Short Communication J. Trad. Med. 21, 182－186, 2004

DNA array analysis of gene expression changes by Choto-san in the ischemic rat brain

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The effects of Choto-san on gene expression in the dementia model rat brain were studied using a DNA microarray system. Choto-san inhibited the expression of 181 genes that has been enhanced by permanent occlusion of the bilateral common carotid arteries (2VO). Choto-san also reversed the expression inhibition of 32 genes induced by 2VO. These results may suggest that Choto-san, which has been therapeutically used as an antidementive drug, shows therapeutic effects through gene expression changes.

Key words Choto-san, dementia, gene expression, DNA microarray, ischemia, rat brain.

Introduction

Choto-san is a "Wakan-yaku (Oriental medicine)" which has recently been used as a therapeutic drug for vascular dementia. The effect has already been confirmed in a double-blind test.[1)] Experimental protocols using animal models have substantiated the antidementive effects of Choto-san. Choto-san improves the scopolamine- and tetrahydrocannabinoid-induced disruption of spatial cognition of the radial maze in rats,[2)] transient cerebral ischemia-induced impairment of water maze performance in mice,[3)] and passive avoidance performance in senescence-accelerated mice.[4)] The molecular mechanisms of Choto-san has also been reported as follows. The involvement of free radical scavenging activity of Choto-san in the prevention of neuronal cell death in the hippocampal CA1 region after ischemic reperfusion in rats has been shown by electron spin resonance.[5)] This free radical-scavenging effect has been also reported in red blood cells[6)] and in NG108-15 cells.[7)] On the other hand, it has been also reported that Choto-san activates nitric oxide synthase to improve cerebral circulation.[8)]

We have demonstrated that rats with chronic central hypoperfusion induced by permanent occlusion of the bilateral common carotid arteries (2VO) are a useful model for investigating dementia. The 2VO rats have progressive and long-lasting cognitive deficits.[9, 10)] This model animals also show the delayed neuronal cell loss in the CA1 hippocampal region,[11)] white matter lesions[12)] and decreased microtuble-associated protein (MAP-2)/increased glial fibrillary acidic protein (GFAP) immunoreactivity[12)] within 7 days. These changes also appear in vascular dementia[13)] and Alzheimer's disease patients.[14)] Furthermore, we have been reported that the mRNA expressions of Alzheimer's disease related factors, amyloid precursor protein, α7 nicotinic receptor and secretase, are enhanced at 4 days after 2VO.[15)]

In this study, a DNA array system showed genes with a change in expression in the 2VO rat brain. Using the same kind of DNA tip, the expression changes induced by Choto-san were also examined under the 2VO condition. The results may suggest the functional intrinsic factors that are involved in dementia.

Materials and Methods

Animals. Male Wistar rats (Sankyo Labo Service, Hamamatsu, Japan) of the age of 14 weeks were used. The animals were housed under a 12-hour light/dark cycle (light on during 7.30am - 7.30pm) at a room temperature of 24 ± 1℃ with a relative humidity of 55 ± 5 %. Food and water were supplied ad libitum.

Extract preparation. Choto-san water extract was prepared from a mixture of 11 medicinal plants according to the following recipe: Aurantii Nobilis pericarpium (Peel of *Citrus unshiu* Markovich) 3 g, Ophiopogonis tuber (root of *Ophiopogon japonicus* Ker-Gawler) 3 g, Pinelliae tuber (tuber of Pinellia ternata Breitenbach) 3 g, Hoelen (fungus of *Poria cocos* Wolf) 3 g, Uncariae Ramulus et Uncus (hooks and branch of *Uncaria rhynchophylla* Miquel, *Uncaria sinensis* Oliver) 3 g, Ginseng radix (root of *Panax ginseng* C.A. Meyer) 2 g, Saposhnikoviae radix (root and rhizome of *Saposhnikovia divaricata* Schischkin) 2 g, Chrysanthemis flos (flower of *Chrysanthemum morifolium* Ramatulle, *Chrysanthemum indicum* Linne) 2 g, Glycyrrhizae radix (root of *Glycyrrhiza uralensis* Fisher, Glycyrrhiza globa Linne) 1 g, Zingiberis rhizoma (rhizome of *Zingiber officinale* Roscoe) 1 g, and Gypsum Fibrosum ($CaSO_4 \cdot 2H_2O$) 5 g. All the components except Ramulus et Uncus were put together and boiled in 280 ml of water for 60 min. Romulus et Uncus was added after 45 min and the boiling was continued for 15 min. The water extract was filtered and then freeze-dried.

2VO operation. The surgery was performed as described previously.[10)] Rats were anesthetized with pentobarbital sodium (30 mg/kg, i.p.). A ventral midline incision was made to expose the common carotid arteries. The arteries were then carefully separated from the adjacent vessels and

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Table 1 Genes which showed markedly enhanced expression by ischemic treatment and decreased expression by Choto-san treatment under an ischemic condition in rat brains.

1) 2VO (+), Cho (−)

GenBank No.	Gene name
NM_007940	epoxide_hydrolase_2,_cytoplasmic
NM_007998	ferrochelatase
NM_008154	G-protein_coupled_receptor_3
NM_008650	methylmalonyl-Coenzyme_A_mutase
NM_008869	phospholipase_A2,_group_IVA_(cytosolic,_calcium-dependent)
NM_008939	protease,_serine,_12_neurotrypsin,_(motopsin)
NM_009479	uroporphyrinogen_III_synthase
NM_010045	Duffy_blood_group
NM_010387	histocompatibility_2,_class_II,_locus_Mb1
NM_010804	myeloid/lymphoid_or_mixed_lineage-leukemia_translocation_to_10_homolog_(Drosophila)
NM_010918	natural_killer_tumor_recognition_sequence
NM_011396	solute_carrier_family_22_(organic_cation_transporter),_member_5
NM_011481	src-related_kinase_lacking_C-terminal_regulatory_tyrosine_and_N-terminal_myristylation_sites
NM_011606	tetranectin_(plasminogen_binding_protein)
NM_012031	sperm_associated_antigen_1
NM_012043	immunoglobulin_superfamily_containing_leucine-rich_repeat
NM_013538	gene_rich_cluster,_C8_gene
NM_016706	coilin
NM_016710	nucleosome_binding_protein_1
NM_016717	selenocysteine_lyase
NM_016885	endomucin
NM_017473	retinol_dehydrogenase_7
NM_019425	glucosamine-phosphate_N-acetyltransferase_1
NM_019804	UDP-Gal:betaGlcNAc_beta_1,4-galactosyltransferase,_polypeptide_4
NM_020035	phosphatidylinositol_glycan,_class_O
NM_020610	RIKEN_cDNA_A330103B05_gene
NM_020614	TATA_box_binding_protein_(Tbp)-associated_factor,_RNA_polymerase_I,_B
NM_021292	Ellis_van_Creveld_gene_homolog_(human)
NM_021334	integrin_alpha_X
NM_021346	zinc_finger_protein_318
NM_021520	solute_carrier_family_28_(sodium-coupled_nucleoside_transporter),_member_2
NM_025898	N-ethylmaleimide_sensitive_fusion_protein_attachment_protein_alpha
NM_025928	RIKEN_cDNA_2600009M07_gene
NM_026064	RIKEN_cDNA_2900073G15_gene
NM_026473	RIKEN_cDNA_2310057H16_gene
NM_026504	DNA_segment,_Chr_5,_ERATO_Doi_33,_expressed
NM_030566	RIKEN_cDNA_2610011A08_gene
NM_054046	differentially_expressed_in_FDCP_8
NM_054089	nuclear_receptor_coactivator_6_interacting_protein

2) 2VO (+), Cho ↓

GenBank No.	Gene name	Cho/2VO ratio
NM_007672	cerebellar_degeneration-related_2	0.45
NM_007700	conserved_helix-loop-helix_ubiquitous_kinase	0.40
NM_008359	interleukin_17_receptor	0.48
NM_008486	alanyl_(membrane)_aminopeptidase	0.34
NM_008929	DnaJ_(Hsp40)_homolog,_subfamily_C,_member_3	0.48
NM_009026	RAS,_dexamethasone-induced_1	0.46
NM_009029	retinoblastoma_1	0.35
NM_009282	stromal_antigen_1	0.44
NM_009502	vinculin	0.45
NM_009621	a_disintegrin-like_and_metalloprotease_(reprolysin_type)_with_thrombospondin_type_1_motif,_1	0.41
NM_009775	benzodiazepine_receptor,_peripheral	0.35
NM_012025	Rac_GTPase-activating_protein_1	0.39
NM_016692	inner_centromere_protein	0.28
NM_017383	contactin_6	0.40
NM_019949	ubiquitin-conjugating_enzyme_8	0.35
NM_022000	GNAS_(guanine_nucleotide_binding_protein,_alpha_stimulating)_complex_locus	0.38
NM_023118	disabled_homolog_2_(Drosophila)	0.28
NM_024469	basic_helix-loop-helix_domain_containing,_class_B3	0.43
NM_026565	RIKEN_cDNA_9430083G14_gene	0.25
NM_028152	MMS19_(MET18_S._cerevisiae)-like	0.42
NM_033134	inositol_polyphosphate-5-phosphatase,_72_kDa	0.43
NM_054071	fibroblast_growth_factor_receptor-like_1	0.49

3) 2VO ↑, Cho ↓

GenBank No.	Gene name	2VO/Sham ratio	Cho/2VO ratio
NM_007778	colony_stimulating_factor_1_(macrophage)	2.56	0.47
NM_010919	NK2_transcription_factor_related,_locus_2_(Drosophila)	2.94	0.27
NM_011599	transducin-like_enhancer_of_split_1,_homolog_of_Drosophila_E(spl)	2.94	0.37
NM_016777	nuclear_autoantigenic_sperm_protein_(histone-binding)	2.86	0.39
NM_017373	nuclear_factor,_interleukin_3,_regulated	3.70	0.39
NM_021463	phosphoribosyl_pyrophosphate_synthetase_1	2.17	0.40
NM_024438	dual_specificity_phosphatase_19	2.80	0.42
NM_031156	insulin_degrading_enzyme	3.33	0.33

4) 2VO ↑, Cho (−)

GenBank No.	Gene name	2VO/Sham ratio
NM_022964	Williams-Beuren_syndrome_chromosome_region_5_homolog_(human)	2.00
NM_026086	RIKEN_cDNA_1600031M04_gene	2.00

This table only shows the 71 markedly changing genes out of 181 candidates which may be involved in the effects of Choto-san on dementia. "2VO (+), Cho(-)" means expression could be detected in the 2VO brain but not in the sham-operated or Choto-san-treated 2VO brain. "2VO ↑, Cho ↓" means expression could be detected in all conditions, but was increased by 2VO and decreased by Choto-san treatment. Other symbols use the same logic. "2VO/Sham ratio" was calculated from the density of fluorescence between 2VO- and sham-operated brain samples. This table only lists the factors which showed over 2.00 ratio value of 2VO/sham and under 0.50 of Choto-san/2VO. Detailed information of this experiment is available from the following net site: http://www.toyama-mpu.ac.jp/riw/shiken/exp-chotosan.html.

Table 2 Genes which showed inhibition expression by ischemic treatment and recovered the expression by Choto-san treatment under an ischemic condition in rat brains.

1) 2VO (−), Cho (+)

GenBank No.	Gene name
NM_008713	nitric_oxide_synthase_3,_endothelial_cell
NM_008803	phosphodiesterase_8A
NM_009256	serine_protease_inhibitor_6
NM_009377	tyrosine_hydroxylase
NM_009612	activin_A_receptor,_type_II-like_1
NM_010600	potassium_voltage-gated_channel,_subfamily_H_(eag-related),_member_1
NM_010718	LIM_motif-containing_protein_kinase_2
NM_013606	myxovirus_(influenza_virus)_resistance_2
NM_015765	heat_shock_protein,_70_kDa_4
NM_016670	Pbx/knotted_1_homeobox
NM_019423	elongation_of_very_long_chain_fatty_acids_(FEN1/Elo2,_SUR4/Elo3,_yeast)-like_2
NM_019635	serine/threonine_kinase_3_(Ste20,_yeast_homolog)
NM_020491	Sjogren's_syndrome/scleroderma_autoantigen_1_homolog_(human)
NM_021487	potassium_voltage-gated_channel,_Isk-related_family,_member_1-like
NM_024258	ubiquitin_specific_protease_16
NM_024283	RIKEN_cDNA_1500015O10_gene
NM_024472	cDNA_sequence_BC002216
NM_024477	cDNA_sequence_BC002262
NM_025409	RIKEN_cDNA_1110057H19_gene
NM_025560	RIKEN_cDNA_1810049H13_gene
NM_026534	RIKEN_cDNA_3110003A22_gene
NM_030719	opposite_strand_transcription_unit_to_Stag3
NM_032398	plasmalemma_vesicle_associated_protein
NM_033526	ataxin-1_ubiquitin-like_interacting_protein
NM_053123	SWI/SNF_related,_matrix_associated,_actin_dependent_regulator_of_chromatin,_subfamily_a,_member_1
NM_053130	protocadherin_beta_5
NM_053155	calmin
NM_080440	solute_carrier_family_8_(sodium/calcium_exchanger),_member_3
NM_130454	RecQ_protein-like_5
NM_133746	RIKEN_cDNA_2810048G17_gene
NM_133823	RIKEN_cDNA_2810018E08_gene

2) 2VO ↓, Cho ↑

GenBank No.	Gene name	2VO/Sham ratio	Cho/2VO ratio
NM_009234	SRY-box_containing_gene_11	0.58	1.87

"2VO (-), Cho(+)" means expression could be detected in the sham-operated and Choto-san-treated 2VO brain but not in the 2VO brain. "2VO ↓, Cho ↑" means expression could be detected in all conditions, but was decreased by 2VO and recovered by Choto-san treatment. Detailed information of this experiment is available from the following net site: http://www.toyama-mpu.ac.jp/riw/shiken/exp-chotosan.html.

nerves. Silk suture (No. 1, Natsume Co. Ltd., Tokyo, Japan) was employed to occlude the arteries. Sham-operated controls received the same operation but without occlusion of the arteries.

Drug administration. There was no drug or vehicle administration during the first 24 hours after the operation. The sham-operated rats were given water and the 2VO rats were given water or 1 g/kg Choto-san once a day at around 4:00 pm for three consecutive days. The animals were decapitated on the fourth day, 18 hrs after the final drug administration. The whole brain was removed and frozen in liquid nitrogen and then kept at -80℃ for further RNA isolation.

RNA isolation and DNA array analysis. Total RNA isolation was performed using Isogen^{R} (Nippon Gene, Toyama, Japan), which is a modification of the methods described by Chomczynski and Sacchi.[16] The RNA was precipitated with isopropanol. The precipitate was resuspended in DEPC-water. The RNA concentration was spectrophotometrically measured at 260 nm. Five of the 20 μg individual brain samples were mixed to make a 2.5 μg/μl solution. The final three samples of the sham-operated, water-administered 2VO and Choto-san administered 2VO rats, were used to certify the total RNA quality by an Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA). The concentrations were 2.91, 2.41 and 2.30 μg/μl, respectively. The RNA of the 2VO sample was labeled by Cy3. The sham-operated and Choto-san-treated samples were labeled by Cy5. Then, the samples were hybridized with an IntelliGene II mouse chip (Takara, Ohtsu, Japan) at 65℃ for 14 hrs to analyze the expression levels by DNA microarray methods.

Results and Discussion

The expression levels of RNAs were assayed by a DNA microarray system using an IntelliGene II mouse chip, which spotted 4,277 genes. No differences could be seen in housekeeping gene expression among the three samples. The 2VO treatment enhanced the expression of 286 (6.7 %) genes and decreased that of 199 (4.7 %) genes. The numbers of genes which changed expression under the 2VO condition by Choto-san were 112 (2.6 %) which increased and 496 (11.6 %) which decreased. Interestingly, among the 286 genes that showed expression stimulation by 2VO, 63 % (181 genes) were inhibited by Choto-san. The genes which showed a remarkable difference are listed in Table 1. On the other hand, among 199 genes which show the expression inhibition by 2VO, 32 genes (16 %) were reversed the expression by Choto-san. All of the array data are available on the web site, http://www.toyama-mpu.ac.jp/riw/shiken/exp-chotosan.html.

Recently, there have been many reports on gene expression changes using the DNA microarray system. The most interesting point of this report is that the gene expression changes induced by Choto-san, a kind of Wakan-yaku, were detected under a pathogenetic condition in permanent ischemic brains. The results indicated that the expression of the 181 genes was enhanced by 2VO and reversed by Choto-san, as a pharmacological effect. These genes should be candidates for factors having intrinsic roles in the generation of dementia. In fact, one of the identified factors, the Williams-Beuren syndrome-related gene (NM 022964), has been reported to be involved in slight dementia.[17] Although further studies are necessary to clarify the molecular pharmacological roles and mechanisms of Choto-san in dementia, this report may also suggest that Choto-san, which has been therapeutically used as an antidementive drug, shows therapeutic effects through gene expression changes.

Acknowledgment

This work was supported in part by a Grant-in-Aid for Exploratory Research to Dr. M. Tohda (#15659157) from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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Japanese abstract

痴呆モデル動物である両側総頸動脈永久結紮ラット脳中での釣藤散による遺伝子発現変化をDNAマイクロアレイ法により検討した。被検4,277種の遺伝子中181種類の発現が脳虚血4日後の脳中で上昇し，釣藤散1g/kg1日1回3日間経口投与により回復することが明らかとなった。また，32種類の発現が，脳虚血により減少し，釣藤散で回復することもわかった。この結果より，釣藤散は関連遺伝子の発現変化を介して抗痴呆効果をあらわしている可能性が考えられる。

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