White spot syndrome virus Manipulates Ubiquitin Gene Expression in *Penaeus monodon*

R. Vidya · P. Gireesh-Babu · K. Pani Prasad

Received: 31 May 2012 / Accepted: 10 September 2012 / Published online: 16 December 2012
© Indian Virological Society 2012

Abstract The present study reports the effect of WSSV on the expression profiles of shrimp ubiquitin gene both at protein and transcript levels at different time points after infection. A gradual decrease in ubiquitin protein levels was observed till 12 hpi (0.25 and 0.23 µg/mg of the total protein in gills and muscle, respectively) with a sudden increase at 18 hpi (1.86 and 1.8 µg/mg of total protein in gills and muscle, respectively). The RT-PCR results showed a gradual decrease in ubiquitin mRNA levels in gill tissue till 12 hpi while a more or less constant mRNA levels were observed in muscle tissue till 12 hpi. However, a fourfold and 16 fold induction in ubiquitin mRNA levels at 18 hpi in gill and muscle, respectively suggests its possible regulation by WSSV in the manifestation of the disease.

Keywords *Penaeus monodon* · Ubiquitin · Expression profiling · WSSV · Semi-quantitative RT-PCR · ELISA

White spot syndrome virus (WSSV) is currently the most serious viral pathogen of shrimp worldwide causing 100 % cumulative mortality in 2–10 days [6]. WSSV, the largest animal virus known to affect cultured shrimp is a sole member of the monotypic family Nimaviridae, genus *Whispovirus* [2]. It has a circular dsDNA genome of about

300 kbp that contains approximately 185 open reading frames (ORFs) [5]. Due to its serious impact on shrimp aquaculture, there is an urgent need to understand WSSV and to unveil the underlying mechanisms involved in WSSV pathogenesis in shrimp. Recent research supports the role of ubiquitin pathway in WSSV infection in shrimps. Interesting feature of this particular interaction is the occurrence of a common RING finger domain in the viral proteins (WSSV199, WSSV222, WSSV249 and WSSV403) and host ubiquitin ligases. It is reported that the ring finger domain of certain viruses act as host E3 ubiquitin protein ligase and sequesters the host ubiquitin conjugating enzyme for viral pathogenesis [8]. Although several studies have been reported on the expression profiles of ubiquitin ligases, no work was reported on the fate of ubiquitin gene expression in response to WSSV infection. This is important because, although the virus can modulate the expression of E2 and E3 ligases in its favour, availability of ubiquitin protein plays a major role in degradation pathway. In this context, the present study was conducted to see whether the virus affects the ubiquitin gene expression in the same way as the E2 ligase. Following WSSV infection in *Penaeus monodon*, the ubiquitin gene expression at mRNA and protein levels was determined by semi-quantitative RT-PCR and quantitative ELISA techniques, respectively.

Penaeus monodon of 10–20 g body weight were procured from a local farm and maintained in 1,000 l fibre-glass tanks with aerated natural sea water of 12 ppt. The animals were fed thrice a day with artificial pellet feed (CP, Thailand). The WSSV crude extract for experimental challenging was prepared by the method of Xie and Yang [9]. A total of five time points after WSSV challenge i.e., 0, 3, 6, 12 and 18 h were selected to quantify the expression levels of ubiquitin gene in comparison to unchallenged

R. Vidya · K. Pani Prasad
Division of Aquatic Environment and Health Management,
Central Institute of Fisheries Education, ICAR, Mumbai, India

P. Gireesh-Babu (✉)
Division of Fish Genetics and Biotechnology, Central Institute
of Fisheries Education, Indian Council of Agricultural Research,
Versova, Mumbai 400061, India
e-mail: girishbiotech@gmail.com

controls. For this, three groups comprising 15 shrimps (10–15 g) each were kept in plastic crates of 25 l capacity with aeration. Two groups of shrimps were injected with 75 μ l of WSSV crude extract at the ventral side of the second abdominal segment. The group injected with same volume of STE buffer served as control. Five shrimps at each time point were collected from all the groups including control. Gill and muscle tissue from the specimens was collected aseptically and stored at -80°C .

Relative quantification of the ubiquitin mRNA at different time points in *P. monodon* challenged with WSSV crude extract was done through RT-PCR. β -actin gene was used as an internal control. Primer sets PmUbq-F: AATACGTCCTCAAGCTGC and PmUbq-R: GG AAGATGTGCGACCTTGTCAG; and Pmactin-F: CCCA GAGCAAGAGAGGTA and Pmactin-R: GCGTATCCT TCGTAGATGGG were used to amplify *P. monodon* ubiquitin and β -actin gene partial sequence, respectively. Total RNA from gill and muscle tissues of experimental animals was isolated using TRIzol[®] method and first strand cDNA synthesized using standard protocols [7]. PCR was carried out in a 25 μ l reaction volume containing 2 μ l cDNA, 10 pmol of each specific primer, 200 μ M of each dNTPs, 0.75 U of *Taq* DNA polymerase and $1\times$ *Taq* buffer containing 1.5 mM MgCl_2 . The cycling parameters included an initial denaturation of 3 min at 94°C followed by 20 s at 94°C , 20 s at 55°C and 30 s at 72°C repeated for 30 cycles with a final extension at 72°C for 5 min. The PCR products were run on 2 % agarose and EtBr stained gels were imaged using Syngene Gel documentation system. DNA quantification was performed by GelQuant software (V 2.0). The intensities of the ubiquitin bands were normalized against β -actin to avoid errors in the sample variation and PCR efficiency. The normalized ubiquitin expression in control was used to calculate the fold change in treated samples. The results showed that there is an initial decrease in ubiquitin transcript levels till 12 h post infection (hpi) in gill tissue and more or less constant in muscle while a sudden increase in the transcript levels was seen in both the tissues at 18 hpi. While the muscle tissue registered a 17-fold increase in the ubiquitin mRNA, the gill tissue showed only fivefold induction (Fig. 1).

To quantify the ubiquitin protein levels in tissue samples, an ELISA test was developed using anti-ubiquitin antibodies raised in rabbit [3]. For this, the ubiquitin protein from shrimp muscle tissue was purified following the protocol of Agell and Mezquita [1]. This differential purification specifically purifies ubiquitin along with other high mobility group proteins that can be separated on 18 % polyacrylamide gel and requires no additional screening. The presence of ubiquitin was further validated by electrophoresing the protein extract along with bovine serum

Fig. 1 Expression levels of ubiquitin mRNA in gill and muscle tissues of healthy and WSSV infected black tiger shrimp, *Penaeus monodon* at 0, 3, 6, 12 and 18 hpi. A semi-quantitative RT-PCR technique is employed using β -actin as reference gene

ubiquitin (Suyog Diagnostics, India) in an 18 % polyacrylamide gel, which showed the co-migration of shrimp ubiquitin with bovine serum ubiquitin. The gel purified shrimp ubiquitin was used to immunize rabbits and produce rabbit anti-ubiquitin immunoglobulins following the method of Haas and Bright [4]. The total immunoglobulin concentration of the antiserum was estimated to be 1.54 mg/ml. The specificity and cross reactivity of ubiquitin antiserum was determined by agarose gel precipitation test. The results show that the anti-ubiquitin antibodies specifically reacted with shrimp ubiquitin while no cross reactivity was observed with goat gamma globulin (data not shown).

The ubiquitin concentration in control group was found to be constant throughout the experimental period, which was 1.431 $\mu\text{g}/\text{mg}$ of total protein in gills and 1.342 $\mu\text{g}/\text{mg}$ of total protein in case of muscle. But in the infected group, there is a reduction in the ubiquitin protein levels from 0 to 12 hpi while there is a sudden increase in the expression levels at 18 hpi over controls in both gill and muscle tissues (Fig. 2). The concentration of ubiquitin fell to a minimum of 0.25 and 0.23 $\mu\text{g}/\text{mg}$ of the total protein in both gill and muscle, respectively at 12 hpi. However, at 18 hpi a sudden increase in the ubiquitin levels compared to control suggests a strong upregulation of the ubiquitin gene. The concentration of the ubiquitin protein was estimated to be 1.85 and 1.67 $\mu\text{g}/\text{mg}$ of the total protein in gills and muscle, respectively at 18 hpi. We have also earlier reported the expression profiles of WSSV RING finger domain gene, WSSV ORF 199 and shrimp ubiquitin conjugating enzyme (PmUbc) genes [5]. A significant increase in PmUbc expression at 24 hpi was observed followed by a decline till 72 hpi. Expression of WSSV199 was observed at 24 hpi in WSSV infected *P. monodon*. Since the up-regulation of PmUbc was observed at 24 hpi where

Fig. 2 Expression levels of ubiquitin protein in gills (a) and muscle (b) tissues of healthy and WSSV infected *Penaeus monodon* as determined by ELISA using anti-ubiquitin polyclonal antibodies produced in rabbit. The ubiquitin protein levels were determined at 0, 3, 6, 12 and 18 h post WSSV infection in *P. monodon*

WSSV199 expression was detected, it can be speculated that these proteins might interact with host ubiquitination pathway for viral pathogenesis. Taken together with the present study it can be concluded that WSSV modulates the host ubiquitination pathway and redirects the host immune proteins for degradation in the 26S proteasome for the progression of disease.

In *L. vannamei* it was found that the expression of ubiquitin conjugating enzyme (PvUbc) gene increases

consistently with time post WSSV challenge and it was suggested that PvUbc expression is induced by WSSV infection [8]. Fang and Kwang [2] also reported that the WSSV 403 expression increases consistently post WSSV infection in *L. vannamei*. The present study reports the ubiquitin gene expression profiles in WSSV challenged shrimp at different time points and stands as a preliminary study to understand the role of ubiquitin in the disease manifestation. Further research is needed to determine the exact relationship between ubiquitin gene expression and WSSV infection and its possible role in disease manifestation.

Acknowledgments The authors would like to acknowledge financial support from the Institute, Indian Council of Agricultural Research, New Delhi for the Masters' fellowship to the first author and Director, CIFE for providing facilities.

References

1. Agell N, Mezquita C. Cellular content of ubiquitin and formation of ubiquitin conjugates during chicken spermatogenesis. *Biochem J*. 1988;250:883–9.
2. Fang H, Kwang J. Identification and characterization of a new E3 ubiquitin ligase in white spot syndrome virus involved in virus latency. *Virology*. 1988;5:151.
3. Gutierrez JA, Guerriero V Jr. Quantitation of Hsp70 in tissues using a competitive enzyme-linked immunosorbent assay. *J Immunol Methods*. 1991;143:81.
4. Haas AL, Bright PM. The immunochemical detection and quantitation of intracellular ubiquitin–protein conjugates. *J Biol Chem*. 1985;260:12464.
5. Jeena K, Pani Prasad K, Pathan MK, Gireesh Babu P. Expression profiling of WSSV ORF 199 and shrimp ubiquitin conjugating enzyme in WSSV Infected *Penaeus monodon*. *Asian-Aust J Anim Sci*. 2012;25:1184–9.
6. Lightner DV. A handbook of shrimp pathology and diagnostic procedures for diseases of cultured penaeid shrimp. Baton Rouge: World Aquaculture Society; 1996. p. 304.
7. Sambrook SJ, Russell DW. Molecular cloning, a laboratory manual. 3rd ed. Cold Spring Harbor: Cold Spring Harbor Laboratory Press; 2001.
8. Wang Z, Chua HK, Gusti AARA, Fang H, Fenner B, Manopo I, Wang H, Kwang J. RING-H2 protein WSSV249 from white spot syndrome virus sequesters a shrimp ubiquitin-conjugating enzyme, PvUbc, for viral pathogenesis. *J Virol*. 2005;79:8764–72.
9. Xie XX, Yang F. Interaction of white spot syndrome virus VP26 protein with actin. *Virology*. 2005;336:93–9.