DIFFERENTIALLY GENE EXPRESSION IN THE BRAIN OF COMMON CARP (*CYPRINUS CARPIO*) RESPONSE TO COLD ACCLIMATION

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Abstract: There are a variety of approaches to identify groups of genes that change in expression in response to a particular stimulus or environment. We here describe the application of suppression subtractive hybridization (SSH) for isolation and identification genes in the brain of common carp (Cyprinus carpio) under cold temperatures. The materials were prepared through cooling the hybrid F2 of purse red carp (cold-tolerant strains) and bighead carp (cold-sensitive species) to different regimes of temperatures. A subtracted cDNA library containing 2000 clones was constructed. About 60 positive clones were identified to express differentially by dot blotting in screening 480 clones. Sequencing 26 clones and aligning in GenBank/EMBL database using blastn searching engine, 15 genes showed higher similarities with 85-98%. These annotated genes contained (1) genes for transcription factors and gene products involved in signal transduction pathways such as zinc-finger protein, brevican; (2) genes involved in lipid metabolism such as Acyl-CoA synthetases, and (3) genes involved in the translational machinery such as cytochrome c oxidase, ependymin glycoprotein. In addition, real-time PCR was also conducted to validate these genes. To sum up, we believe this study will make an important contribution to elucidate the possible mechanisms on fish cold tolerance at a molecular level.

Key words: cold tolerance, suppression subtractive hybridization (SSH), real-time PCR, Cyprinus carpio

1. INTRODUCTION

Temperature has been recognized as a major environmental factor at the molecular, cellular, tissue, organism and ecosystem levels of biological hierarchy. Low temperature may make the cell threaten by a number of physiological and developmental changes. For aquatic ectotherms, visually observed changes associated with decreased temperature include changes in behavior and coloration. As the decline of temperature, fish exhibits a general decline in activity, respiration capacity, immune capacity and an abrupt loss of equilibrium. In addition, many fish will cease feeding and its muscle become more rigid until to death. It is very necessary to improve the ability of cold tolerance in fish, which may be expected to enlarge the culture areas of some thermophilic fish and improve their disease-resistant ability. It is also important for the study of cold tolerance in all other organisms.

Common carp (*Cyprinus carpio*) is a major kind of fish in the aquaculture industry around China, which can survive at the temperature ranging from 0□ to 35□. Many documents reported common carp could alter enzyme activities, membrane fluidities and behaviors to adapt to lower temperature. It is reported that lower temperature had an effect on lipid composition of cell membrane (Yeo et al., 1997). With the decline of temperature, fish produce lots of long-chain unsaturated fatty acid, which will promote membrane fluidity of cells. Cossins et al. (2002) also verified the importance of desaturase induction in the inducible cold tolerance of carp. As for the enzyme activities, Brown (1960) brought forth that there are two pathways of glucose metabolism and activities of LDH isoenzyme increased response to cold acclimation in carp liver. Few studies reported the effect of temperature on behavioural decision-making under predation risk. Fraser et al.(1993) found that Atlantic salmon juvenile use low water temperature as a cue to switch from diurnal to nocturnal foraging. At present, many cold-induced genes have been isolated in teleost fish, including *cytochrome c oxidase*, *cytochrome P450*, *antifreeze protein*, *methallothionein-1*, *carnitine palmitoyltransferase I*, *adenine nucleotide translocase* and *ependimin* (Hardewig et al., 1999; Kloepper-Sams &Stegeman, 1992; Pickett et al., 1983; Beattie et al., 1996; Rodnick & Sidell, 1994; Roussel et al., 2000; Tang et al., 1999). The brain is the organ that senses temperature and makes instructions to cold acclimation. It is possible that cold-induced genes in the brain contribute to the control and regulation of the acclimation responses. Besides of the cold-induced alternations in the composition of the phospholipids, the release of neurotransmitters and hormones in the brain has also been reported (Yeo et al., 1997; Tiku et al., 1996; Poli et al., 1997). Moreover, studies on the activities of *AchE* in brain showed that *AchE* of some thermo fish becomes unstable at low temperature and possibly exists two isomers at different temperature (Baldwin & Hochachka, 1997).

However, little knowledge are known on the molecular mechanism under temperature acclimation.

In the present study, we set out an experimental fish system and describe the application of suppression subtractive hybridization (SSH) on isolation and identification of the cold tolerance-related genes in the brain of common carp in order to further understand the molecular process involved.

2. MATERIALS AND METHODS

2.1 Construction of experimental fish system

Bighead carp (♀, cold sensible) and red purse carp (♂, cold tolerance) were selected to be the parents of experimental fish. Considering that bighead carp cannot survive safely while red purse carp can survive in winter, segregation in F_2 of the crosses may exhibit cold tolerant and cold sensitive respectively. Therefore, F_2 was chosen as the experimental fish system in this study.

2.2 Cold acclimation of experimental fish

The hybrids of F_2 weigned ranging from 58-70mg were maintained in a temperature-controlling aquarium at 16□ for one week. Then water temperature was decreased to 10□ and 4□ at a rate of 2□ per hour, and maintained for 5 days separately (Fig.1). Brain tissues were collected from 5 fish of each temperature-controlling point under 10□ and 4□ and stored in liquid nitrogen quickly.

2.3 RNA preparation

Brain tissues were ground with a mortal and pestle, and then homogenized in Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA). Total RNA was extracted according to the manufacturer's instructions. The cDNA of cold-treated tissues was synthesized by long distance PCR method using SMART PCR cDNA Synthesis Kit (Clontech).

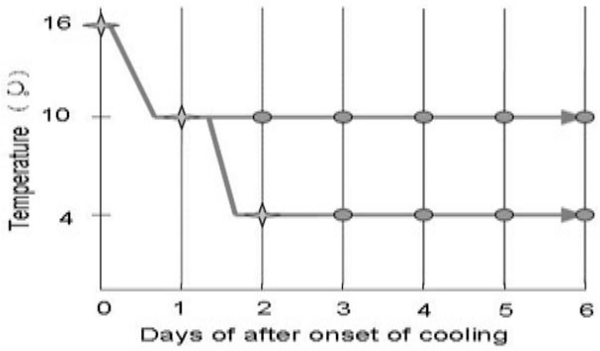

Fig.1: Schematic diagram showing the cooling time course and sampling regime used
Notes: Samples collected from temperature-controlling point represented by solid asterisk.

2.4 Construction of subtracted cDNA library

The mixed brain samples from 10 and 4 were selected to be the object of study. SSH was carried out using Clontech PCR-Select cDNA Subtraction Kit and Advantage Klen-Taq Polymerase Mix (Clontech). One microgram each of prepared tester and driver ds cDNA were digested by Rsa enzyme,and the tester cDNA was separated into half parts and then each was ligated to adaptor 1 and adaptor 2R in separated ligation reaction mixture at 16 overnight. The reaction was stopped by EDTA/glycogen and ligase was inactivated by heating the sample at 72 for 5min. For the first hybridization, an excess amount of driver cDNA was added to each tester cDNA (ligated with adaptor 1 and 2R) in separate sample. After denaturation at 98 for 1.5min, the first hybridization was performed in a hybridization buffer at 68 for 8h. The two samples from the first hybridization were mixed and fresh denatured driver cDNA were added. In the second hybridization, new bybrid molecules corresponding to differentially expressed cDNA with different adaptors on each end were formed. Two PCR reactions were performed using different primers to selectively amplify the differentially expressed sequences. The PCR products were cloned into pMD 18 T-vector (Takara). The subtracted cDNA library was constructed after transformation into DH5α *Escherichia coli* strain.

2.5 Screening of differentially expressed clones by dot blotting

After purification by phenol-chloroform extraction, PCR products of forward- and reverse-subtracted cDNAs were used to be the probes labeled by α-^{32}P dATP separately. 480 clones were selected at random from the

forward subtracted library enriched for cold-related sequences and the cDNA inserts were amplified. The PCR products were then blotted onto membranes together with control cDNAs and probed with the forward and reverse subtracted cDNA pools. 26 of positive clones screened were sequenced and performed alignment in the GenBank/EMBL database using blastn searching engine.

2.6 Real-time PCR

Another group of fish produced through inducing the gynogenesis of bighead carp was conducted the same temperature decreasing experiments described above. The purpose of this experiment is to further validate the genes isolated by SSH. Two tissues of livers and intestines were sampled and prepared single strand cDNA from three temperature points of 16℃, 10℃ and 4℃, respectively. Parts of sequences obtained by SSH were used to design primer pairs with software Beacon Designer (version 4.0). Real-time PCR was carried out in the Rotor-Gene 3000 PCR (CORBETT) using QuantiTect™ SYBR® Green PCR kit (QIAGEN).In this study, double standard curves of comparative quantitation method was used to analyze the differences of gene expression at different temperature points.

3. RESULTS AND ANALYSIS

3.1 Selection of the experimental fish

In this study, the mixed brain samples from 10□ and 4□were selected to be the object of study. It is reported that low temperature firstly have an effect on the central nervous system of fish. In addition, carp can live at 10-16□ normally while abnormally even to death at 4□. Therefore, it is hopefully to find cold-related genes through comparing these two temperature points.

3.2 Experimental key steps: the quality of total RNA, adaptor ligation efficiency and subtracted hybridized efficiency

Total RNA was extracted by Trizol reagents and visualized on 2% agarose gel. The value of OD260/280 was more than 2.0. It is very important to

perform the ligation well before subtracted hybridization. The results show a successful ligation and subtraction using α-tubulin gene as the control(Fig.2).

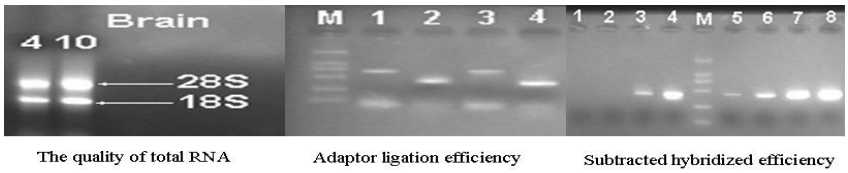

Fig. 2: The results of three key steps of this study

Notes: PCR was performed on subtracted cDNA(Lane1-4) and unsubtracted cDNA(Lane5-8); M: DNA Marker DL2000; Lane 1,5: 18 cycles; Lane 2,6: 23 cycles; Lane 3,7: 28 cycles; Lane 4,8: 33 cycles.

3.3 Dot blotting analysis

Sixty of the 480 clones were screened positive by differential screening with forward and reverse subtracted probes and 26 cDNA clones were selected for sequencing. As figure 3 showed, duplicate dot blots hybridized with forward (A, C) and reverse subtracted cDNA probes (B, D), respectively.

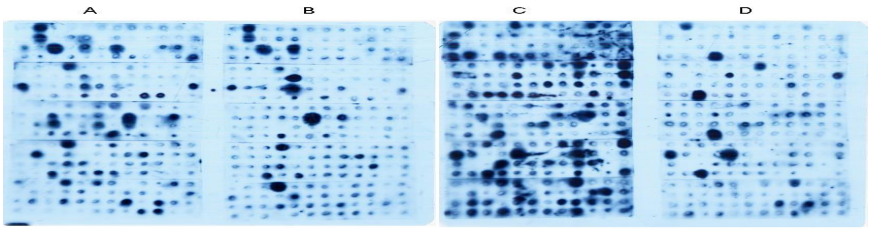

Fig. 3: Differential screening of SSH-selected cDNA clones with forward and reverse subtracted probes.

Notes: A,C: probe was prepared by the forward SSH PCR□products; B,D: probe was prepared by the reverse SSH PCR□products

3.4 Sequence analysis

To characterize the 26 differentially expressed genes, we used blastn searching engine to find homological genes in GenBank/EMBL database. Fifteen genes show more than 85% homology to known genes, the remaining genes are unkown function. The major categories of differentially expressed genes in this study included (1) genes for transcription factors and gene products involved in signal transduction pathways such as zinc-finger protein, brevican; (2) genes involved in lipid metabolism such as Acyl-CoA

synthetases and (3) genes involved in the translational machinery such as cytochrome c oxidase, ependymin glcoprotein.

3.5 Validation of real-time PCR

Two putative genes (4℃) isolated by SSH named LKE-25 (col-t 22) and LKE-62 (col-t 29) were applied to amplify the target genes, 18srRNA of common carp was used to amplify the housekeeping gene (Table 1). Double standard curves of real-time PCR were constructed individually and showed that the values of relative coefficients (R) are >0.99, most values are even beyond 0.999. Melt curves were also conducted and showed that the primer pairs are very specific in this study. The results of real-time PCR demonstrated that the concentration of LKE-25 decreases generally with the temperature decreased in different tissues, while, the concentration of LKE-62 is on the rise in livers and intestines with the temperature increased. According to our results, we considered LKE-62 is a up-regulated gene and LKE-25 is a down-regulated gene at lower temperature.

Table 1. Characterization of primers for real-time PCR

Primers	Primers sequence (5′→3′)	Annealing temperature	Melt temperature	Cycles
LKE-25	F:CATAGCCGATCAACGAACC R:TAGAAACTGACCTGGATTGC	55	65-95	35
LKE-62	F:CTTCGTGGAGTGTGGCTAATC R:CGGTTACATAGGAATGGTCTGAG	55	65-95	35
18srRNA	F:CCTGTCGCCGCTGAATACC R:TCGCTTTCGTCCGTCTTGC	55	65-95	35

4. DISCUSSIONS

There are many studies reported the influences of low temperature in common carp, but mainly focued on the changes of membrane fluidity, lipid composition and enzyme activities. Brain is the most important organ during cold acclimation that can sense temperature and make instructions. Thus, it is necessary to do some research on the response to low temperature in the brain. By cold acclimation, the concentration of unsaturated fatty acid in the membrane increases, thereby promotes membrane fluidity (Roy et al., 1997). In addition, it is reported that ependymin (EPD) expressed increasingly in the brain and play an important role in fish to cold acclimation at an early stage. Up to now, a system analysis of differentially expressed genes in the brain is not available.

In the present study, some cold-induced genes in the brain of common carp were examined using SSH and validated by real-time PCR. It is resulted that 12.5% clones of the subtracted library showed differentially expression at low temperature. Among 26 differentially expressed genes, fifteen genes

showed more than 85% homology to known genes while the remaining were still unknown. Because of the large numbers of unknown genes involved in cold acclimation, it is not yet possible to draw a clear picture of the molecular events that lead to adaptation or tolerance to adverse environmental conditions. Ju et al. (2002) hypothesized that a number of molecular events must occur to prepare the organism for environmental stresses including a cascade of signal transduction, activation of transcriptional factors that direct synthesis of new proteins to cope with low temperature. Many of these molecular events are rapid and transitory, but some of them may be persistently induced.

Brevican is a brain-specific proteoglycan that has been suggested to play a role in central nervous system fiber tract development (Gary et al., 1998). Besides preventing the formation of new synapses, it has been speculated that brevican might function as an insulator, sealing the synapses and preventing loss of transmitter substances from the synaptic cleft to the periphery. As the decline of temperature, brevican expressed up regulated that may prevent the formation of new synapses and lead to the low immunity of organisms. Arnab et al. (2004) isolated an intronless gene from rice encoding a zinc-finger protein and found this gene over-expressed after cold stress. However, here we characterized zinc-finger protein down regulated. It is necessary to make a further study.

Recent studies suggest that the long-chain acyl-CoA synthetases (ACS) may play a role in channeling fatty acids either toward complex lipid synthesis and storage or toward oxidation. Acyl-CoA binding protein (ACBP) was involved in fatty acid elongation and membrane assembly and organization (Gaigg et al., 2001). The detection of this gene showed that there must be some changes of cell membrane exposure to low temperature.

As for the genes involved in the translational machinery, several studies have shown that acclimation to low temperature provokes a compensatory increase of cytochrome c oxidase activity in fish tissues(Battersby et al., 1998; Thillart & Modderkolk, 1978; Wodtke, 1981). Ependymin encoding glycoprotein (EPN) is probably associated with collagen fibrils and has the capacity to bind calcium and results in a conformational transition. It is speculated by Tang et al. (1999) that EPN plays an important role in the cold acclimation of fish. The increase of EPN under cold stress not only prevents the nerve system from damage but also promotes its regeneration.

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