



# Cloning and characterization of prion protein coding genes of Japanese seabass (*Lateolabrax japonicus*) and Japanese flounder (*Paralichthys olivaceus*)

Meijie Liao<sup>a,1</sup>, Zhiwen Zhang<sup>a,b,1</sup>, Guanpin Yang<sup>a,\*</sup>, Xiuqin Sun<sup>b</sup>, Guiwei Zou<sup>c</sup>,  
Qiwei Wei<sup>c</sup>, Dengqiang Wang<sup>c</sup>

<sup>a</sup>College of Marine Life Sciences, Ocean University of China, Qingdao 266003, PR China

<sup>b</sup>The First Institute of Oceanography, National Oceanic Administration of China, Qingdao 266061, PR China

<sup>c</sup>Changjiang Fishery Institute, Chinese Academy of Fishery, Jingzhou 434000, PR China

Received 12 September 2004; received in revised form 5 January 2005; accepted 23 January 2005

## Abstract

Complementary DNA (cDNA) sequences of Japanese seabass (*Lateolabrax japonicus*) and Japanese flounder (*Paralichthys olivaceus*) prion protein (PrP) encoding genes were cloned and characterized through RT-PCR approach. The PrPs of two fish species consist of 507 and 497 amino acid residues with an estimated molecular weight of about 54 and 52 kilodaltons, respectively. The deduced amino acid sequences of two sequences are about 65% similar to those of Japanese pufferfish (*Fugu rubripes*, stPrP-1) and Atlantic salmon (*Salmo salar*) in average, and bear the structural features of PrPs including a signal sequence, tandem repeats, a hydrophobic region, glycosylation sites, two cystein residues potentially involved in the formation of a disulphide bridge and a glycosyl phosphatidylinositol anchor site. The isolation of two PrP encoding genes will facilitate the exploration concerning the evolution, structure and function of PrPs and possible transmissible spongiform encephalopathies (TSE) in fish.

© 2005 Elsevier B.V. All rights reserved.

**Keywords:** *Lateolabrax japonicus*; *Paralichthys olivaceus*; Prion; Prion protein; Transmissible spongiform encephalopathy

## 1. Introduction

Proteinaceous infectious agents (prions) are the principle causing transmissible spongiform encephalopathies (TSE) of animals. Usually, prions are referred to the aggregates of prion protein (PrP), a normal membrane binding glycoprotein with unknown physiological function, found in fish,

\* Corresponding author. Tel.: +86 532 2031636; fax: +86 532 2032276.

E-mail address: [yguanpin@mail.ouc.edu.cn](mailto:yguanpin@mail.ouc.edu.cn) (G. Yang).

<sup>1</sup> The authors contributed equal data to this work.

reptiles, amphibians, birds and mammals. According to the protein only replication hypothesis, prions can initiate and catalyze the transformation of cellular PrPs into themselves (Bolton et al., 1982). Recently, it was found that prion replication might depend on RNA molecules as well (Deleault et al., 2003). Many properties of prions, such as their structure and cellular function remain to be unclear (Aguzzi and Polymenidou, 2004). The amino acid sequences of PrPs from different animal classes are diverse obviously, however, they hold conservative structural features including a signal sequence, tandem repeats, a hydrophobic region, glycosylation sites, a disulphide bridge and a glycosyl phosphatidylinositol anchor site (Suzuki et al., 2002).

Fish, birds and mammals are the most important meat sources for human consumption. Non-fish prions in feed containing prion-contaminated meat and bone meal may infect cultured fish. Fish prions may develop and spread in the processing and utilization of feed containing trash fish meal and low value fish meal. In order to assure our food security and sustainable fishery, fish PrPs and possible fish prions and TSE should be attended to intensively. In addition, the studies on fish PrPs will facilitate our understanding of the evolution, structure and function of PrPs and the susceptibility of fish to non-fish prions.

Comparative genomics is a powerful method for the isolation and characterization of novel genes (Premzl et al., 2003; Strumbo et al., 2001). Expressed sequence tags (ESTs) of Japanese pufferfish (*Fugu rubripes*) and Atlantic salmon (*Salmo salar*) PrPs have been identified through homologue searching, and then complete cDNA sequences cloned with rapid amplification of cDNA ends (RACE) (Oidtmann et al., 2003; Rivera-Milla et al., 2003). However, EST data are not available for the majority of fish species, thus it is not feasible to trace PrP encoding gene tags of these fish species in such a way. Since PrP amino acid sequences, especially in some structural regions, are relatively conserved within closely related organisms, degenerate PCR primers for fish PrP cDNA fragment amplification were designed in this study and used to amplify and clone Japanese seabass and Japanese flounder PrP cDNA fragments. Starting from these fragments, complete PrP cDNAs of two fish species were isolated with RACE. Japanese seabass

(*Lateolabrax japonicus*) and Japanese flounder (*Paralichthys olivaceus*) are two representatives of the diverse fish species commercially cultured currently in China. The cloning of their PrP encoding genes will facilitate the investigation of possible fish prions and fish TSEs.

## 2. Materials and methods

### 2.1. Materials

Total RNA extraction kit was purchased from Shanghai Sangon Biotechnology, China. M-MLV RTase cDNA Synthesis Kit, cDNA PCR Library Kit, TaKaRa Ex Taq™ HS polymerase and pMD18-T vector were purchased from Dalian Takara Biotechnology, China. Live Japanese seabass and Japanese flounder individuals were purchased from local aquatic product market of Qingdao, China.

### 2.2. Methods

Total RNAs were isolated from the brain tissues of Japanese seabass and Japanese flounder according to kit manufacturer's instruction and separated in 1% agarose gel. The quality of total RNA was judged according to the intensity and sharpness of 18 S and 28 S rRNA bands. The concentration of total RNA was estimated according to the intensity of quantitative yeast tRNA marker band on the adjacent lane. cDNA was synthesized from 10 µg total RNA and linked with adaptors by using M-MLV RTase cDNA Synthesis Kit in combination with cDNA PCR Library Kit, precipitated together with yeast tRNA carrier, dissolved in 20 µL water and stored at -20 °C.

Two primers, stF: 5'-GGG GG(A/T) TA(C/T) CCA AA(C/T) CAG AA(C/T) CC-3' and stR: 5'-CGA GGG AAA CGC CCA AG(G/T) CC(A/G) TA-3', were designed according to relatively conserved tandem repeat and hydrophobic region of Japanese pufferfish and Atlantic salmon PrPs. The middle fragments of Japanese seabass and Japanese flounder PrP cDNAs were amplified from 1 µL cDNA mixture. Thermocycling conditions were 30 cycles of 94 °C 1 min, 55 °C 1 min and 72 °C 2 min. Amplification volume was 50 µL. The PCR product was separated in

Table 1  
Similarities between any two of known fish PrPs including two obtained in this study (%)

	1	2	3	4	5
1 Fugu stPrP-1	100				
2 Fugu stPrP-2	15	100			
3 Atlantic salmon	61	9	100		
4 Japanese flounder	68	16	63	100	
5 Japanese seabass	66	13	62	75	100

GenBank accession numbers of Japanese pufferfish (*Fugu rubripes*) PrPs are AY141106 (stPrP-1) and AY188583 (stPrP-2) respectively, that of Atlantic salmon (*Salmo salar*) is AY141107, and those of Japanese seabass (*Lateolabrax japonicus*) and Japanese flounder (*Paralichthys olivaceus*) are obtained in this study.

agarose gel. Multiple bands were recovered from gel and ligated with pMD18-T vector separately. The ligation product was introduced into competent *E. coli* JM109. The recombinants were selected out through

the re-amplification of the middle fragments of Japanese seabass and Japanese flounder PrP cDNAs with universal sequencing primers on the vector or stF and stR. At least 4 clones were sequenced per band.

Two additional primers, stF2: 5'-GTC A(T)AC AGG TTT TGC CAA AAA AGC C-3' and stR2: 5'-ATC(T) CCA GCC ACA GCA CCG ACAA(G) C-3', were designed to anneal to the middle fragments of Japanese seabass and Japanese flounder PrP cDNAs. Nested PCRs were conducted by using stF, stF2 and RA adaptor primer to amplify the 3' end, and stR, stR2 and CA adaptor primer the 5' end of PrP cDNAs of two fish species, respectively. Recovering, cloning, insert-containing recombinant selecting and sequencing of two ends were done the same of the middle fragments.

Amino acid sequences of Japanese seabass and Japanese flounder PrPs were deduced, and accord-

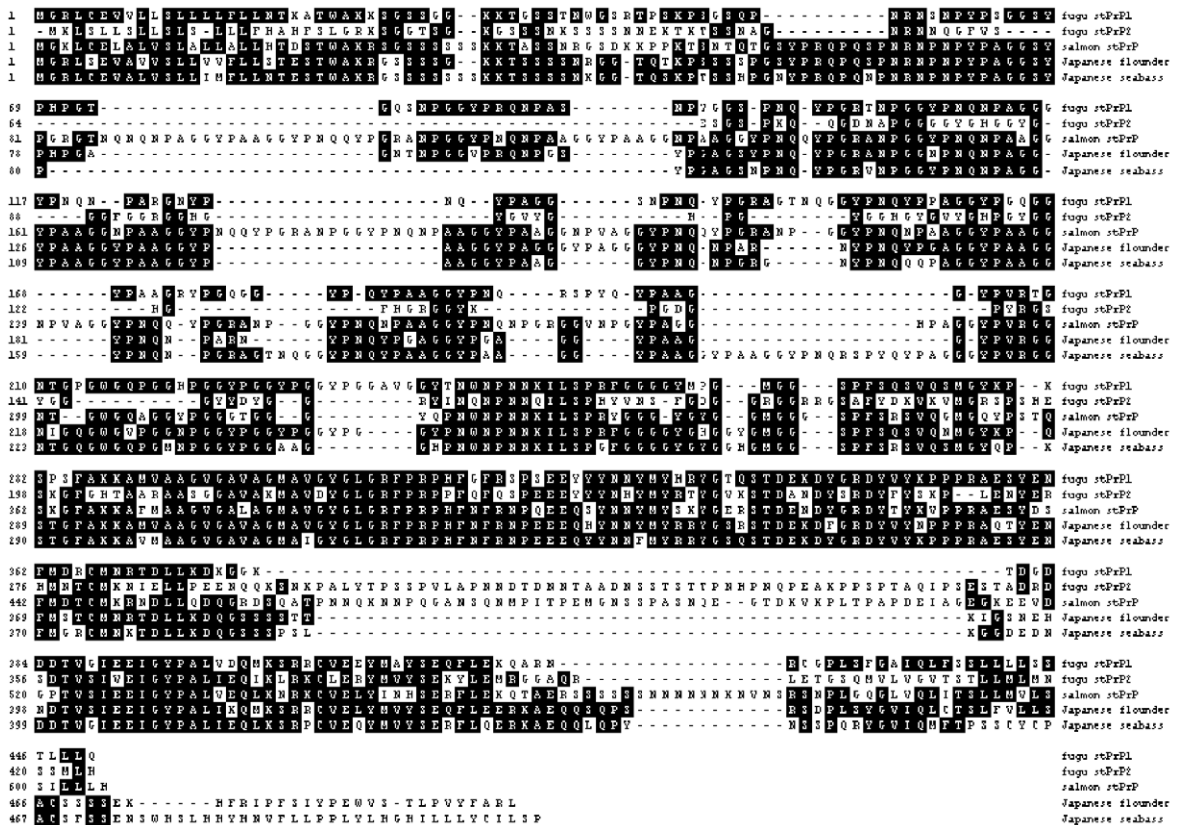


Fig. 1. The alignment of known fish PrP amino acid sequences including two obtained in this study. GenBank accession numbers of Fugu stPrPs are AY141106 (stPrP-1) and AY188583 (stPrP-2), respectively, that of salmon is AY141107, and those of Japanese seabass and Japanese flounder are AY854951 and AY854950, respectively.

ingly, the secondary structures, molecular weights and isoelectric points of two PrPs were predicted using DNASTar software. The signal sequences of two PrPs were predicted by using the prediction tool SignalP V1.1 at <http://www.cbs.dtu.dk/services/SignalP/>, and putative glycosyl phosphatidylinositol (GPI) anchor sites were analyzed with Big-PI Predictor at [http://www.mendel.imp.univie.ac.at/sat/gpi/gpi\\_server.html](http://www.mendel.imp.univie.ac.at/sat/gpi/gpi_server.html). Trans-membrane regions were determined with DAS at <http://www.sbc.su.se/~miklos/DAS/maindas.html>. Alignments, phylogenetic tree construction and sequence similarity analysis were carried out with DAMBE at <http://www.aixl.uottawa.ca/> in combination with MEGA 2 V2.1 at <http://www.megasoftware.net/>.

### 3. Results

PrP of Japanese seabass consists of 507 amino acid residues with the predicted molecular weight of 54 kD and isoelectric point at 9.07. PrP of Japanese flounder is

497 amino acid residues in length, weighing about 52 kD and be neutral when pH value is about 9.44. Japanese seabass PrP cDNA and Japanese flounder PrP cDNA sequences have been submitted to GenBank and accession numbers, AY854951 and AY854950, have been assigned to them respectively.

The similarity between PrP amino acid sequences of two fish species is about 75%, and the average similarity of these two PrPs with those of Japanese pufferfish (stPrP-1) and Atlantic salmon is about 65%, similar to 61% between Japanese pufferfish (stPrP-1) and Atlantic salmon (Table 1). In contrast, the average similarity of these two PrPs with that of Japanese pufferfish stPrP-2 is about 15%, similar to those between Japanese pufferfish stPrP-1 and atPrP-2, and between Atlantic salmon PrP and Japanese pufferfish stPrP-2. Among 3 PrPs synthesized by Japanese pufferfish (*F. rubripes*) genome, only stPrP-1 is closest to tetrapod PrPs in structural relatedness, while stPrP-2 and fugu prion-like protein have diversified in their evolution and are hardly expressed in brain tissue, although they appear to be more

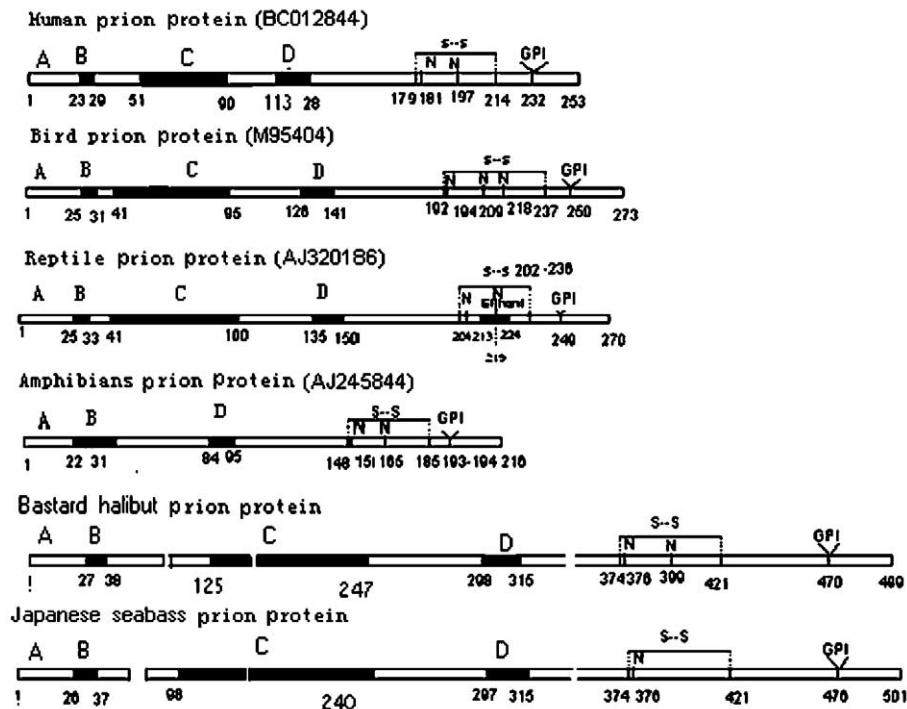


Fig. 2. Schematic presentation PrP structural features. (A) Signal sequence; (B) basic region; (C) tandem repeat of short peptide; (D) hydrophobic region. Please note that (C) is fragmented in order to reduce the length of fish PrPs.

closely linked to tetrapod PrPs in genomic localization (Oidtmann et al., 2003). Thus, Japanese pufferfish stPrP-2 is excluded in the identification of PrPs of two fish species.

The alignment of fish PrP amino acid sequences showed that many regions of fish PrPs are conservative, especially tandem peptide repeats and hydrophobic region, where our two PCR primers locate (Fig. 1). Japanese seabass PrP bears 3 helices and 3 putative trans-membrane regions with a signal sequence, a lysine serial, a tandem repeat of short peptide sequence and hydrophobic regions located between amino acid residues 1 and 25, 26 and 37, 98 and 240, 297 and 315, respectively, a glycosylation site and a GPI anchor site at 376 and 476 residues, respectively, and a potential disulfide bond between residues 374 and 421. Japanese flounder PrP is characterized by 3 helices and 3 putative trans-membrane regions with a signal sequence, a lysine serial, a tandem repeat of short peptide sequences and a hydrophobic region locating between 1 and 26, 27 and 36, 125 and 247, 298 and 315 amino acid residues, respectively, two glycosylation sites at 376

and 399 residues and a GPI anchor site at 470 residues, and a potential disulfide bridge between 374 and 421 residues. Judged from the sequence similarity and the structural features, both sequences we identified are PrP cDNAs encoding homologues to mammalian PrPs.

PrPs are relatively conserved in closely related species but highly variable between different animal classes. Phylogenetic analysis showed that fish PrPs cluster into one clade supported by very high bootstrap values, and the two PrPs identified in this study are similar to those of known fish species, but not to those of other animals including mammals, amphibians, reptiles and birds (Figs. 2 and 3).

#### 4. Discussion

Although PrP sequences of different animal classes are thoroughly diverse, PrPs of fish, amphibians, reptiles, birds and mammals bear conservative structural characters including a signal region, a tandem repeat, a hydrophobic region, a few glycosylation

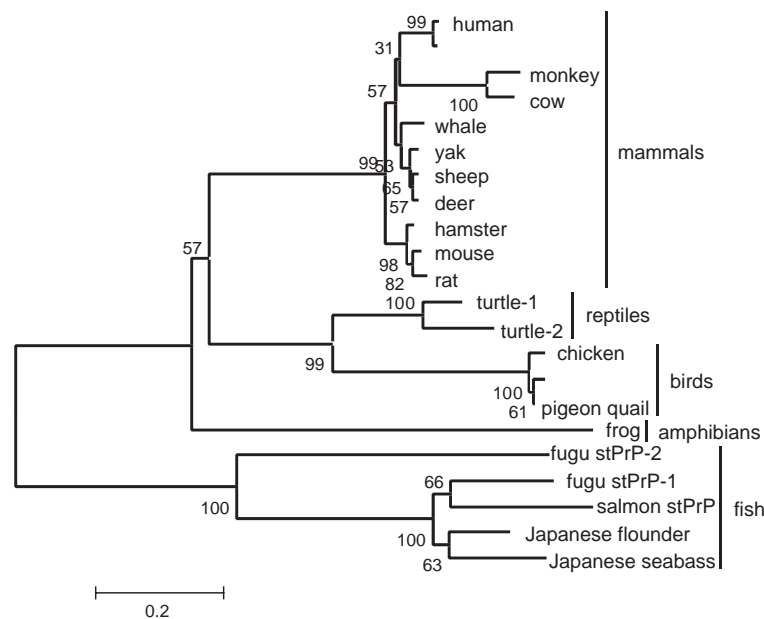


Fig. 3. Phylogenetic relationships of vertebrate PrPs. The accession numbers: Human, BC012844; Chimpanzee, U08296; Monkey, AY382293; Hamster, M33958; Mouse, BC006703; Rat, BC072692; Whale, AY133054; Sheep, AY350266; Deer, AY367637; Cow, AY720678; Frog, AJ320186; Turtle-1, AB088368; Turtle-2, AJ245488; Chicken, M95404; Pigeon, AF283319; Pigeon, AF256081; Quail, AF256081; Fugu stPrP-1, AY141106; Fugu stPrP-2, AY188583; Salmon, AY141107; Japanese flounder and Japanese seabass PrP sequences are obtained in this study. Bootstrap values are based on 2500 repeats.

sites, a GPI anchor site and 2 cystein residues possibly involved in the formation of a disulfide bridge (Suzuki et al., 2002). The PrP of Japanese seabass shares 75% amino acid sequence identity with that of Japanese flounder. The sequence identity of the newly identified 2 PrPs with those of Japanese pufferfish (stPrP-1) and Atlantic salmon is about 65% in average, similar to 61% between Japanese pufferfish (stPrP-1) and Atlantic salmon. Both PrP sequences presented here bear the structural features of known PrPs, with the sequence identity significantly higher than those of different animal classes (data not shown). According to these findings, we believe that the cDNA sequences we isolated from Japanese seabass and Japanese flounder are the homologues of prion protein encoding gene (*prnp*) of other animals.

The amino acid sequences of PrPs of closely related animals are diverse, which are more pronounced between PrPs of animals in different classes. Such sequence variations make the isolation of PrP encoding genes with the approaches like RT-PCR very difficult. Upon the cloning of hamster PrP encoding gene, its homologues of mammals including mouse and human are isolated using sequence homology based methods (Simoinc et al., 2000). However, the cloning of PrP genes of birds, reptiles and amphibians (Oesch et al., 1985; Simoinc et al., 2000; Harris et al., 1991) has been proven to be very difficult through such ways. Comparative genomics provide a convenient gateway or a powerful tool to find the homologues of known genes and sometime novel ones (Premzl et al., 2003; Strumbo et al., 2001). Through searching and comparison of genomic DNA, EST, cDNA and protein sequences in databases, we can find new genes, deduce their structures, chromosomal locations and possible functions. In fact, the genes encoding PrPs or PrP-like proteins of pufferfish, zebrafish and *Xenopus laevis* are identified in such a way (Premzl et al., 2003; Strumbo et al., 2001; Oidtmann et al., 2003; Rivera-Milla et al., 2003). However, data like ESTs and genomic sequences are not available for the majority of fish species, and the chances of finding target genes from a small number of ESTs is very low. Since the PrP sequences, especially in some regions, are relatively conservative within closely related species, degenerate primers corresponding to the tandem repeat and hydrophobic regions were

designed in this study, and used to amplify the middle parts of Japanese seabass and Japanese flounder PrP cDNAs. Starting from them, both 3' and 5' ends of cDNAs were isolated with RACE method. The successful isolation of PrP cDNAs from these two fish species has proven the feasibility of PrP encoding gene cloning of closely related organisms. The cDNA sequences of two fish PrPs will support the evolutionary analysis and the structural and functional decipherments of PrPs, and the exploration of possible fish TSEs as well.

Prion proteins are the products encoded by *prnp* gene or its homologues (Aguzzi and Polymenidou, 2004; Si et al., 2003a,b; Uptain and Lindquist, 2002). As an eukaryotic gene, *prnp* is spliced into exons and introns, however, PrP is encoded by only one exon. Pseudogenes of *prnp* have been found (O'Rourke et al., 2004; Lee et al., 1998). Proteins, similar to PrP in some characters but not all, have been reported, which are called PrP-like proteins (Suzuki et al., 2002; Premzl et al., 2003). We found that the length of tandem repeat peptide and repeat number, the length of hydrophobic region and the length of spacers between conservative structural features, and thus the total length of fish PrPs are longer than those of mammals, birds, amphibians and reptiles obviously. We believe that PrP may have a core sequence and follow some rules in its evolution. In addition, at least 3 PrP related elements exists in *F. rubripes* genome, although only one is closest to tetrapod PrPs in structural relatedness and expresses mainly in brain tissue (Oidtmann et al., 2003). Since gene duplication is common in fish, Japanese seabass and Japanese flounder genomes should harbor more PrP related elements. However, these questions wait for further investigations and more data.

### Acknowledgements

This research was financially supported by National Natural Science Foundation of China (approval number: 40176028), National Key R and D Program ("973" Program) of China (approval number: G1999012005) and Cooperative Project of The Key Lab of Freshwater Germplasm and Biotechnology of Chinese Ministry of Agriculture (approval number: LFB20040503).

## References

- Aguzzi A., Polymenidou M., 2004. Mammalian prion biology: one century of evolving concepts. *Cell* 116, 313–327.
- Bolton D.C., McKinley M.P., Prusiner S.B., 1982. Identification of a protein that purifies with the scrapie prion. *Science* 218, 1309–1311.
- Deleault N., Lucassen R.W., Supattapone S., 2003. RNA molecules stimulate prion protein conversion. *Nature* 425, 717–720.
- Harris D.A., Falls D.L., Johnson F.A., Fishbach G.D., 1991. A prion-like protein from chicken brain copurifies with an acetylcholine receptor-inducing activity. *Proceedings of the National Academy of Sciences of the United States of America* 88, 7664–7668.
- Lee I.Y., Westaway D., Smit A.F.A., Wang K., Seto J., Chen L., Acharya C., Ankener M., Baskin D., Cooper C., Yao H., Prusiner S.B., Hood L.E., 1998. Complete genome sequence and analysis of the prion gene region from three mammalian species. *Genome Research* 8, 1022–1037.
- Oesch B., Westaway D., Waelchli M., McKinley M.P., Kent S.B.H., Aebersold R.H., Barry R.A., Tempst P., Teplow D.B., Hood L.E., Prusiner S.B., Weissmann C., 1985. A cellular gene encodes scrapie PrP 27–30 protein. *Cell* 40, 735–746.
- Oidtmann B., Simon D., Holtkamp N., Hoffmann R., Baier M., 2003. Identification of cDNAs from Japanese pufferfish (*Fugu rubripes*) and Atlantic salmon (*Salmo salar*) coding for homologous to tetrapod prion proteins. *FEBS Letters* 538, 96–100.
- O'Rourke K.I., Spraker T.R., Hamburg L.K., Besser T.E., Brayton K.A., Knowles D.P., 2004. Polymorphisms in the prion precursor functional gene but not the pseudogene are associated with susceptibility to chronic wasting disease in white-tailed deer. *Journal of General Virology* 85, 1339–1346.
- Premzl M., Sangiorgio L., Strumbo B., Graves J.A.M., Simonic T., Gready J.E., 2003. Shadoo, a new protein highly conserved from fish to mammals and with similarity to prion protein. *Gene* 314, 89–102.
- Rivera-Milla E., Stuermer C.A.O., Malaga-Trillo E., 2003. An evolutionary basis for scrapie disease: identification of a fish prion mRNA. *Trends in Genetics* 19 (2), 72–75.
- Si K., Lindquist S., Kandel E.R., 2003. A neuronal isoform of the *Aplysia* CPEB has prion-like properties. *Cell* 1156, 879–891.
- Si K., Giustetto M., Etkin A., Hsu R., Janisiewicz A.M., Miniaci M.C., Kim J.H., Zhu H.X., Kandel E.R., 2003. A neuronal isoform of CPEB regulates local protein synthesis and stabilizes synapse-specific long-term facilitation in *Aplysia*. *Cell* 115, 893–904.
- Simoinc T., Duga S., Strumbo B., Asselta R., Cecilian F., Ronchi S., 2000. cDNA cloning of turtle prion protein. *FEBS Letters* 469, 33–38.
- Strumbo B., Ronchi S., Bolis L.C., Simonic T., 2001. Molecular cloning of the cDNA coding for *Xenopus laevis* prion protein. *FEBS Letters* 508, 170–174.
- Suzuki T., Kurokawa T., Hashimoto H., Sugiyama M., 2002. cDNA sequence and tissue expression of *Fugu rubripes* prion protein-like: a candidate for the teleost orthologue of tetrapod PrPs. *Biochemical and Biophysical Research Communications* 294, 912–917.
- Uptain S.M., Lindquist S., 2002. Prions as proprotein-based genetic elements. *Annual Review of Microbiology* 56, 703–741.