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EXPERIMENTALLY INDUCED DISEASE

# Tissue Distribution of the 27.8 kDa Receptor and its Dynamic Expression in Response to Lymphocystis Disease Virus Infection in Flounder (*Paralichthys olivaceus*)

**R.-H. Wu[*], X.-Q. Tang[*], X.-Z. Sheng[*] and W.-B. Zhan[*,†]**

[*] *Laboratory of Pathology and Immunology of Aquatic Animals, Ocean University of China, 5 Yushan Road, Qingdao, PR China and* [†] *Function Laboratory for Marine Fisheries Science and Food Production Processes, Qingdao National Laboratory for Marine Science and Technology, No. 1 Wenhai Road, Aoshanwei Town, Jimo, Qingdao 266071, China*

## Summary

Lymphocystis disease virus (LCDV) enters and infects the gill cells of flounder (*Paralichthys olivaceus*) via a 27.8 kDa membrane protein receptor. In the present study, immunohistochemistry was performed to locate the tissue distribution of this molecule in healthy flounder and showed that it was widely distributed in the tissues tested. Indirect enzyme-linked immunosorbent assay (ELISA) showed that the expression of the receptor in healthy flounder was highest in the gills and stomach, then in the skin, intestine and liver, followed by the spleen, head kidney, heart, ovary and brain and finally the kidney. On LCDV infection, ELISA indicated that the expression of the receptor, as determined by ELISA, was significantly upregulated in all tissues of LCDV-infected flounder compared with controls, but this expression decreased over the 4 weeks post infection. In contrast, real-time quantitative polymerase chain reaction demonstrated that the copy number of the LCDV gene in the tissues increased with time post infection, and that viral loads were higher in the tissues with higher expressions of the receptor. These results point to a correlation between high expression of the 27.8 kDa receptor and efficient LCDV propagation. The wide tissue distribution of the receptor might be one reason why LCDV can infect various tissues leading to systemic infection.

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*Keywords:* 27.8 kDa receptor; flounder; lymphocystis disease virus; tissue distribution

## Introduction

Lymphocystis disease virus (LCDV) is the causative agent of lymphocystis disease, which affects over 140 different wild and cultured fish species worldwide, including those of commercial importance such as flounder (*Paralichthys olivaceus*) (Kitamura *et al.*, 2007). The diseased fish develop characteristically hypertrophied cells and form tumour-like nodules on the skin, fins, mouth, gills and internal organs, causing significant economic loss (Fuji *et al.*, 2006; Sheng *et al.*, 2007; Cano *et al.*, 2009a). Advances have been made in understanding the epidemiology, diagnosis, prophylaxis and treatment of lymphocystis disease (Basurco *et al.*, 1990; Bowden *et al.*, 1995; Li *et al.*, 2010; Sheng *et al.*, 2013; Sun *et al.*, 2013), as well as the target tissues, reservoir and vector of LCDV (Kvitt *et al.*, 2008; Cano *et al.*, 2009b, 2013); however, current understanding is limited regarding the pathogenesis of LCDV infection.

Viral receptors are the primary determinants of tissue tropism and play important roles in the pathogenicity of viruses (O'Donnell *et al.*, 2009). A 27.8 kDa protein from flounder gill (FG) cells, a cell line developed by Tong *et al.* (1997), was identified as a cellular receptor for LCDV. Evidence supporting this

Correspondence to: X.-Z. Sheng (e-mail: xzsheng@ouc.edu.cn).

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hypothesis included the following observations: (1) the 27.8 kDa protein could be coprecipitated with LCDV (Wang *et al*., 2011); (2) LCDV infection of FG cells was inhibited by polyclonal and monoclonal antibodies (MAbs) specific for the 27.8 kDa protein (Wang *et al*., 2011; Sheng *et al*., 2012); and (3) expression of the 27.8 kDa protein was upregulated on FG cells and conferred sensitivity to LCDV infection (Wu *et al*., 2015). Further studies on the tissue distribution of the 27.8 kDa receptor (27.8R) as well as its expression during viral infection in flounder will improve understanding of the tissue tropism and pathogenesis of LCDV.

The aims of the present study were to investigate the tissue distribution of the 27.8R in healthy flounder by immunohistochemistry and to characterize the dynamic expression of the 27.8R induced by LCDV infection by indirect enzyme-linked immunosorbent assay (ELISA). Additionally, the copy number of a target gene from LCDV was determined by quantitative real-time polymerase chain reaction (q RT-PCR).

## Materials and Methods

### *Fish*

Healthy flounder, 700–900 g in weight, were obtained from a fish farm in Qingdao, Shandong province, China. A total of 48 fish were kept in rearing tanks supplied with aerated running seawater at 18 ± 1°C and fed daily with dry food pellets for 1 week before LCDV infection.

### *Monoclonal Antibodies*

A mixture of two mouse monoclonal antibodies (MAbs) specific for the 27.8R (clone numbers 3D9 and 2G11 mixed in equal volumes 1:1) (Sheng *et al*., 2012) and the white spot syndrome virus (WSSV) (clone number 1D5; Zhan *et al*., 1999) were produced by our laboratory, and the same batches of antibodies as described in Wu *et al*. (2015) were used. The MAbs are of the IgG1 subclass.

### *Immunohistochemistry*

Immunohistochemistry (IHC) was carried out to characterize the tissue distribution of the 27.8R in healthy flounder. Tissues from three healthy flounder, including gill, stomach, intestine, skin, liver, ovary, brain, spleen, kidney, head kidney and heart, were fixed in Bouin's fixative and rinsed with 70% alcohol. Subsequently, the tissues were dehydrated and embedded in paraffin wax. Sections (7 μm) were mounted on poly-L-lysine-coated slides. After dewaxing in xylene and rehydration in graded ethanol, antigen retrieval was carried out as described by Hirayama *et al*. (2002). Sections were pretreated with 0.5 M EDTA for 1 h at room temperature to inhibit endogenous alkaline phosphatase (AP) activity, and were incubated with blocking buffer containing bovine serum albumin (BSA) 3 % (w/v) for 1 h to block non-specific antibody binding. Incubation with anti-27.8R MAb (1 in 1,000 dilution) was in a humid chamber for 1 h at 37°C. As negative controls, anti-27.8R MAb was replaced by phosphate buffered saline (PBS, pH 7.4) and also by anti-WSSV MAb (1 in 1,000 dilution). After washing three times with PBS, the sections were incubated with biotinylated horse anti-mouse IgG (Vector Laboratories, Burlingame, California, USA; 1 in 300 dilution) and streptavidin–alkaline phosphatase (SA–AP; Boehringer Mannheim Biochemicals, Indianapolis, Indiana, USA) for 1 h at 37°C, respectively. After three washes in PBS, the slides were incubated with AP-Red™ substrate kit (Zymed, San Francisco, California, USA) and counterstained with Mayer's haematoxylin for 10 min, and then examined under a light microscope (Olympus).

### *LCDV Infection and Sampling*

LCDV particles were isolated, purified and stored in our laboratory (Cheng *et al*., 2006; Sheng *et al*., 2012) and the concentration of LCDV protein was determined by the Bradford method (Bradford, 1976). Before infection, the 11 tissues listed above were sampled from three individual healthy fish. For infection, a total of 42 fish were divided randomly into two groups. Group 1 was injected intramuscularly with 300 μl purified LCDV (diluted in PBS, 100 μg per fish) and group 2 was injected intramuscularly with an equal volume of sterile PBS. The same 11 tissues were sampled from three individuals at 0 h, 3 h, 12 h, 3 days, 1 week, 2 weeks and 4 weeks post infection (hpi, dpi or wpi, respectively). The membrane protein of each tissue was pooled from three fish in each group for detecting the expression of the 27.8R by ELISA. Total DNA was extracted from the sampled tissues for LCDV quantification by qRT-PCR using the TIANamp Marine Animals DNA Kit™ (Tiangen, Beijing, PR China) according to the manufacturer's instructions.

### *Membrane Protein Extraction and Enzyme-linked Immunosorbent Assay*

Tissue membrane protein was extracted according to the method of Wang *et al*. (2011). Tissues were homogenized in lysis buffer (250 mM sucrose, 20 mM Tris–HCl, pH 8.0, 137 mM NaCl, 10% glycerol and 1% NP-40) containing the Roche Complete protease

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