Fish & Shellfish Immunology 30 (2011) 1170–1177

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Short sequence report

Construction and characterization of a cDNA library from shark regenerated hepatic tissue

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ARTICLE INFO

Article history:
Received 30 September 2010
Received in revised form
21 February 2011
Accepted 26 February 2011
Available online 5 March 2011

Keywords:
Shark
Liver regeneration
cDNA library
Expressed sequence tags (ESTs)
Gene profile

ABSTRACT

Sharks are a type of fish with a full cartilaginous skeleton and have big livers. To better understand liver regeneration in sharks and screening for the important genes participated in disease-defense, in this study, a cDNA library of regenerated liver tissues of shark, *Chiloscyllium plagiosum*, was constructed. A total of 2103 expressed sequence tags (ESTs), which represents 997 unique genes, were sequenced. Among these genes, 434 (43.53%) of them showed significant similarity (E-values $< 10^{-5}$) to the sequences in NCBI Nt database, 685 (68.71%) of these unique genes showed significant similarity (E-values $< 10^{-5}$) to the sequences in NCBI Nr database, and 662 (66.40%) of these unique genes showed significant similarity (E-values $< 10^{-5}$) to the Swiss-Prot database. Preliminary analysis of unique genes according to COG database showed that unigenes were further grouped into 21 functional categories including inorganic ion transport and metabolism, energy production and conversion, posttranslational modification, protein turnover and chaperones, general function prediction only, translation, and ribosomal structure and biogenesis. Several possible candidate genes involved in liver regeneration were selected to analyze their expression with relative quantification real-time PCR. This study may contribute to our better understanding of the molecular mechanism of regeneration in shark liver. Furthermore, the EST cataloguing and profiling of shark will be also benefited to the functional genomic research in this marine species.

1. Introduction

The liver is one of the key organs in animals for detoxification and other functions. Additionally, it is also well known for its powerful regenerative capability [1]. The mechanism of liver regeneration has been an object of curiosity in related research areas for many years [2,3]. It is presumed that insight research of the molecular mechanism of liver regeneration can help us understand more about the theory of liver regeneration and to find the therapeutic targets for drug design and screening. A number of experimental models and techniques have been used to study the mechanism of liver regeneration at the level of organic, cellular and molecular basis in mice [3], rats [4], baboons [5], dogs [6] and humans [7] in the past two decades. However, little is known about shark liver regeneration.

Sharks, being different from other animals, have large livers and powerful digestive abilities [8], so we presumed that the bigger liver might play an important role in its anti-disease capabilities. To better understand liver regeneration and its relationship to disease-defense in sharks, the cDNA library of regenerated hepatic tissues was constructed with *Chiloscyllium plagiosum*, one of the most popular sharks distributed along the coastal line of southeast China. The randomly selected 2395 clones were sequenced to get 2103 expressed sequence tags (ESTs), where these ESTs represent the expressing profile of shark regenerated hepatic tissues. They were further analyzed using various gene blast tools, and ESTs of known and putative function were then grouped into different functional categories. The sequence information of the cDNA library may provide a useful tool for us to understanding the molecular mechanisms of liver regeneration in shark, as well as identify the functional genes in cartilaginous fishes.

2. Materials and methods

2.1. Animal

All the experimental protocols using animals were approved by the Animal Ethical Committee of China Pharmaceutical University.

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doi:10.1016/j.fsi.2011.02.025

C. plagiosum, one kind of sharks distributed widely along the Eastern China Sea and South China Sea, was obtained from Huimingqiao sea-products market of Nanjing, China, and authenticated by Professor Wen Wang, College of Life Sciences, Nanjing Normal University. The voucher specimen was deposited in the Department of Marine Bioresources, China Pharmaceutical University, Nanjing, P. R. China. The sharks were maintained at the sea-products market for almost one week in artificial seawater under the similar temperature of sea. After transfer to the department, the shark was kept in our lab for 10 days before it was subjected to hepatectomy.

2.2. Partial hepatectomy

Resection of the left hepatic lobes (40%) of the shark liver was performed as described by Higgins and Anderson [9]. Twenty-four hours after partial hepatectomy, the right hepatic lobes of the liver were removed from sharks, and immediately frozen in liquid nitrogen, then stored in −80 °C for total RNA extraction.

2.3. Extraction of total RNA and isolation of mRNA

Total RNA extraction followed the method described by Chomczynski [10,11], and its quality was assessed by agarose gel electrophoresis under non-denaturing conditions. Poly(A^+) RNA was purified with the Poly(A)tract mRNA Isolation Systems kit (Promega, Madison, WI, USA), as described in the manual.

2.4. cDNA library construction

Generation of the cDNA library was carried out according to the protocol of SMART cDNA synthesis by long-distance PCR (LD-PCR) described in the commercial kit Clontech Creator™ SMART™ cDNA Library Construction Kit (Clontech Laboratories, Palo Alto, CA, USA). After Proteinase K digestion and *Sfi* I digestion, cDNA size fraction was performed using QIAEXIIGEL Extraction Kit (Qiagen, Hilden, Germany). cDNA fractions longer than 1000 bp were precipitated by ethanol and glycogen. The cDNAs were ligated into pDNR-LIB vectors (Clontech Laboratories) with T4 DNA ligase (Takara Bio, Otsu, Japan). The constructs were then electro-transformated into *Escherichia coli* DH10B competent cells (Invitrogen, Carlsbad, CA, USA) with Bio-Rad Electroporator (Bio-Rad, Hercules, CA, USA). All the procedures followed the Creator SMART cDNA Library Construction Kit User Manual (Clontech Laboratories).

2.5. DNA sequencing and sequence analysis

The plasmids extracted from randomly selected 2395 clones were subjected to sequencing reaction using primer M13 and the MEGABace DYEnamic ET DYE terminator kit (GE Healthcare, Piscataway, NJ, USA) according to the manual instruction. The sequencing of 5′-termini of the randomly selected clones was performed in a MegaBACE 2000 sequencer (GE Healthcare). All the sequencing was completed in Beijing Genomics Institute (BGI, Beijing, China).

Raw single-pass sequence data was trimmed of the vector sequences, with low quality or sequences less than 100 bp being discarded. The remaining 2103 sequences were subjected to data analysis. Individual tentatively genes were compared with nucleotide and protein sequences by Blastn, Blastx (http://www.ncbi.nlm.nih.gov/BLAST/) and Swiss-Prot database (http://www.expasy.ch/sprot/) to search for similarity. The database-matched ESTs (E-value $< 10^{-5}$) were assigned to 21 groups according to their functional classification compared with Clusters of Orthologous Groups of proteins (COGs) database (http://www.ncbi.nlm.nih.gov/COG/). Kyoto Encyclopedia of Genes and Genomes (Kegg) (http://www.genome.jp/kegg/) Pathways were also used to uncover the molecular interaction and reaction networks for metabolism and various cellular processes of the cDNA library.

2.6. Real-time PCR analysis

Total RNA was extracted from all 7 tissues sampled (shark regenerated liver for 0 h, 1 h, 6 h, 12 h, 24 h, 48 h and 72 h) according to a standard guanidium isothiocyanate extraction procedure, followed by a DNase I treatment. After RNA quantification, 1 μg of total RNA was reverse transcribed using RevertAid™ First Strand cDNA Synthesis kit (Fermantas, Burlington, Canada).

All reactions were carried out using a Bio-Rad iCycler Real-time PCR (Bio-Rad, Hercules, CA, USA) and SYBR *Premix Ex Taq*™ (Takara Bio, Otsu, Japan). The reverse transcription product was submitted to real-time PCR in a final volume of 20 μl containing 10 μm of each specific primer (Table 1) and 1X SYBR *Premix Ex Taq*™. The amplification was carried out as follow: initial enzyme activation at 94 °C for 15 min, then 40 cycles of 94 °C for 15 s and 60 °C for 1 min. All primer pairs tested generated a unique peak in the dissociation curve with PCR efficiency (E) between 99% and 100%. Relative quantification (RQ) of each gene expression was calculated according to comparative Ct method using the formula: $RQ = 1 + E^{-\Delta Ct} = 2^{-\Delta Ct}$, with E = 100% and the expression of 18s rRNA as the endogenous control (Table 1). Comparison of mRNA expression variation was performed using analysis of variance followed by a Student's t test ($P < 0.05$).

3. Results

3.1. Properties of the cDNA library

The high-quality, directional cDNA library of shark regenerated hepatic tissues was constructed in this study. The primary titer of the library was counted for 10^6 pfu and the insertion efficiency was assessed as about 95.5%. The sizes of the inserts, based on the randomly selected 22 recombinant plasmids in the primary library, were ranging from 500 bp to 3000 bp as measured by PCR amplification (Fig. 1), indicating the cDNA library should fulfill the requirements for EST analysis.

3.2. Sequence analysis of the cDNA library

Single-pass sequencing was done on 2395 randomly selected bacterial clones from the 5′ ends of the cDNA library. After heading and tailing vectors, poor quality sequences and sequences shorter than 100 bp were removed, and the final number of ESTs was 2103, with an average length of 502 nucleotides.

To predict the roles of the corresponding gene products, the original sequences were assembled and edited to 217 contigs and 780 singletons, representing 997 tentatively unique genes. Hence,

Table 1
Primers used in real-time PCR experiment.

Gene	Primers (sense and antisense)
18s rRNA	Forward 5′-GACTCAACACGGGAAACCTCA -3′
	Reverse 5′-CAGACAAATCGCTCCACCAA -3′
Ras	Forward 5′-TGAAGTGGATGGCAAACAGG -3′
	Reverse 5′-ATCAGTGTCAGGATAGGAGAGTGGT -3′
FABP	Forward 5′- GGCAGAGGAATTTGACGAA -3′
	Reverse 5′- TGCCATCCTTCAATTCTCTAA -3′
HSS	Forward 5′-GATTGTGGAGCAAATTGGAA-3′
	Reverse 5′-GGCAATGTGGTCATCAACTT-3′
HGF	Forward 5′- AGAGACCTGCCACCTTGAAC-3′
	Reverse 5′ -TGGCATCACCAGGATATTCA-3′