



The Chinese traditional medicine ‘bushen yinao pian’ increased ageing-related gene *lrpap-1* expression in the cerebral tissues of accelerated senescence-prone mouse 8 (SAMP8/Ta)*

Chong Zhang^{1,2}, Guisheng Liu¹, Jinyan Cheng¹, Jingang Wang³ & Qingxuan Chen^{1**}

1. The Institute of Genetics and Developmental Biology, Chinese Academy of sciences, Beijing, 100080.

2. The Graduated School of Chinese Academy of Sciences, Beijing, 100039.

3. Lingtai Bichen Medical Technology Co. Ltd. Beijing, 100086.

Abstract

The molecular mechanism of the Chinese traditional medicine ‘bushen yinao pian’ (a complex prescription used for clinical anti-ageing in China over twenty years) are shown here by analyzing the gene expression in cerebral tissue between test and control patients of the accelerated senescence-prone strain mouse SAMP8/Ta. The result shows that this complex prescription increased expression the low-density lipoprotein related-receptor associated protein-1 (LRPAP-1), an ageing-related gene, which functions as a chaperon or escort protein in the intracellular transport of low-density lipoprotein related-receptor, a transporter of amyloid beta protein (A β P). It indicated that the Chinese traditional medicine ‘bushen yinao pian’ attribute to anti-ageing role by increasing *LRPAP-1* expression.

Aging is a progressive physiological changes in an organism that lead to senescence, or a decline of biological functions and of the organism’s ability to adapt to metabolic stress. Accelerated senescence-prone strain mice (SAMP8/Ta) are used as a murine model in ageing research, which have short life span and ageing-related deficits in learning and cognitive abilities, emotional disorder, abnormal circadian rhythms and impaired immune response¹. In China, the traditional medicine ‘bushen yinao pian’ (a complex prescription used for anti-ageing) had been used over twenty years in clinic. Most of its component had been proved to be anti-ageing^{2,3}. But the mechanism of its anti-aging is still unclear.

To peer this, we applied mRNA differential display reverse translation PCR (DDRT-PCR) to analyze the effect of the ‘bushen yinao pian’ on the gene expression changes in the cerebral tissue of accelerated senescence-prone (SAMP8/Ta) mice (a strain that develops early senescence). Six arbitrary 5’primers and three anchor 3’primers were used in this study to compare the test male individuals with the same litter control male individuals. Part of DDRT-PCR results refers to figure as (Fig. 1). Only six distinctive bands displayed to be different between the test and control patients. One of the fragments is a part of the low-density lipoprotein receptor-related protein associated protein-1 (LRPAP-1) gene, which is an ageing-related gene we interested in this article, increased expression in the test individuals.

*This work was supported by the State Basic Development Program (Grant No.2000016107), and Lingtai Bichen Medical Technology Co. Ltd.

** Corresponding author: Tel.+86-10-62553160; Fax: +86-10-62551951; E-mail. qingxuanchen@yahoo.com

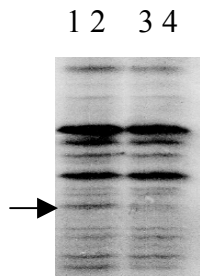


Fig.1: The DDRT-PCR result.

1,2 from test patients.

3,4 from control patients.

The row shows the differential display band.

After screened the cDNA library with 3' Irapap-1 fragment as a probe, the full-length Irapap-1 cDNA sequence was cloned, which contains 1452bp and encoded 360 amino acids. It refers to figures as (Fig. 2).

```

      M A P   R R E   R V S   T L P R   L Q L   L V L   L L L P
GCTTCAGAGG AAGATGGCGC CTCGAAGAGA GAGGGTCTCT ACGCTGCCCC GGCTCCAAC TGTAGTGCTG TTGTTGCTGC
  L M L   V P Q   P I A G   H G G   K Y S   R E K N   E P E   M A A
CGCTGATGCT TGTGCCCCAG CCCATAGCAG GCCATGGCGG CAAGTACTCG CGAGAGAAGA ACGAGCCGGA GATGGCCGCC
  K R E S   G E E   F R M   E K L N   Q L W   E K A   K R L H   L S P
AAGCGGAGT  CCGGGGAGGA GTTCCGCATG GAGAAGCTGA ACCAGCTATG GGAGAAGGCC AAGCGGCTTC ATCTGTCTCC
  V R L   A E L H   S D L   K I Q   E R D E   L N W   K K L   K V E G
TGTGAGGCTG GCCGAGCTGC ATTCTGACCT GAAGATACAA GAGAGGGATG AACTCAACTG GAAAAAGCTG AAGGTGGAAG
  L D K   D G E   K E A K   L I H   N L N   V I L A   R Y G   L D G
GCTTGATAA  GGATGGGGAG AAAGAAGCAA AACTGATCCA CAACCTCAAC GTCATCCTGG CCAGATACGG ACTGGATGGG
  R K D A   Q M V   H S N   A L N E   D T Q   D E L   G D P R   L E K
AGGAAGGACG CCCAGATGGT GCACAGCAAC GCCCTCAATG AAGACACCCA GGATGAGCTG GGGGACCCCA GGCTGGAAAA
  L W H   K A K T   S G K   F S S   E E L D   K L W   R E F   L H Y K
GCTGTGGCAC AAGGCAAAGA CATCAGGGAA ATTCTCCAGT GAAGAGCTGG ACAAGCTGTG GAGAGAGTTT CTGCATTACA
  E K I   Q E Y   N V L L   D T L   S R A   E E G Y   E N L   L S P
AAGAGAAGAT CCAGGAGTAC AATGTGCTGC TAGACACACT GAGCAGAGCT GAAGAAGGTT ATGAGAACCT TCTCAGTCCC
  S D M A   H I K   S D T   L I S K   H S E   L K D   R L R S   I N Q
TCGGACATGG CCCACATCAA GAGCGACACC CTGATCAGCA AGCACAGTGA GCTGAAGGAC AGACTGCGCA GTATCAACCA
  G L D   R L R K   V S H   Q G Y   G S T T   E F E   E P R   A I D L
GGGCTTGAC  CGCCTGCGGA AGGTCAGCCA CCAGGGCTAT GGCTCCACCA CTGAGTTTGA AGAGCCCGG GCGATAGATC
  W D L   A Q S   A N F T   E K E   L E S   F R E E   L K H   F E A
TGTGGGACCT GGCTCAGTCT GCCAACTTCA CTGAGAAGGA ACTGGAGTCA TTCAGGGAGG AGCTCAAGCA CTTTGAGGCC
  K I E K   H N H   Y Q K   Q L E I   S H Q   K L K   H V E S   I G D
AAAAATTGAAA AGCACAACCA CTACCAGAAG CAGCTGGAGA TTTCCACCA GAAGCTGAAG CACGTGGAGA GCATCGGCGA
  P E H   I S R N   K E K   Y V L   L E E K   T K E   L G Y   K V K K
CCCCGAGCAC ATCAGCCGCA ACAAGGAGAA ATACGTGCTG CTGGAGGAGA AGACCAAGGA GCTGGGCTAC AAGTGAAGA
  H L Q   D L S   S R V S   R A R   H N E   L *
AGCATCTACA GGACCTGTCT AGCAGGGTCT CAAGGGCTCG GCACAATGAG CTCTGAGGAC CAGAAGCCAC CAGCAGCAGC
  CTAGAGAGAA CACTTGAAGA CACCGGGAGC TGTACGATG TCATCGGCTT GCATAGACCT GAGGTGACTG GTGTGGCTGA
CCACCGTGGC AAGGAGGATC CCTTGAATA CCAAGCTGAT CCTACAGTGG CTGGCAAGGA CTTATTTTCT TTCAAGCAAG
  TGTAGTTGTC ACCACCCTGG ACGAGGGCCT TGGGTACCGC TACCAGTGAG ATAGGACTGG ACTCCGAGCT GCAGCACAAC
AGTTTATATT GAAATCACAT AAACCTGCCT GCCACTGGAA ACATTCTGTA CAGAGTCCTT AAATACATGG CAGAGTTTTG
AAAAAAAAAA AA

```

Fig 2. The cDNA sequence of *LRPAP-1* and its encoded protein sequence.

To verified the result, reverse northern blot had been used to analysis the samples of the test and control patients. The results refer to figure as (Fig. 3a, b and c).

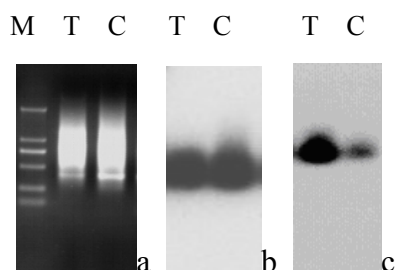


Figure 3. The results of virtual northern blot.

M, marker DL2000.

T, sample from test group.

C, sample from control group.

a, electrophoresis result of amplified cDNA.

b, hybridization result of β -actin.

c, hybridization result of *lrpap-1*.

The SAMP8 is a strain, which develops impaired learning and memory with aging that correlates with an age-related increase in levels of amyloid beta protein (A β P), and cognitive defects^{4,5}. A β P is thought to play a causal role in the development of Alzheimer's disease⁶ (AD). As is the case for any peptide, the levels of A β P are a balance between its rates of synthesis and degradation⁷. Both of these rates had postulated to be altered in AD. Additionally, the level of A β P in the central nervous system (CNS) is affected by its rate of clearance from the brain⁸. A β P is cleared from the brain by two major mechanisms. First, A β P in the cerebrospinal fluid is transferred to blood across arachnoid villi and lymphatic drainage along with reabsorption of the cerebrospinal fluid (CSF). This is a passive mechanism and affects all substances found in the CSF. Second, A β P₁₋₄₀ is transported across brain capillaries by a saturable efflux transporter system^{8,9}. This transporter, tentatively identified as LDL receptor-related protein⁹-1, has been suggested to be impaired in AD¹⁰. Such impairment could be a factor in the accumulation of A β P in the CNS and the onset of AD.

The low-density lipoprotein receptor-associated protein (LRPAP-1) is predominantly localized in the endoplasmic reticulum, where it associates with the LRP¹¹, and involved in the amount of mature LRP expressed on liver and brain¹². RAP-deficient mice show a reduced amount of mature LRP in liver and brain. The LRPAP-1 functions as a chaperon or escort protein in the intracellular transport of LRP, by preventing it binding to the nascent receptor¹³.

Here we assumed that the Chinese traditional medicine 'bushen yinao pian' retard the ageing progress by increasing *LRPAP-1* expression to facilitate the LRP maturity, which is an efflux transporter of A β P, ulteriorly alleviate the development of senile-related pathophenotypes.

Material and Methods

Medicine. The 'bushen yinao pian' (a complex prescription used for anti-ageing in china) was offered by Lingtai Bichen Medical Technology Co. Ltd. (Mudangjiang city, China), which is composed of 16 Chinese traditional medicine.

Those are include in as follows: Ginseng root (*Radix Ginseng*), Pilose Deerhorn (*Cornu Cervi Pantotrichum*), fruit of Chinese Magnoliavine (*Fructus Schisandrae*), fruit of Hairystamen Wolfberry (*Fructus Lycii*), prepared rhizome of Adhesive Rehmannia (*Radix Rehmanniae Preparata*), seed of Spine Date (*Semen Ziziphi Juiubae*), root of Chinese Angelica (*Radix Angelicae Sinensis*), root of Ningpo Figwort (*Radix Scrophulariae*), root of Creeping Liriope (*Herba Ophiopogonis*), etc.

Animal feed. Senescence-Accelerated Mouse prone strain 8/Ta (SAMP8/Ta) were offered by Kyoto University of Japan and housed in the experimental animal center of our Institute under the same condition in a clean facility on a 12-hours light/dark cycle. The control group mice were given a standard commercial pellet diet (Feed-Processing Plant of the Experimental Animal Center, the Academy of Military Medicine Sciences, Beijing, PR China) and the test group mice given the medicine appended pellet diet, which is added 1% 'bushen yiniao pian' (a complex prescription of Chinese traditional medicine for anti-ageing) in the standard commercial pellet diet (Feed-Processing Plant of the Experimental Animal Center, the Academy of Military Medicine Sciences, Beijing, China), and tap water ad libitum.

Total RNA isolation. After three moon feed, the male mice used for the experiment were decapitated, and the cerebral tissues were taken out. Cut out about 30mg cerebral tissue of each mouse, then according to the RNeasy mini Kit (Qiagen Inc. Valencia.) protocol to isolate total RNA, and the total RNA samples are treated with RQ1 RNase-Free DNase (Promga Co., Madison.), stored at -20°C before using.

DDRT-PCR. To detect gene expression change between the test group and the control group, samples of the same litter mice were compared according to the modified protocol of Liang and Pardee¹⁴. Briefly as follow: In each PCR tube (Axygen Scientific Inc., Union city), add 2 µl of total RNA(1µg)、2 µl of 10X RT buffer (New England Biolabs Inc., Hertfordshire, England, UK), 1.6µl of 2.5mM dNTPs (Promga), 2µl one of three DDRT 3'Primers (SBS, Beijing, PR China. The sequences are AAGCTTTTTTTTTTTTA, AAGCTTTTTTTTTTTTC, and AAGCTTTTTTTTTTTTG respectively), 0.5µl of RNasin Ribonuclease Inhibitor (40iu/µl) (Promga), and add double distilled water to 19µl. 65°C for 5 minutes, 42 °C for 10 minutes, then in each tube add 1µl of M-MuLV Reverse Transcriptase (200iu/µl) (New England Biolabs Inc.); 37°C for 50 minutes、75°C for 5minutes on Thermocycler (Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany).

Set up the PCR system for amplification of the RT products as follow: 2µl of RT product, 2µl of 10X Taq DNA Polymerase Buffer (Promga), 2µl of 25mM MgCl₂, 1.6µl of 2.5mM dNTPs, 2µl one of the 5' Primer (2µM) (SBS. The sequences are ACAGAGCACA, CACAGTTTGC, CCACAGAGTA, GGAAGTCCGT, GGCAAGTCAC, and AGGACCGCTA), 2µl of 3' Primer (2µM) (SBS), 0.4µl of Taq DNA Polymerase (5iu/µl) (Promga), 0.5µl of α-³²p-dCTP (10µCi/µl) (Yahuei Co. Ltd., Beijing, PR China), add water to 20µl. 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. After electrophoresis on 6% Urea denature polyacrylamide gel, the gel exposed to an X-ray film (Fuji Photo Film Co., Ltd. Tokyo, Japan) for 72 hours at -20°C.

Recovery and reamplification of cDNA fragment. Locate bands of interesting and cut out the located bands from the gel. Soak it in 100µl double distilled water for 10 minutes at room temperature. Boil for 15 minutes. Spin and transfer the supernatant to a new microfuge tube. Add 10µl of 3M sodium acetate (PH5.2), 2.5µl of Glycogen (20mg/ml) (Sigma-Aldrich Fine Chemicals, St. Louis, Missouri), 450µl of ethanol. -20°C overnight, spin for 10 minutes at 4°C. And rinse the pellet with 200µl ice-cold 85% ethanol. Dissolve the pellet in 10µl double distilled water.

Reamplify the recovered product as follow: 4µl of recovered product, 4µl of 10X Taq DNA Polymerase Buffer, 4µl of 25mM MgCl₂, 3.2µl of 2.5mM dNTPs, 4µl of 5'Primer (2µM), 4µl of 3'Primer (2µM), 0.8µl of Taq DNA Polymerase (5iu/µl), add double distilled water to 40µl. The PCR parameters of reamplification are 94°C for 10 minutes; 94°C for 1 minute, 40°C for 2minutes, 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, 20cycles; 72°C for 5 minutes. After reamplified, a part of products was verified by using 1.2% agarose (Biowest, Distributed by shanghai yito enterprise Co. Ltd., Shanghai, PR China) gel electrophoresis.

Subcloning of reamplified cDNA fragments and sequence analysis. The rest part of reamplified products were purified with Wizard[®] PCR Preps DNA Purification System (Promga) according to the manual protocol, and ligated into pGEM-T Easy Vector (Promga) according to the manual. Then the ligated products are transformed into E. coil DH.5α competent cell (Tianwei Time Co. Ltd., Beijing, PR China) and cultured on LB/amp/IPTG/X-Gal plates to obtain single positive clone of aim fragment. The plasmid DNA was purified with UltraPure plasmid DNA mini purification kit (SBS) according to the manual, and identified by PCR with the same primers and parameters of the insert fragment, and electrophoresis on 1.2% agarose gel. Those subcloned fragments sequenced by Bioasia Technology Co. Ltd. (Shanghai, PR China). And the sequences are compared with the national center of Biotechnology Information (NCBI) nonredundant sequence database using the BLASTX and BLASTN program.

cDNA library construction and screening. Total RNAs were extracted from test patient's cerebral tissue with RNeasy Mini kit, then constructed cDNA library with SMART cDNA Library construction Kit (BD Biosciences. Clontech Inc., Palo Alto) as protocol described and routine screening were carried with 3'-Irpap1 fragment as a probe.

Virtual northern blot analysis. The cDNA was verified with virtual northern blot according to the method of Hung¹⁵ et al. Briefly, reverse transcript the total mRNA with powerscript reverse transcriptase (BD Biosciences. Clontech Inc.), then amplify it with Advantage 2 PCR kit (BD Biosciences. Clontech Inc.) as protocol described. After electrophoresis on 1.2% Agarose, transferred of amplified cDNA to nitrocellulose membrane (Schleicher & Schuell Inc., Keene), and hybrid to

³²P-labeled fragment, which labeled with random primer DNA labeling kit (TaKaRa Biotechnology Co. Ltd., Dalian, PR China) as protocol descript.

Reference

1. Takeda, T., Hosokawa, M. & Higuchi, K. Senescence-accelerated mouse (SAM): a novel murine model of accelerated senescence. *J. Am. Geriatr. Soc.* **39**(9), 911–919 (1991).
2. Liu, C.X. & Xiao, P.G. Recent advances on ginseng research in China. *J. Ethnopharmacol.* **36**(1), 27-38 (1992).
3. Xiao, P.G., Xing, S.T., & Wang, L.W. Immunological aspects of Chinese medicinal plants as antiageing drugs. *J. Ethnopharmacol.* **38**(2-3), 167-175 (1993).
4. Flood, J.F. & Morley, J.E. Learning and memory in the SAMP8 mouse. *Neurosci. Biobehav. Rev.* **22**(1), 1–20 (1998).
5. Nomura, Y., Yamanaka, Y., Kitamura, Y., Arima, T., Ohnuki, T., Oomura, Y., Sasaki, K., Nagashima, K. & Ihara, Y. Senescence-accelerated mouse: neurochemical studies on aging. *Ann NY Acad Sci* **786**, 410–418 (1996).
6. Banks, W.A. & Morley, J.E. Memories are made of this: recent advances in understanding cognitive impairments and dementia. *J. Gerontol.* **58**(4), 314–321 (2003).
7. Sambamurti, K., Greigh, N.H. & Lahiri, D.K. Advances in the cellular and molecular biology of the beta-amyloid protein in Alzheimer's disease. *Neuromolecular Med.* **1**(1), 1–31 (2002).
8. DeMattos, R.B., Bales, K.R., Cummins, D.J., Paul, S.M. & Holtzman, D.M. Brain to plasma amyloid- β efflux: a measure of brain amyloid burden in a mouse model of Alzheimer's disease. *Science* **295**(5563), 2264-2267 (2002).
9. Shibata, M., Yamada, S., Kumar, S.R., Calero, M., Bading, J., Frangione, B., Holtzman, D.M., Miller, C.A., Strickland, D.K., Ghiso, J. & Zlokovic, B.V. Clearance of Alzheimer's amyloid- β_{1-40} peptide from brain by LDL receptor-related protein-1 at the blood-brain barrier. *J. Clin. Invest.* **106**(12), 1489–1499 (2000).
10. Rosenberg, R.N. The molecular and genetic basis of AD: the end of the beginning: the 2000 Wartenberg lecture. *Neurology* **54**(11), 2045–2054 (2000).
11. Krieger, M. & Herz, J. Structures and functions of multiligand lipoprotein receptors: macrophage scavenger receptors and LDL receptorrelated protein (LRP). *Annu. Rev. Biochem.* **63**, 601–637 (1994).
12. Sanchez, L., Alvarez, V., Gonzalez, P., Gonzalez, I., Alvarez, R. & Coto, E. Variation in the LRP-associated protein gene (LRPAP1) is associated with late-onset Alzheimer disease. *Am. J. Med. Genet.* **105**(1): 76-8 (2001).
13. Willnow, T.E., Armstrong, S.A., Hammer, R.E. & Herz, J. Functional expression of low-density lipoprotein receptor-related protein is controlled by receptor-associated protein in vivo. *Proc. Natl. Acad. Sci. U.S.A.* **92**(10), 4537–4541 (1995).
14. Liang, P. & Pardee, A.B. Different display a general protocol. *Mol. Biotech.*

- 10**(3), 261-267 (1998).
15. Hung, H.L., Song, F. & Gewirtz, A. A method for identifying differentially expressed genes in rare populations of primary human hematopoietic cells. *Leukemia*. **13**(2): 295-297 (1999).