Effect of the Chinese Traditional Medicine “Bushen Yinao Pian” on the Cerebral Gene Expression of the Senescence-Accelerated Mouse Prone 8/Ta

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Abstract: The effect of Chinese traditional medicine “Bushen Yinao Pian,” a complex prescription used for anti-aging, on the cerebral gene expression of the senescence-accelerated mouse prone 8/Ta (SAMP8/Ta) had been studied with messenger ribonuclear acids reverse transcription differential display polymerase chain reaction (mRNA DDRT-PCR). Eight differential displayed bands had been discerned and sequenced. The sequences of those fragments are matched to adipocyte-specific protein-5; low density lipoprotein receptor-related protein associated protein-1; reticulon-3; cysteine and histidine-rich domain (CHORD)-containing, zinc-binding protein-1; cytochrome c oxidase subunit-2 (Cox-2); cytochrome c gene, MC1; DNA sequence from clone RP23-72M11 on chromosome X, respectively and a novel sequence fragment. Most of these genes are aging-related. It can be proved that the “Bushen Yinao Pian” truly has anti-aging function.

Keywords: Chinese Traditional Medicine; Anti-Aging; Senescence-Accelerated Prone Mouse; DDRT-PCR.

Introduction

Aging is a progressive physiological change in an organism leading to senescence, or a decline of both biological functions and of the organism’s ability to adapt to metabolic stress. Three mechanisms of aging are involved: (1) gene damage accumulations in the organism, (2) the programmed senescence controlled by biological clock genes, and...
(3) gradual erosion of chromosome ends causes the state change of its telomeres. On the molecular level, all three mechanisms are related to gene expression changes or gene mutations of the organisms (Karlseder et al., 2002; Kirkwood and Austad, 2000; Jazwinski, 1996).

The senescence-accelerated mice (SAM) was developed by the Chest Disease Research Institute, Kyoto University, Japan, in 1970. Among them, the life-span of the accelerated senescence-prone strain (SAMP) is shorter by 27% than the accelerated senescence-resistant strain (SAMR), which is used as the control. SAM is the only mammalian animal model used in the study of the mechanism of senescence acceleration (Zhang et al., 2002). At present, using the SAM mice for molecular mechanism is one of the hot topics in aging research.

Many Chinese traditional medicines have anti-aging effects. The “Bushen Yinao Pian” (a complex prescription), which is produced according to the Chinese traditional theory, had been proven to be effective in clinic for over 20 years. But its molecular mechanism is still elusive.

In this study, messenger ribonucleic acids reverse transcription differential display polymerase chain reaction (mRNA DDRT-PCR) was used to analyze the mRNA profile changes in the cerebral tissues of the SAMP8/Ta between the “Bushen Yinao Pian” given group and the control group. The aim of this study is to investigate whether the medicine has an anti-aging effect on the gene expression levels in SAMP8/Ta.

Material and Methods

Medicine

The Chinese traditional medicine “Bushen Yinao Pian” (a complex prescription used for anti-aging in China) was provided by Lingtai Bichen Medical Technology Co. Ltd. (Mudangjiang City, China), which is composed of: Ginseng root (Radix Ginseng), Pilose Anter (Cornu cervi Pantotrichum), Tuckahoe (Poria), rhizome of Common Yam (Rhizoma Dioscoreae), prepared rhizome of Adhesive Rehmannia (Radix Rehamanniae preparatum), root of Chinese Angelica (Radix Angelicae sinensis), rhizome of Chuanxiong Ligusticum (Rhizoma Ligustici chuanxiong), fruit of Malaytea scurfpea (Fructus Psoraleae), root of Common Epipremnum (Radix seu Caulis Epipremni pinnati), fruit of Hairystamen Wolfberry (Fructus Lycii), root of Ningpo Figwort (Radix Scrophulariae), root of Creeping Lrioipe (Radix Ophiopogonis seu Liriopis), fruit of Chinese Magnoliavine (Fructus Schisandraceae), seed of Spine Date (Semen Ziziphi spinosae), and Cinnabar (Cinnabaris).

Animal Breeding

Accelerated senescence-prone mice strain 8/Ta (SAMP8/Ta) were provided by Kyoto University, Japan, and housed in the experimental animal center of our institute in a clean facility on a 12-hour light/dark cycle. Two-month-old mice were grouped randomly into two groups (the numbers of male and female are equal in each group). Each group
contained 30 mice. The control group was given a standard commercial pellet diet (Feed-Processing Plant of the Experimental Animal Center, the Academy of Military Medicine Sciences, Beijing, China). The test group was given the medicine appended pellet diet, 1% “Bushen Yinao Pian” was added to the standard commercial pellet diet (Feed-Processing Plant of the Experimental Animal Center, the Academy of Military Medicine Sciences, Beijing, China). Both groups were given tap water *ad libitum*.

**Total RNA Isolation**

After a three-month feeding period, the mice were decapitated, and the cerebral tissues were obtained. Total RNA was isolated form cerebral tissue (30 mg/mouse) according to the RNeasy mini Kit (Qiagen Inc. Valencia.) protocol, treated with RQ1 RNase-Free DNase (Promga Co., Madison), and stored at −20°C before using.

**DDRT-PCR**

To detect gene expression changes between the test and the control groups, samples of the same gender mice were compared according to the modified protocol of Liang and Pardee (1998) as described (Zhang et al., 2004). In each PCR tube (Axygen Scientific Inc., Union City), 2 µl of total RNA (1 µg/mouse), 2 µl of 10x RT buffer, 1.6 µl of 2.5 mM dNTPs (Promega), 2 µl one of three DDRT 3′ primers (SBS, Beijing, China). The sequences are AAGCTTTTTTTTTTTTA, AAGCTTTTTTTTTTTTC, and AAGCTTTTTTTTTTTTG, respectively), 0.5 µl of RNasin ribonuclease inhibitor (40 iu/µl, Promega), and double distilled water were added to 19 µl. It was incubated at 65°C for 5 minutes, 42°C for 10 minutes, and then 1 µl of M-MuLV reverse transcriptase (200 iu/µl) (New England Biolabs Inc., Hertfordshire, England, UK) was added and continued to incubate at 37°C for 50 minutes and 75°C for 5 minutes on Thermocycler (Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany).

The RT products was amplified by PCR system as follows: 2 µl of RT product, 2 µl of 10X Taq DNA polymerase buffer, 2 µl of 25 mM MgCl₂, 1.6 µl of 2.5 mM dNTPs, 2 µl one of the 5′ primer (2 µM) (SBS, the sequences are ACAGAGCACA, CACAGTTTGC, CCACAGAGTA, GGAACTCCGT, GGCAAGTCAC, and AGGACCGCTA), 2 µl of 3′ primer (2 µM) (SBS), 0.4 µl of Taq DNA polymerase (5 iu/µl, Promega), 0.5 µl of α-32p-dCTP (10 μCi/µl) (Yahuei Co. Ltd., Beijing, China), and added water to 20 µl. The parameters of PCR are as follows: 94°C for 10 minutes; 94°C for 1 minute, 40°C for 2 minutes, 72°C for 1 minute, 40 cycles; 72°C for 5 minutes on thermocycler.

To eliminate individual difference, two products from different individuals of the test group (3 and 4 in Fig. 1) and two products from different individuals of the control group (1 and 2 in Fig. 1) were loaded side by side onto the 6% urea denature polyacrylamide gel. After electrophoresis, the gel was exposed to an X-ray film (Fuji Photo Film Co., Ltd. Tokyo, Japan) for 72 hours at −20°C.
Recovery and Re-amplification of cDNA Fragment

The differential displayed bands were cut out from the gel, soaked in 100 µl double distilled water for 10 minutes at room temperature and then boiled for 15 minutes. After the centrifugation, 10 µl of 3M sodium acetate (pH 5.2), 2.5 µl of glycogen (20 mg/ml) (Sigma-Aldrich Fine Chemicals, St. Louis, Missouri), 450 µl of ethanol were added to the supernatant and stored at −20°C overnight. The DNA was recovered by centrifugation.

The recovered product was re-amplified as follows: 4 µl of recovered product, 4 µl of 10x Taq DNA polymerase buffer, 4 µl of 25 mM MgCl₂, 3.2 µl of 2.5 mM dNTPs, 4 µl of 5′ primer (2 µM), 4 µl of 3′ primer (2 µM), 0.8 µl of Taq DNA polymerase (5 iu/µl), and added double distilled water to 40 µl. The PCR parameters of re-amplification are 94°C for 10 minutes; 94°C for 1 minute, 40°C for 2 minutes, 72°C for 1 minute, 20 cycles; 72°C for 5 minutes; 94°C for 10 minutes; 94°C for 1 minute, 40°C for 2 minutes, 72°C for 1 minute, 20 cycles; 72°C for 5 minutes. A part of products was verified by using 1.2% agarose (Biowest, Distributed by Shanghai Yito enterprise Co. Ltd., Shanghai, China) gel electrophoresis.

Subcloning of Re-amplified cDNA Fragments and Sequence Analysis

The remaining part of re-amplified products was purified with Wizard® PCR Preps DNA Purification System (Promega) according to protocol, and ligated into pGEM-T Easy Vector (Promega) according to the manual as well. The ligated products were transformed into E. coli DH.5α competent cells (Tianwei Time Co. Ltd., Beijing, China) and cultured on LB/amp/IPTG/X-Gal plates to obtain positive clones of aim fragment. The plasmid DNA was purified with UltraPure plasmid DNA mini purification kit (SBS) according to the manual, and the insert was confirmed by PCR with the same primers and parameters,
and electrophoresis on 1.2% agarose gel. The subcloned fragments were sequenced in Bioasia Technology Co. Ltd. (Shanghai, China). The sequences were compared with the National Center of Biotechnology Information (NCBI) non-redundant sequence database using the BLASTX and BLASTN programs.

**Virtual Northern Blot Analysis**

The cDNA was verified with virtual northern blot according to the method of Hung et al. (1999). Briefly, the total RNA was reverse-transcribed with powerscript reverse transcriptase (BD Biosciences Clontech Inc.), and amplified with Advantage 2 PCR kit (BD Biosciences Clontech Inc.) according to the protocol. After electrophoresis on 1.2% agarose, amplified cDNA was transferred to a nitrocellulose membrane (Schleicher & Schuell Inc., Keene), and hybrid with $^{32}$P-labeled fragment, labeled with random primer DNA labeling kit (TaKaRa Biotechnology Co. Ltd., Dalian, China).

**Results**

In this study, we applied mRNA differential display reverse transcription PCR (DDRT-PCR) to analyze the profiles of the gene expression in the cerebral tissues of the mice. Six arbitrary 5′ primers and three anchor 3′ primers were used to compare between the “Bushen Yinao Pian” group and the control group. Part of DDRT-PCR results were shown in Fig. 1.

### Table 1. Features of Sequenced Clone and Results of BLAST Search

<table>
<thead>
<tr>
<th>No.</th>
<th>Size (bp)</th>
<th>DNA Homology (BLAST)</th>
<th>Accession No.</th>
<th>Identity (%)</th>
<th>Overlap Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>p7</td>
<td>190</td>
<td>Adipocyte-specific protein-5</td>
<td>AK080481.1</td>
<td>100%</td>
<td>172/172</td>
</tr>
<tr>
<td>p13</td>
<td>144</td>
<td>Mus musculus low density lipoprotein receptor-related protein associated protein-1</td>
<td>BC059887.1</td>
<td>100%</td>
<td>125/125</td>
</tr>
<tr>
<td>p25</td>
<td>265</td>
<td>Reticulon-3</td>
<td>AK088670.1</td>
<td>99%</td>
<td>153/154</td>
</tr>
<tr>
<td>p39</td>
<td>539</td>
<td>Mus musculus cysteine and histidine-rich domain (CHORD)-containing, zinc-binding protein-1, mRNA</td>
<td>BC018374.1</td>
<td>99%</td>
<td>520/522</td>
</tr>
<tr>
<td>p40</td>
<td>415</td>
<td>Mus musculus cytochrome c oxidase subunit-2 (Cox-2) mRNA</td>
<td>AF378830.1</td>
<td>99%</td>
<td>392/393</td>
</tr>
<tr>
<td>p41</td>
<td>210</td>
<td>Novel</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p51</td>
<td>329</td>
<td>Mouse DNA sequence from clone RP23-72M11 on chromosome X</td>
<td>AL731705.11</td>
<td>99%</td>
<td>308/309</td>
</tr>
<tr>
<td>p52</td>
<td>314</td>
<td>Mouse cytochrome c gene (MC1)</td>
<td>X01756.1</td>
<td>99%</td>
<td>297/299</td>
</tr>
</tbody>
</table>

Notes for Table 1: The backgrounds of fragments in this table are as follows: The fragments p13, p39, p40, p52 come from the test group and p25, p51 from the control group of the male mice; the fragments p41 comes from the test group and p7 from the control group of the female mice.
The differential displayed fragments were re-amplified, ligated, and sequenced. In this study, 29 bands were found distinct differential displayed (DD) on the film, and 23 bands were recovered. Among them, eight bands were confirmed to be positive. The homologies of the sequences were searched in NCBI-nr by using the BLASTN program (Table 1). Virtual northern blotting (Hung et al., 1999) was used to further confirm the result of DDRT-PCR. In these eight bands, the expression of reticulon-3 decreased only in the male mice of the test group (Figs. 2 and 3) and the expression of another sequence, which is homology with mouse DNA sequence from clone RP23-72M11 on chromosome X, decreased in both genders of the test group. The expressions of low density lipoprotein receptor-related protein associated protein-1 (Fig. 5), cytochrome c (MC1, Fig. 2) and a novel fragment, increased in both genders of the test group, and the other two expressions, the cysteine and histidine-rich domain (CHORD)-containing, zinc-binding protein-1 and cytochrome c oxidase subunit-2 (Cox-2), increased only in the male mice of the test group. The expression of adipocyte-specific protein-5 decreased only in the female mice of the test group (Fig. 4).

**Discussion**

Aging is a common biological phenomenon in almost all organisms. In China, using TCM for anti-aging has a long history, and has accumulated an abundance of data in the clinic.

![Figure 2](image-url)
Figure 3. Virtual northern blot results of the gene expression decreased in both genders of the test group. (a) The gel electrophoresis results of cDNA amplification with advantage 2 PCR kit. (b) The virtual northern blot results with the fragment of reticulon-3. M, DL2000 molecular marker; T1, T2, T3, T4, samples from individual male test group; C1, C2, C3, C4, samples from individual male control group mouse, respectively.

Figure 4. Virtual northern blot result of the gene expression decreased in one gender of the test group. (a) The gel electrophoresis results of cDNA amplification with advantage 2 PCR kit. (b) The virtual northern blot results with the fragment of adipocyte-specific protein-5. M, DL2000 molecular marker; MC, sample from male control group; MT sample from male test group; FC, sample from female control group mouse; FT, sample from test group female mouse.
In the “herbal medicine of the agricultural God’s scripture” (the first pharmacological book in China), 85 herbal medicines had been described to have anti-aging functions. The “Bushen Yinao Pian,” made of 15 different Chinese medical herbs, has been proven to be effective in the clinic for anti-aging.

In this investigation, by using the DDRT-PCR method to compare the gene expression profiles between medicine given group and control group, eight positive DD bands had been obtained. Their sequences were analyzed for homology using the BLASTN program in NCBI-nr. The results showed that seven of them have sequence homology to adipocyte-specific protein-5, low density lipoprotein receptor-related protein associated protein-1, reticulon-3, cysteine and histidine-rich domain (CHORD)-containing zinc-binding protein-1, cytochrome c oxidase subunit-2 (Cox-2), cytochrome c gene (MC1), DNA sequence from clone PR23-72M11 on mouse chromosome X, respectively, and one of them is a novel sequence.

The adipocyte-specific protein signals the size of the adipose tissue mass to hypothalamic regions, thereby influencing energy metabolism (Oberkofler et al., 1997). Adipocyte-specific protein-5 is a lipid binding protein (McGowan et al., 1997), its functions are supposed to be involved in fatty acid (FA) uptake, transport, and targeting. They may modulate FA concentration and influence the function of enzymes, membranes, ion channels, receptors, gene expression and cellular growth and differentiation (Veerkamp...
and Zimmerman, 2001). Ruan et al. (2002) found a global reduction of gene expression in adipocyte with its insulin responsiveness decrease. Aging is associated with a decrease in glucose tolerance. And the glucose tolerance has a reverse-relation with the insulin responsiveness (Craig et al., 1989). The results of this investigation showed that “Bushen Yinao Pian” decrease adipocyte-specific protein-5 mRNA transcripts in the cerebral tissues of the SAMP8/Ta, which may indicate that the adipose tissue mass decrease in the cerebral tissue and play an anti-aging function.

Low density lipoprotein receptor-related protein associated protein-1 (LRPAP-1) is a member of the LDL receptor family, which mediates endocytosis and signal transduction of many extracellular ligands, and participates in lipoprotein metabolism, protease regulation, embryonic development and the pathogenesis of disease (e.g. Alzheimer’s disease). It universally inhibits ligand interaction with these receptors. Under physiological conditions, LRPAP-1 serves as a molecular chaperone/escort protein for these receptors to prevent premature interaction of ligands with the receptors and thereby ensures their safe passage through the secretory pathway (Bu, 2001). In Sprague-Dawley rat, Field and Gibbons (2000) found that the old rats (age 15 months) expressed 43% ± 7% of the level of LDL receptor and 45% ± 16% of the corresponding level of LRP compared to the young animals (age 2 months). This investigation showed that the LRPAP-1 expression increased in the mouse cerebral tissues of the medicine given group. It indicated that this medicine affects the physiological functions by enhancing LRPAP-1 expression to facilitate LDL receptor-related protein maturity.

Neuroendocrine-specific protein (NSP) reticulons (RTN) are endoplasmic reticulum-associated protein complexes, which are localized in the endoplasmic reticulum (ER) and identified as markers for neuroendocrine differentiation (Huang et al., 2004). Reticulon-3 is thought to be an inhibitor for neurite outgrowth, restricting the regenerative capabilities of the mammalian CNS after injury (Oertle et al., 2003), and is a member of a novel p53-independent pathway of apoptosis regulated by Golgi/endoplasmic reticulum protein interactions (Di Sano et al., 2003). It is reported that programmed cell death (PCD) increases with age (Itzhaki et al., 2003). In this investigation, we found that this medicine decreases reticulon-3 expression. The medicine may contribute to anti-aging by decreasing reticulon-3 expression, which inhibits the apoptosis of neurons and alleviates the injury of nerve caused by neurodegeneration in the aging process.

Zinc-binding protein-1 is a plasma membrane transporter, which transports intracellular Zn$^{2+}$ out of cells. It is potentially present in all neurons; is one of three pathways of zinc exit or entry in neurons; and is important for cellular zinc homeostasis (Colvin et al., 2000). Tsuda et al. (1997) reported that zinc-binding protein expression became detectable exclusively in pyramidal neurons of the CA1 region after ischemia, and it is thought to be related to the fate of the neurons after transient ischemia. In this investigation, we found that the cysteine and histidine-rich domain (CHORD)-containing zinc-binding protein-1 expression is higher in medicine-given mouse cerebral tissues than in control mouse cerebral tissues. It indicated that this medicine could enhance the tolerance of neurons to ischemia/hypoxia of the cerebrum in the aging process.
The decline of mitochondrial oxidative phosphorylation causes the decrease of maximum ATP production ability, and is one of the driving forces of the aging process itself (Gadaleta et al., 1998). Cytochrome C oxidase subunit-2 (Cox-2) and cytochrome C (MC1) are two important members of the mitochondrial electron transport chain, which are the vital locations in the energy generation of cells. The free radical theory (Harman, 1956) proposed that the degeneration in aging process is mainly related to the damage caused by free radicals. The production of superoxide radicals and hydrogen peroxide by NADH-ubiquinone reductase and ubiquinol-cytochrome C reductase in the mitochondrial electron transport chain plays an important role in neurodegeneration (Cadenas et al., 1977). In this investigation, the expression level of Cox-2 and MC1 is higher in the cerebral tissues of medicine-given mice than that of the control. It indicated that the medicine could promote energy metabolism of the cerebral cells, and decrease electron accumulation in reductive coenzyme Q and the production of free radical to ameliorate the aging procession.

The sex difference is an important factor that affects animal life-span. It affects not only on the structural and physiological levels but also on the molecular level. For example, the expression level of superoxide dismutase (SOD) in the motor neurons of flies is genotype- and sex-specific (Spencer et al., 2003). In mice, adiponectin levels increase during sexual maturation by four-fold in males and ten-fold in females (Combs et al., 2003). In the pituitary gland of mouse brain, consisting of both endocrine and neural elements, EGF and TGF-α mRNA levels were significantly higher in males than in females (Lazar and Blum, 1992). In this investigation, the gene of adipocyte-specific protein-5 also expressed differently with genders, of which expression level is lower in male mice than in female mice. It was suggested that there are some differences in the aging mechanism between the genders, and this is probably the reason why TCM has different effects on gene expression in different genders.

In this study, DDRT-PCR was used to analyze the effect of the TCM “Bushen Yinao Pian” on the mRNA profiles in the cerebral tissues of SAMP8/Ta. Those affected genes are involved in many life processes, which include material transport, energy metabolism, etc. The data of this study demonstrated that TCM has anti-aging functions through regulating the level of related gene expression.

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