

Evidences that Intake of L-valine May Affect the Lifespan-specific Local Gene Network Pattern in *Caenorhabditis Elegans*

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Abstract. The purpose of this study is to preliminarily investigate whether branched-chain amino acids (BCAAs) prolong the lifespan of model organism *Caenorhabditis elegans* (*C. elegans*) through modifying the ageing-related local gene network (LGN) pattern. After synchronization adult *C. elegans* were fed with living *E. coli* strain OP50 on nematode growth medium (NGM) agar plates with or without BCAAs (L-valine, L-leucine, L-isoleucine). L-valine extended the average lifespan of *C. elegans* significantly at low concentration (0.1×), but the other two amino acids affected lifespan slightly. Quantitative polymerase chain reaction (QPCR) was employed to quantify expression levels of thirteen genes that are closely related with BCAAs metabolic modules in the LGN. It was found that the thirteen genes' expression levels were differentially affected (some up-regulated, some down-regulated, and some had over 10-fold changes in the expression level) by intake of 0.1×Val, suggesting that the extension of lifespan through 0.1×Val was possibly through some uncharacterized lifespan-specific local gene network pattern change resulted from differential expression-level changes of a group of genes.

Introduction

C. elegans has been a good model organism for lifespan studies [1]. Various compounds were demonstrated to be strongly linked to longevity in different model organisms. In 2007, Bass TM et al testified resveratrol in some trials caused slight increases in lifespan in *C. elegans* in both wild-type and sir-2.1 mutant populations [2, 3]. Later on in 2008, Kimberley Evason et al indicated valproic acid could not only be in favor of life extension, but also delay age-related degeneration of physiologic processes that mediate body movement [4]. Notably in 2012 Paola V et al reported that very low concentration (1 mM) of ethanol could improve the starvation survival of L1 larvae [5]. In the same year Kim Zarse et al found L-theanine extended *C. elegans* lifespan at a wide range of concentration from 100 nM to 10 micromolar [6]. These results demonstrated that small chemicals or nutrients can modulate ageing-related metabolism equilibrium or regulate some ageing-related signaling pathways in *C. elegans*.

Recent intriguing reports indicated that BCAA-enriched mixture consisting of L-valine, L-isoleucine, and L-leucine could steadily increase the average lifespan of mice [7]. This was likely the consequence of increased mitochondrial biogenesis and reduced oxidative stress in cardiac and skeletal muscles via eNOS-mediated mechanisms [7]. The increased survival

with BCAAs in *Saccharomyces cerevisiae* was also reported in 2009 by Alvers A.L et al [8]. But so far the role of BCAAs in affecting lifespan in the model organism *C. elegans* has not been thoroughly studied.

In this report we tested the impact of L-valine, L-leucine and L-isoleucine on the lifespan of *C. elegans* at different concentrations, and then measured the expression levels of thirteen genes that are closely linked with BCAAs metabolic modules. Our results provided some new evidence that the extension of lifespan through 0.1×Val was possibly through lifespan-specific local gene network pattern change resulted from differential expression-level changes of a dozen of genes.

Methods

Compounds

L-valine, L-leucine, and L-isoleucine were obtained from Sangon (Shanghai). For each amino acid, the final concentrations in NGM agar plates of (1×) were $C_{\text{Val}(1\times)}=0.649\text{mM}$, $C_{\text{Leu}(1\times)}=2.88\text{mM}$, and $C_{\text{Ile}(1\times)}=0.577\text{mM}$.

Ageing Related Local Gene Network

Construction of ageing-related local gene network was started with 18 genes with known functional association with lifespan determination in *C. elegans*. WormNet provided a tool to construct functional connections among a group of genes. The 18 lifespan-related genes were found to link total 200 genes. All these 218 genes (not shown) were then employed to construct local gene network in KEGG database using KGML software [9-11], but only 16 genes were able to be linked with each other as LGN within known biological pathways. Interestingly, the constructed LGN was well connected with amino acids metabolic modules. In the LGN, there are about 13 genes have distinct closeness with the metabolic modules of branched-chain amino acids (Fig. 1 and Table 1, all related information can be checked in KEGG database).

Maintenance and Cultivation of Nematodes

The *C. elegans* strain for the experiment was wild-type N2. All experiments operations related with nematodes were performed exactly as the literatures previously described [12-15]. The *C. elegans* were cultured onto NGM agar plates at 22°C and fresh living *E. coli* OP50 bacteria were used as the only food source.

Lifespan Assay

After synchronization L4 larvae were cultured on NGM agar plates (with and without L-valine, L-leucine and L-isoleucine). The beginning of *C. elegans* hatching was recorded as the day zero, and then nematodes were transferred daily to fresh plates to until all died. The survival and death of nematodes were cultivated on different plates was recorded in detail every day. SPSS Statistics 19.0 software was used for statistical analysis after the end of experiments.

Motility

We divided motility into four classes, where “4” animals move spontaneously and smoothly, leaving sinusoidal and symmetric tracks; “3” animals move spontaneously at lower speed; “2” animals only move slowly when prodded with a platinum wire; “1” animals only move the mouth or tail. Worms after adulthood were observed and recorded daily.

Table 1. QPCR primers for 13 genes that are closely related with BCAAs metabolism in LGN

No.	Gene name	Primer	Primer sequence(5'-3')
1	alh-9	q1-q2	5'-GCTCCAGCAAATAACTCCCC-3' 5'-TGTTCAAATCAGCATCCTCGT-3'
2	sptl-2	q3-q4	5'-CTGGAATGTGCTTGAACC-3' 5'-TTGTGCTCAAATACTTTTGTAGAA-3'
3	cyp-33E1	q5-q6	5'-AAATGTTCCGTAAGCAGGC-3' 5'-TTCATAGTAACTCCGACTTCCTT-3'
4	unc-25	q7-q8	5'-GGGAAGCAGTTGGATGGG-3' 5'-TTCTCCTGGTAAAATAGTTGGC-3'
5	cts-1	q9-q10	5'-GGTAGCACCAGTAGTTCAAAA-3' 5'-CGGAGAAGGAAAGGTATGGAT-3'
6	C14E2.2	q11-q12	5'-AGGAGACCACTTTCCAGGCT-3' 5'-CGAACGAGGTGATCGACTTG-3'
7	C32F10.8	q13-q14	5'-AAGATGGAGTATGCTGTTTCGTG-3' 5'-ATTGCGATCCTTGAGCGTAG-3'
8	odc-1	q17-q18	5'-TGTATCTGACCCAAGTCTACG-3' 5'-TCTCCACATTTCCTCGCTC-3'
9	C05D2.3	q19-q20	5'-TACTGGGATGGAATAAGGACC-3' 5'-GCTCTATAGCGTCGATCAAAA-3'
10	vrs-2	q21-q22	5'-CTGCTGAAATGGGATGAGGA-3' 5'-GTGGTTGCCTGAGTGAAGTTAT-3'
11	ctl-3	q23-q24	5'-AGACACCACTTCTCGTTCGTT-3' 5'-GGTAATGCGTGTCCGTGTAG-3'
12	C28h8.11	q25-q26	5'-CGTGTCCGTATTTGGGTTCT-3' 5'-AAGCATCCGCAGTTTATGA-3'
13	T22H6.2	q27-q28	5'-GGTAGCCTAATGGTTATTTTCG-3' 5'-CACGCACTGTCTACGTGTTTC-3'
	GAPDH		5'-ATGCCAAAGCCAAGTGTGTCG-3' 5'-TGTTGGAGTATCCGAAGTCTGTTAT-3'

Detection of Gene Expression

Total RNA was extracted from nematodes fed amino acids extending lifespan significantly using UNIQ-10 column Trizol total RNA extraction kit (Sangon, Shanghai) and was reverse transcribed into cDNA using AMV first strand cDNA synthesis kit (Sangon). Thirteen genes that are closely related with BCAAs metabolic modules in the LGN were selected for real-time quantitative PCR (QPCR) analysis. Primers were listed in Table 1. QPCR with a final volume of 12uL contained 6uL 2×NPK62 QPCR buffer (GREDBIO), 0.2uL cDNA, 1.5uL primer pair, 4.3uL ddH₂O was performed on QPCR machine TP810 R-1447 (Takara). QPCR reaction began with a denaturation step at 94°C for 2min, followed by 65 cycles of amplification at 94°C for 30s, 60°C for 1min and 72°C for 1min. PCR ended with 72°C for 5min. At the end of the amplification, a melting analysis was performed.

Results and Discussion

Construction of Aging-related Local Gene Network of *C. elegans*

A local gene network was constructed that included both known ageing-related genes (such as age-1, akt-1 and pdk-1) and BCAAs metabolic modules. Especially, L-valine (C00183) is located directly in the LGN. Thirteen genes (Table 1) in the LGN that have been rarely studied were selected for gene expression measurement since they are very close to BCAAs metabolic modules.

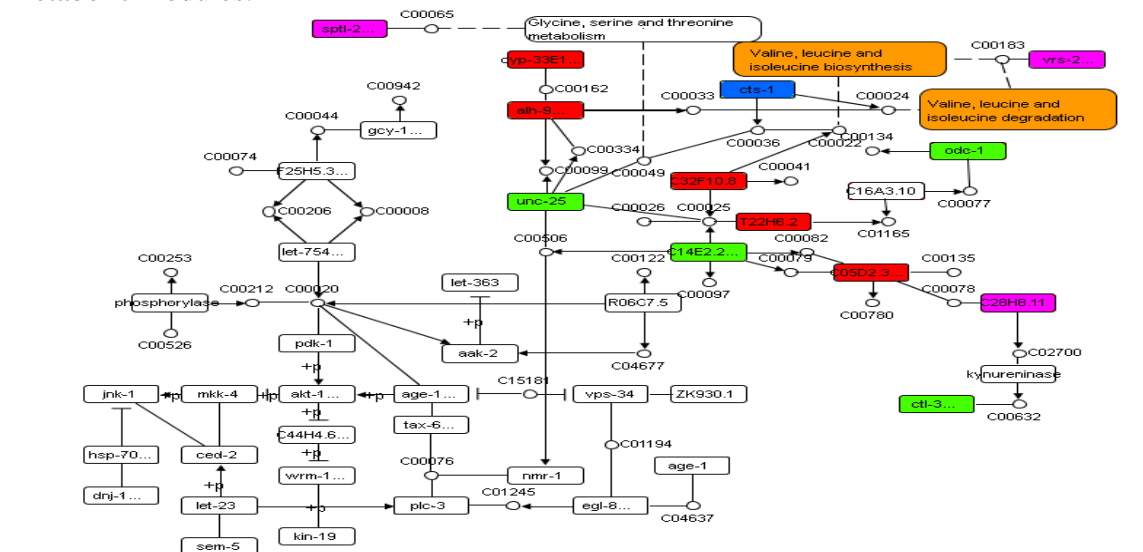


Figure 1. Ageing-related local gene network of *C. elegans* generated with KGML tools. Possible lifespan-related genes and metabolic modules are connected with each other by biochemical pathways. Small chemicals related with metabolic modules or signaling pathways were labeled by circle. Colored boxes represent the 13 genes that are close to BCAAs metabolic modules and subjected to qPCR analysis. Red: up-regulated then down-regulated; Green: down-regulated then up-regulated; Pink: up-regulated; Blue: down-regulated.

BCAAs Affect Lifespan in *C. elegans*

Applying L-valine, L-leucine, L-isoleucine separately at three different concentrations (0.1×, 1× and 10×) to *C. elegans* using the above-mentioned methods affects lifespan (Table 2). The results showed that 0.1×Val could extend the average lifespan significantly ($P=0.001<0.01$), and also increase the physiological activity (crawling speed, Fig. 2) while Leu and Ile had no significant impact on *C. elegans* survival.

L-valine Influence the Expression of Aging-related Genes

RNA was extracted from nematodes fed with 0.1×Val after 4d, 8d and 12d, then the expression of lifespan-related genes in LGN was detected by QPCR. We used a comparative method ($2^{-\Delta\Delta Ct}$) for quantitative statistics analysis and regarded GAPDH as reference gene with the prerequisite that all primer pairs had similar amplification efficiency (close to 1.0). Fig.3 demonstrated QPCR results for 13 selected genes. Five genes (alh-9, cyp-33E1, C32f10.8, C05D2.3 and T22H6.2, red-colored in Fig1) were first up-regulated then down-regulated; Four genes (unc-25, C14E2.2, ctl-3 and odc-1, green-colored in Fig1) were down-regulated first then up-regulated; Three genes (sptl-2, vrs-2 and C28h8.11, colored pink

in Fig1) were differentially up-regulated while one gene (cts-1, colored blue in Fig1) was down-regulated.

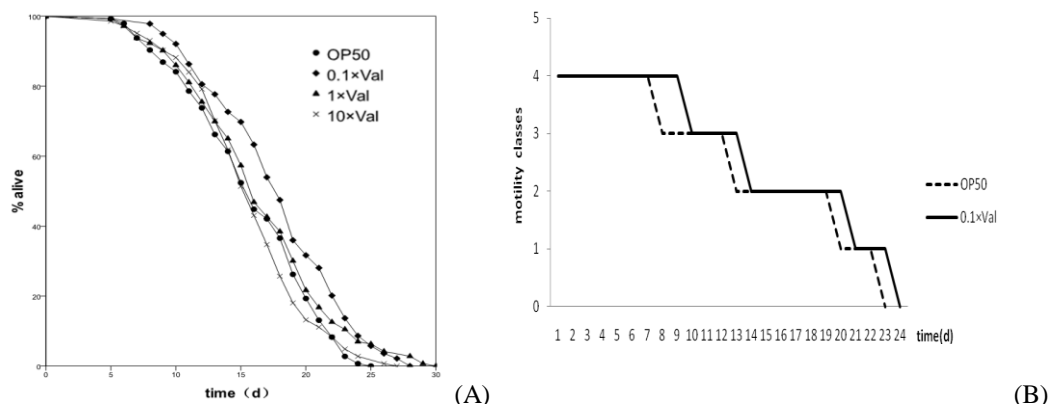


Figure 2. The low concentration of L-valine affected the lifespan and motility of adult *C. elegans*.

(A)Kaplan-Meier survival curves. (B) Motility assay results for 0.1xL-valine.

Table 2. Summary table of lifespan assays (** P<0.01)

Treatment	Average lifespan (d) (\pm SD)	P value (vs. OP50)	Number of experiments	Number of nematodes
Control	15.786 \pm 0.406	--	3	145
0.1xVal	17.842 \pm 0.428	0.001**	3	139
1xVal	16.524 \pm 0.458	0.228	3	143
10xVal	15.743 \pm 0.379	0.938	3	144
0.1xLeu	15.156 \pm 0.419	0.280	3	141
1xLeu	15.464 \pm 0.438	0.590	3	140
10xLeu	16.313 \pm 0.484	0.402	3	134
0.1xIle	15.336 \pm 0.512	0.488	3	134
1x Ile	15.979 \pm 0.412	0.740	3	140
10x Ile	15.216 \pm 0.484	0.366	3	139

Summary

Results in this study indicated that L-valine significantly extends the average lifespan of *C. elegans* at low concentration (0.1x). Besides, the metabolic modules of branched-chain amino acids are directly associated with the ageing-related local gene network, providing a convenience that LGN can be employed as a platform to investigate molecular mechanisms of lifespan determination.

BCAAs (L-valine, L-isoleucine, and L-leucine) has the function of extending lifespan of mice because of increased mitochondrial biogenesis and reduced oxidative stress in cardiac and skeletal muscles via eNOS-mediated mechanisms [7]. Reports indicated most long-lived mutants containing mitochondrial ETC mutants, IIS mutants, and translation mutants all have increased levels of BCAAs in *C. elegans* [12, 13]. TOR pathway mutants, dietary restriction mutants and other translation mutants were also reported to have an under-appreciated function of amino-acid metabolism in longevity [14].

L-valine directly appears in the lifespan-related LGN, and participates in several signaling pathways such as valine-leucine-isoleucine degradation, valine-leucine-isoleucine biosynthesis,

penicillin and cephalosporin biosynthesis, cyanoamino acid metabolism and so on. What is the role of these signaling pathways for ageing regulation or lifespan determination is unknown.

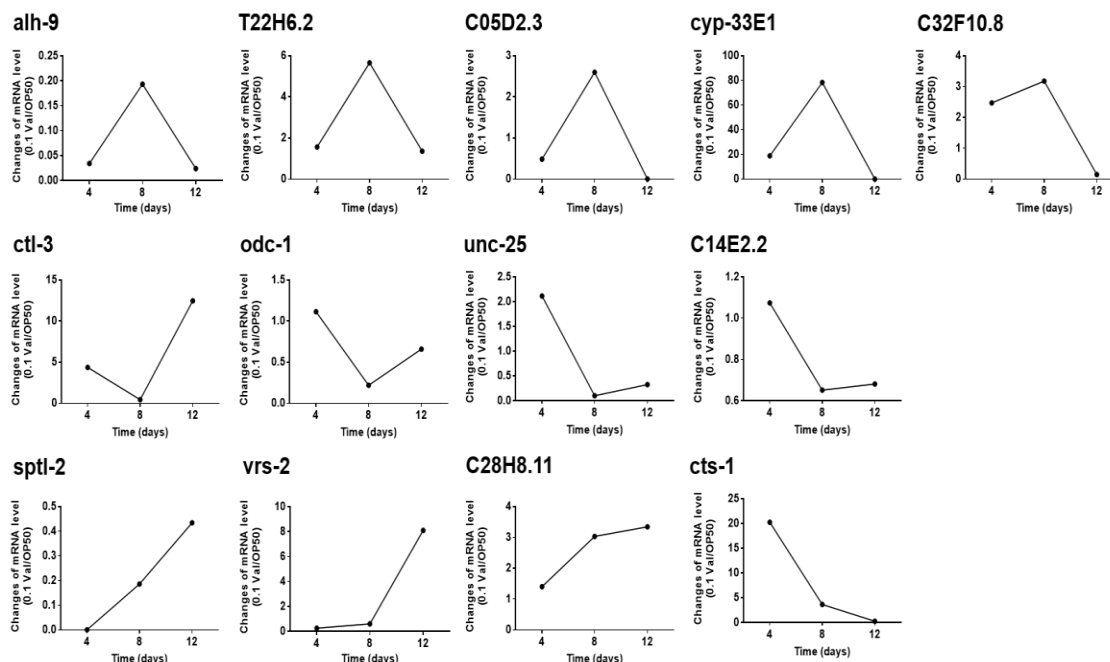


Figure 3. QPCR results of expression level changes for 13 genes closely linked with BCAAs metabolic modules. Also see labels in Figure.1.

Thirteen selected genes that are closely linked with BCAAs metabolic modules in the LGN were subjected to gene expression quantification in this study, while the LGN has at least 39 genes. The thirteen genes didn't include any one of the 18 LGN-construction-starting genes with known lifespan-related biological functions (See Methods). There are several genes such as *let-363*, *pdh-1* and *akt-1* that are involved in longevity-related mTOR signaling pathway. Besides, *let-363* encodes a rapamycin complex-associated protein. Next step is to systematically investigate the gene expression states in the larger local gene network.

One potentially important aspect is molecular network equilibrium theory. In this theory, different metabolic equilibrium states have different logistics distribution and lead to different network patterns or physiological phenotypes. Local gene network is actually a local molecular network in which proteins, enzymes, nutrients, metabolites and their subcellular localizations are key factors that determine the equilibrium states of local molecular network. Theoretically, expression-level changes in some genes in the LGN can lead to significant metabolic trend change or network pattern change. Intake of L-valine may change the pattern of ageing-related local molecular network, and this point of view also needs future detailed experimental evaluation.

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