissue profiling has been challenging for several reasons beyond these quality issues (Mirnics et al., 2001, 2006). Polygenic, epigenetic, and environmental factors affect gene profiling (Mirnics et al., 2001). A presumed narrow range of gene expression in brain tissue due to homeostatic mechanisms restricts the fold-change of differential gene expression observed in microarray analyses of psychiatric disorders (Mirnics and Pevsner, 2004). Another complication reported was the dynamic range of gene expression of the transcriptome. For example, in the hippocampus there was about a 2000-fold difference between highly expressed genes compared to rare transcripts (Evans et al., 2002). Furthermore, RNA transcription was significantly regulated in the opposite direction to protein (Greenbaum et al., 2003), possibly due to mRNA stability, mRNA turn-over, mRNA steady-state transcription differences, and translation differences. Additionally, pharmacological treatments affected the transcriptome and since most patients with severe psychiatric disorders received medication, while controls do not receive psychiatric drugs, this further complicated interpretation of gene expression results.

In an effort to obtain well-characterized samples, which can be utilized for data analysis, investigators have begun to assess quality parameters thought to be important in postmortem brain tissue studies. One parameter often examined is the clinical quality. One aspect of clinical quality is to obtain correct retrospective psychiatric diagnosis (Deep-Soboslay et al., 2005). Medical records and next-of-kin interviews were complementary methods for confirmation of the diagnosis of cases and also useful to account for the lack of a psychiatric history in the controls (Brent et al., 1993; Kelly, 1996; Isometsa, 2001; Deep-Soboslay et al., 2005). Another facet of clinical quality is the agonal state of the patient. There is no consensus method for assessing agonal state (Hardy et al., 1985; Tomita et al., 2004) and thus, the precise effects on microarray quality have yet to be decided (Johnston et al., 1998; Buesa et al., 2004; Iwamoto et al., 2005). pH might be a more objective measure of clinical and tissue quality (Johnston et al., 1998) because pH was inversely correlated to the agonal state (i.e. the sum of the number of agonal factors as described in Tomita et al., 2004) and the duration of agonal state (measured in minute, hour, day). In order to assess this correlation correctly of pH and gene expression profiling, the data should be approached with caution because not all mRNA levels were affected by pH (Barton et al., 1993; Preece and Cairns, 2003; Buesa et al., 2004). For example, about 28% of mitochondrial-related transcripts were affected by pH (Vawter et al., 2006). Many researchers have used pH measurements as a substitute for clinical assessments of agonal state and duration (Johnston et al., 1998; Preece and Cairns, 2003; Buesa et al., 2004; Mirnics et al., 2004). Previous analyses showed that agonal factors were not perfect predictors of microarray and tissue quality and thus, other methods are being developed to assess microarray and sample quality (Li et al., 2005).

Differences in agonal state are clearly associated with differences in both pH and RNA quality (Harrison et al., 1991; Hynd et al., 2003; Tomita et al., 2004). Acidosis in human postmortem brain tissue can be caused by agonal factors such as coma, hypoxia, pyrexia, seizures, dehydration, hypoglycemia, multiple organ failure, head injury, and ingestion of neurotoxic substances, which can affect RNA integrity (Hardy et al., 1985; Harrison et al., 1991, 1995; Barton et al., 1993; Morrison-Bogorad and Pardue, 1995; Hynd et al., 2003). Along with agonal factors per se, rapidity of death played a role in the outcome of the tissue quality (Harrison et al., 1991; Hynd et al., 2003; Tomita et al., 2004). The influence of agonal factors on alterations of neurochemicals in human brain was initially demonstrated by researchers that measured the level of the inhibitory neurotransmitter (GABA) and the biosynthetic enzyme level of glutamic acid decarboxylase (GAD) in schizophrenia and Huntington's chorea (Bird et al., 1977; Spokes, 1979; Spokes and Iversen, 1979; Spokes et al., 1980). GABA was decreased in control brains due to hypoxia and long-term illness, but was even more reduced in Huntington's cases (Bird et al., 1977; Spokes, 1979; Spokes and Iversen, 1979; Spokes et al., 1980). Hypoxia was a key complication of major agonal events (Buesa et al., 2004) and it affected gene expression in human postmortem brain (Burke et al., 1991). Hypoxia was reported to 'cause' a reduction in pH (Hardy et al., 1985; Kingsbury et al., 1995; Corbett et al., 1996) possibly by increasing tissue lactate (Hardy et al., 1985; Yates et al., 1990). A decreased pH was also associated with an increased mitochondrial DNA copy number and an increased number of mitochondrial DNA transcripts in postmortem human brain (Vawter et al., 2006). However, lower pH was correlated with long agonal duration, thereby leading to speculation that mtDNA copy number and mtDNA transcription is influenced by pH as well as events occurring in the agonal phase. These results suggest that pH is a useful monitor for agonal events. However, it cannot be stated that lower pH will decrease RNA quality due to an effect such as acid hydrolysis within the pH range commonly observed in postmortem brain. Others have reported that a lower pH was associated with compromised RNA integrity (Harrison et al., 1995). Thus, pH and RNA integrity are strongly correlated

measures but it cannot be assumed that this represents a 'cause and effect' relationship.

Another factor used to assess postmortem samples is tissue quality. Different parameters have been used to assess tissue quality, notably brain pH, gross neuropathological examination, postmortem interval (PMI), and freezer time. Brain pH, as discussed above, has been shown to be related to agonal state and RNA integrity (Harrison et al., 1995; Kingsbury et al., 1995; Li et al., 2004; Tomita et al., 2004). Postmortem human brain tissue was evaluated using housekeeping gene expression by reverse transcription real-time PCR. The results showed pH was significantly correlated with the gene expression score for four housekeeping genes (Miller et al., 2004). Furthermore, when hippocampal gene expression profiles were examined in schizophrenia, pH was found to contribute to a variation in expression that was greater than any other factor evaluated (Mexal et al., 2006). Additionally, it was reported that pH affected the gene expression of mitochondrial-related genes (Li et al., 2004; Iwamoto et al., 2005; Vawter et al., 2006) and if the effects of pH were not controlled in a post hoc analysis, the gene expression profile results would be a reflection of pH (Li et al., 2004; Mexal et al., 2006; Vawter et al., 2006). It appears that not all mitochondrial-related transcripts are affected to the same degree by pH, however large pH effects were seen in non-mitochondrial-related gene expression (Vawter et al., 2006).

As a surrogate measure of tissue quality, pH, was reported to be stable in brain tissue after death and during freezer storage (Buesa et al., 2004). Different regions of the brain may be used for pH measurements, as stable pH measurements in 10 brain regions for three subjects were shown (Johnston et al., 1998). Cortex has been suggested to be a usable surrogate tissue for pH measurements (Mexal et al., 2006). The range and average pH measurement of postmortem brain collections varies between brain banks. Part of this variability may be due to the method of pH measurement (Preece and Cairns, 2003; Miller et al., 2004; Middleton et al., 2005; Torrey et al., 2005). Although, a more likely explanation is that this variation is due to the observation that agonal factors are variably related to pH, as discussed above.

Human studies have not shown a clear relationship between PMI and RNA quality (Harrison et al., 1995; Cummings et al., 2001; Catts et al., 2005). On the other hand, animal studies, which are more carefully controlled, have shown that an increase in the length of the PMI decreased total RNA amounts (Taylor et al., 1986). A recent study of murine samples reported that increased PMI was associated with decreased pH and decreased RNA integrity measured by 28S/18S ratio (Catts et al., 2005). The acceptable maximum PMI for human studies was reported as 36–48 h (Johnson et al., 1986; Barton et al., 1993; Soverchia et al., 2005). Furthermore, brain refrigeration following death will ultimately slow autolysis and maintain pH homeostasis (Buesa et al., 2004).

Tissue freezer storage time following autopsy was shown to cause degradation of the poly(A) tail region of RNA (Johnston et al., 1998). The loss of the poly(A) tail was thought to result in loss of the rest of the message (Bernstein and Ross, 1989), as this caused an exonuclease involved in RNA degradation to assemble (Ford et al., 1997; Yang et al., 2003; Wilusz and Wilusz, 2004). Another concern, was that loss of the poly(A) tail can impede the priming of the oligo-dT in the cDNA synthesis step of the microarray protocol. Furthermore, while the RNases involved in degradation may not be active at low temperatures, when a sample is subjected to freeze–thaw cycles it may result in degradation (Johnston et al., 1998).

A third aspect of sample assessment for gene expression profiling is the RNA quality, which is critical for subsequent microarray analysis (Dumur et al., 2004). Various criteria have been used to evaluate the integrity of RNA (Buesa et al., 2004; Dumur et al., 2004; Miller et al., 2004). The total RNA ratio, as determined by the Agilent Bioanalyzer, measures the fraction of the area in the 18S and 28S regions. These areas are compared to the total area under the curve and the result is the ratio of large molecules to small molecules. This reading is not sufficient to serve as a universal integrity number. It is now suggested that this reading is better when accompanied with the RNAA integrity number (RIN) algorithm. The RIN algorithm does not use the ratio of the ribosomal bands to determine integrity rather; it uses the entire electrophoretic trace. This tool allows for a robust and reliable prediction of RNA integrity (Schroeder et al., 2006). Another reported advantage of the Agilent Bioanalyzer was reliability and efficiency compared to standard agarose gel (Grissom et al., 2005).

Additionally, cRNA synthesized by in vitro transcription can be assessed as a measure of RNA quality by observing the median length on the Agilent Bioanalyzer (Dumur et al., 2004; Ryan et al., 2004) and by spectrophotometer to gauge the A_{260}/A_{280} absorbance measurements. The synthesis of high quality cDNA and cRNA were associated with the quality of the initial total RNA (Dumur et al., 2004; Carter et al., 2005).

The stability of human postmortem brain tissue mRNA for use in microarray analyses and real time PCR was shown to be an essential prerequisite for further downstream molecular analysis (Bahn et al., 2001; Lipska et al., 2006). For example, when RNA was manually degraded, it was shown that 75% of the differential gene expression was actually due to RNA integrity differences between the samples (Auer et al., 2003). Furthermore, the gene expression patterns showed that RNA degradation led to both up and down regulated genes (Auer et al., 2003; Lee et al., 2005). This was demonstrated by examining degraded total RNA samples at different time points and comparing the results to non-degraded RNA. At each time point, there were a significant number of genes that showed increased expression in the degraded samples when compared to the non-degraded RNA samples. One explanation for this is that RNA fragmentation may have caused a more efficient synthesis of cDNA. However, the RNases active during the freeze–thaw cycles are unpredictable and consequently lead to varying degrees of degradation (Grissom et al., 2005).

RNA degradation can be complex due to the structure of RNA (Hollams et al., 2002) and the sequence of the 3′-untranslated region (UTR) (Berger et al., 2005). The 3′-UTR sequence may have altered the stability of some RNA transcripts (Berger

et al., 2005) that harbor the AU-rich elements (AREs) and iron-responsive elements (IREs), both of which play a role in destabilizing some RNAs (Hollams et al., 2002). mRNA degradation occurred from the 3′ end, the 5′ end, or from internal positions (Buesa et al., 2004). The loss of the 5′ cap led to 5′ → 3′ decay and loss of the poly(A) tail led to 3′ → 5′ decay by exonuclease activity (Buesa et al., 2004; Fritz et al., 2004; Wilusz and Wilusz, 2004), but the predominant degradation pathway in mammals was not determined (Yang et al., 2003; Wilusz and Wilusz, 2004). It has been observed that low degradation caused a reduction in transcript length, but did not reduce the total amount of transcripts (Ryan et al., 2004). However, when genes were organized by functional classes the variation of decay rates between classes of mRNAs was significantly different (Yang et al., 2003).

Affymetrix gene expression probes on the U133 series of chips were designed toward the 3′ end and also further toward the 5′ end of several housekeeping genes (GAPDH and ACTB). The ratio of 3′/5′ expression was evaluated as a measure of transcript degradation. Although, because the site of degradation is unknown, in theory, it is possible to have a low 3′/5′ ratio (meaning relatively intact RNA) in a slightly degraded sample (Dumur et al., 2004; Ryan et al., 2004). Several *studies* have reported that slight RNA degradation does not have a substantial effect on the number of genes detected in the "Present Call" reading on the Affymetrix arrays (Schoor et al., 2003; Ryan et al., 2004; Lee et al., 2005). These studies examined only a small subset of the probesets on the arrays due to the restrictions in the design of each experiment (Schoor et al., 2003; Lee et al., 2005) and furthermore, it was shown that for each transcript the exact mechanism of RNA degradation was unclear (Ryan et al., 2004).

An often-overlooked aspect of discussions of degradation of mRNA is that frequently a spurious increased expression was found using microarray technology (Bahn et al., 2001; Auer et al., 2003; Schoor et al., 2003; Buesa et al., 2004; Ryan et al., 2004; Lee et al., 2005). RNA degradation was induced by an in vitro perturbation experiment and a list of 31 genes was found to be significantly different due to RNA degradation alone (Auer et al., 2003). Our group recently published a list of genes found to be affected by pH in three brain regions (Vawter et al., 2006). The complete Excel table can be downloaded here: http://pritzkerneuropsych.org/data/archive/File022206.aspx. We considered agonal-pH sensitive genes in a control group analysis only, and found 570 genes that were dysregulated across two or more brain regions (DLPFC, ACC, or CB) meeting a fold-change criteria of ±1.25 and in the top 5% ranked differential gene expression values. This data suggested labile transcripts in postmortem tissue may be used advantageously to indicate degradation and/or an imbalance due to agonal-pH factors. Implementing a protocol to qualitatively assess RNA integrity significantly improved the quality of microarray data (Carter et al., 2005).

The final quality parameter in gene expression profiling is the microarray quality. Affymetrix MAS 5.1 software determines whether each transcript was reliably detected using a percent present call (%PC) and a scaling factor (SF), which adjusted the average signal intensity to a preset constant. The %PC and SF obtained from the microarray results were used as gross indicators of RNA degradation or abundance (Ryan et al., 2004; Lee et al., 2005). When RNA was experimentally degraded the %PC was 40%, and with intact RNA the %PC was 54% (Lee et al., 2005). Thus, chips with lower present calls in a sample set must be treated with caution during analysis if the differences are significant this could be due to true biological differences or quality covariables. Prior reports have shown that differences in mRNA quality produced significant changes in microarray %PC and SF (Ryan et al., 2004; Lee et al., 2005).

Agonal factors, pH, and RNA integrity were each related to the post hoc microarray measure called the average correlation index (ACI), which was a chip quality indicator (Tomita et al., 2004). Another method for examining microarray similarity involved hierarchical clustering of samples and gene expression results (Li et al., 2004; Iwamoto et al., 2005; Mexal et al., 2006). Hierarchical clustering of samples by pH was independently replicated in a set of 105 DLPFC (Iwamoto et al., 2005) and a set of 24 human hippocampal samples (Mexal et al., 2006). Not surprisingly, the composition of groups based upon pH and agonal factor states was recommended as criteria for matching samples in human postmortem studies of single gene and protein expression, for examples see (Hardy et al., 1985; Harrison et al., 1991; Kingsbury et al., 1995; Johnston et al., 1998; Preece and Cairns, 2003).

Postmortem brain tissue is a limited resource and a major effort has been put forth to obtain well-characterized subjects. By evaluating the above quality factors in this paper the results will aid in future study design, analysis, and result interpretation. The present study was undertaken to address four broad quality indicators for evaluating the quality of postmortem samples for gene expression profiling.

2. Methods

2.1. Quality control indicators

Quality control indicators were analyzed for 98 anterior cingulate (ACC) and 91 matched cerebellum (CB) samples with microarray profiling results using U133A and U133P Affymetrix chips. Included in the 98 ACC samples were bipolar disorder ($n = 16$), schizophrenia ($n = 19$), control ($n = 42$), and major depression ($n = 18$) samples. However, diagnostic groupings were not used to assess quality. *Clinical quality* was assessed by agonal risk and agonal duration that yielded agonal factor scores (AFS). *RNA quality* was determined based on the 28S/18S rRNA readings and the RNA integrity number (RIN) both obtained from the Agilent Bioanalyzer. The 3′/5′ glyceraldehyde phosphate dehydrogenase (GAPDH) and β-actin (ACTB) housekeeping gene ratios were from the Affymetrix Microarray Suite 5.1 (MAS 5.1) report. Standard denaturing agarose gels were also run for RNA quality, and the AffyRNAdeg software program to compute RNA degradation (see Section 2.4 below). RNA degradation was evaluated using four quality indicators in two brain regions. *Tissue quality* was assessed by pH measurement, post mortem interval (PMI) and freezer time vari-

ables. *Microarray chip quality* was evaluated using the percent present call, scaling factor, average correlation index (ACI) of the chip (Tomita et al., 2004), and by gene clustering after array processing (Type 1/Type 2) (Li et al., 2004; Iwamoto et al., 2005; Mexal et al., 2006).

2.2. Agonal factor score (AFS)

We calculated the AFS based on data collected for each subject, which included the patient's physical health, medication use, psychopathology, substance abuse and details of death. This information was obtained from the medical examiner's conclusions, coroner's investigation, medical records and family interviews. Agonal risk and agonal duration scores were summed to give the final AFS for each subject as described in a prior study (Tomita et al., 2004).

2.3. pH measurements

Brain pH measurements were taken using a 50–100 mg piece of frozen cerebellar cortical slice. The frozen tissue was mixed with 1.0 mm glass beads (BioSpec Products, Bartlesville, OK) and distilled deionized water to form a 10% (w/v) solution. This solution was homogenized by shaking with a Bead-Beater (Biospec Products) for 60 s at 4 °C. The homogenate was then centrifuged at 5000 rpm for 2 min at 4 °C and then equilibrated to room temperature for 10 s and the pH was measured. The pH meter (Corning, Cypress, CA) was calibrated with three standard buffer solutions (pH 4, 7, and 10). The pH was measured in a second laboratory by the same the technique on the same samples and the results were highly concordant between the laboratories ($r=0.97$, $n=10$). This was also repeated in a third laboratory ($r=0.99$, $n=7$). To use a tissue for a single point calibration of our pH measurement technique, we subjected postmortem non-human primate brain cerebella to the same measurement pH method and found the average pH was 7.24 ± 0.15 ($n=6$). This non-human primate experiment had an absence of agonal factors and had a short PMI, which may explain why a higher average pH was found, compared to postmortem human brain collections.

2.4. RNA quality measurements

(1) Total RNA was extracted from ACC ($n=98$) and CB ($n=91$) and evaluated on Agilent Technologies 2100 BioAnalyzer (Palo Alto, CA) to obtain the 28S/18S ratio.
(2) The RNA integrity number (RIN) was determined using Agilent Technologies 2100 Expert Software. The RIN is a software tool used to aid in the estimation of RNA integrity and to compare RNA integrity across samples (Imbeaud et al., 2005). This tool reads the entire electropherogram rather that just the 28S/18S ribosomal bands. The RIN reflected the presence and absence of degradation products; where higher RNA degradation was assigned a lower RIN value.
(3) A measure of RNA integrity was acquired via the microarray gene chip analysis based on the 3′/5′ ratio of signal intensities of the probe sets for the housekeeping genes glyceraldehyde phosphate dehydrogenase (GAPDH) and β-actin (ACTB) derived from Affymetrix MAS 5.1 signal intensity.
(4) A post hoc microarray measure of RNA degradation was proposed which utilized the R program function AffyRNAdeg (R program, Function to assess RNA degradation in Affymetrix GeneChip data) (Cope, 2005). This measurement was based on the fact that Affymetrix arrays have individual probes tiled in a 3′ to 5′ direction along the transcript, therefore an algorithm was created to measure the decay of transcript abundance, i.e. the signal decline within each gene on the Affymetrix GeneChip. The calculations were executed based on the assumption that primer transcription starts at the 3′ end and therefore, probe intensities should be lower at the 5′ end of a probeset compared to the 3′ end if RNA is degraded. This program calculates the average probe intensity based on location in the probeset and produces a plot of the means for each chip by probe. The slope of this graph was used as a measure of the severity of degradation (Gautier et al., 2004).
(5) Total RNA samples were run on a denaturing agarose gel according to a protocol from Ambion (Austin, TX). The agarose gel was quantitated on the BioRad ChemiDoc System and the 28S/18S ratio was determined. The Agilent 28S/18S readings were compared with the results of the same set of samples run on the agarose gel by a paired *t*-test.

2.5. Microarray chip quality

In the current sample, differential gene expression was determined using GC content robust multi-array average (gcRMA) (Wu et al., 2003). We applied a Unigene 4 custom chip definition file (Dai et al., 2005), which is available at: http://brainarray.mbni.med.umich.edu/Brainarray/Database/CustomCDF/CDF_download_v4.asp. RMA was used to process the cel files for signal intensity and the signal intensity was used for gene clustering (Type 1/Type 2) after array processing (Li et al., 2004; Iwamoto et al., 2005; Mexal et al., 2006). The percent present call, the scaling factor and the average correlation index (ACI) of the chip were determined using MAS 5 generated values (Tomita et al., 2004). Pathway enrichment of differential gene expression was examined by gene set enrichment analysis (GSEA) (Mootha et al., 2003a,b).

The relationship of the quality parameters to the total variance was estimated by two methods. The receiver operator characteristic plots of sensitivity and specificity were used to determine the inter-relationships of the quality covariables in determining microarray outcome. This was determined quantitatively by measuring the area under the ROC curve and comparing different quality variables. However, this provided an assessment related to microarray outcomes, so a direct approach was to look at the total variation accounted for by each quality covariable across all genes in ANCOVA analyses. The *F*-ratios for each covariate was averaged across all probesets on the Affymetrix U133A platform.

3. Results

3.1. Clinical quality

The current sample consisted of anterior cingulate ACC ($n=98$) and matched cerebellum CB ($n=91$) samples, i.e. 91 subjects had data for two brain regions (Table 1). Cases were included in the present data in which both medical records and next-of-kin interviews were obtained (Table 1). The controls were also ascertained with the same method so a similar level of rigor was applied to the controls. We did not assess case–control differences but assessed all samples together to minimize analyses, and to maintain power with a large number of subjects. The subjects were diagnosed as bipolar disorder (16%), major depression (18%), schizophrenia (20%) or controls (46%).

We established two groups of subjects based upon agonal factor scores (AFS = 0 versus AFS > 0) and compared these groups for differences in tissue, RNA, and microarray quality. The three categories of quality indicators (tissue pH, RNA quality, and microarray quality) were significantly different between AFS = 0 and AFS > 0 samples for both brain regions (Fig. 1; Table 2).

An over-representation analysis revealed that the mitochondrial pathway of gene expression was affected by AFS. Analysis of ACC showed that mitochondrial enzymes were significantly over-represented when comparing AFS = 0 and AFS > 0 (Table 3). Agonal duration and pH were significantly correlated ($r=-0.43$, $p<0.0001$). These results ($n=98$) agreed with our previously published data which included 40 subjects (Vawter et al., 2006).

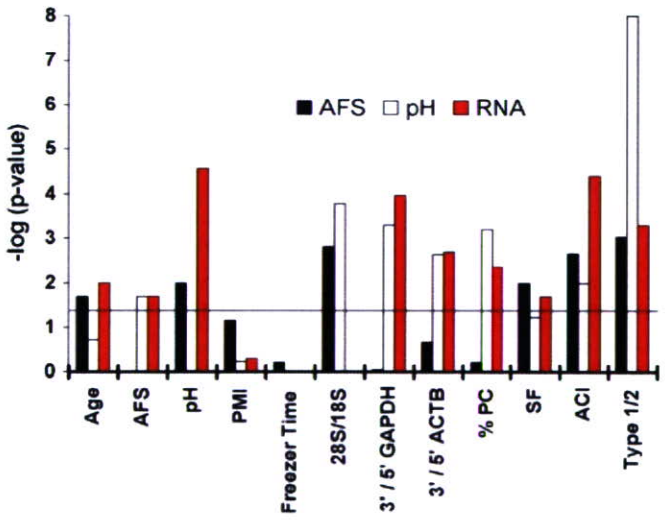

Fig. 1. The significance of comparing groups separated by differences in high vs. low RNA quality, clinical quality, and tissue quality is shown for multiple variables commonly used in controlling gene expression profiling experiments and in particular microarray results (%PC, SF, ACI, and Type 1/2). The data is from Table 2 for the anterior cingulate cortex and shows on the *y*-axis the significance of *t*-test (*p*-value transformed by −log 10) and the *x*-axis shows the individual variables. The abbreviations are same as shown in Table 1. The legend shows three different groups (AFS, pH, and RNA).

Table 1
Summary of four quality control measures for brain samples from anterior cingulate ($n=98$) and cerebellum ($n=91$)

Region	Clinical		Tissue			RNA				Microarray chip			
	Age	AFS 0/AFS > 0[a]	pH	PMI (hours)	Freeze time (years)	28S/18S ratio	3′/5′ GAPDH[b]	3′/5′ ACTB[c]	RIN[d]	% PC[e]	SF[f]	ACI[g]	Type 1/2[h]
Anterior cingulate cortex (ACC) ($N=98$)	51.98 (13.40)	75/23	6.82 (0.24)	24.14 (8.63)	2.96 (3.34)	1.80 (0.37)	1.68 (0.70)	2.93 (1.80)	5.95 (1.35)	43.02 (5.22)	1.42 (1.57)	0.94 (0.06)	71/27
Cerebellum ($N=91$)	51.64 (13.20)	75/16	6.83 (0.24)	23.72 (8.41)	3.15 (3.40)	1.89 (0.46)	1.70 (0.77)	3.55 (3.03)	6.58 (1.66)	43.94 (5.48)	2.57 (4.16)	0.96 (0.05)	64/27

The categories of the four quality control indicators are displayed for each brain region. There were 91 common.
[a] AFS = agonal factor score, number refers to the number of subjects with AFS = 0/AFS > 0.
[b] GAPDH = glyceraldehyde phosphate dehydrogenase.
[c] ACTB = β-actin.
[d] RIN = RNA integrity number.
[e] %PC = percent present call.
[f] SF = scaling factor.
[g] ACI = average correlation index.
[h] Type 1/2 = hierarchical clustering group membership which is defined by cluster membership.