Immunohistochemical Research of the Expression of Nile Tilapia (Oreochromis niloticus) NCCR-1 in Various Tissues

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Abstract It is well known that non-specific cytotoxic cell is a kind of lymphocyte in teleosts, which plays an important role in nonspecific immunity by up-regulating inflammation response and releasing cytokines. NCCs are equivalents of NK cells in mammals. NCC in fishes distributes in tissues and organs with spontaneous cytotoxicity which can kill various target cells such as allogenic or heterogeneous tumor cells, parasitic protozoan and cells induced by pathogens. NCCR-1 is a landmark receptor on NCCs. This study sectioned brain, head kidney, liver and spleen of Nile Tilapia (Oreochromis niloticus) and stained NCCR-1 as well. The study obtained the distribution of NCCs in tissues via immunohistochemical analysis. Furthermore, we also isolated and obtained NCC of head kidney through Percoll density gradient centrifugation, stained by immunohistochemistry and tagged with FITC. The distribution was the same as the staining result in tissues. This study observed the distribution characteristics of NCC in tissues more clearly than qPCR technique, which might lay the foundation for further studies on the function of NCC.

Keywords Nile tilapia (Oreochromis niloticus); NCCR-1; Immunohistochemistry

Background Non-specific cytotoxic cell (NCC) is a kind of lymphocyte in teleost fish, which can induce nonspecific immune response in fish under adverse environment or pathogen stimulation, and its effect is equivalent to the NK (natural killer cell) cell in mammals (Jaso-Friedmann et al., 2004). The effector target cells of NCC include tumor cells and parasitic protozoa, which indirectly resist virus invasion by killing viral-infected abnormal cells (Graves et al., 1985). In addition, through cytokine regulation, fish NCC can also cause inflammatory reactions like NK cells in mammals (Jaso-Friedmann et al., 2001). At present, NCC has been confirmed to exist in many organs or tissues of teleost fishes, including the head-kidney (Mulero et al., 1994), spleen (Wood and Cheers, 1985), liver (Barnes et al., 1977) and peripheral blood (Seppola et al., 2007).

NCCR-1 is a marker receptor on NCC. Studies by Jaso-Friedmann et al. (2004) predicted that NCCR-1 might have three signal domains: antibody binding domain, signal transduction domain and transcription activation domain. The existing reports about NCCR-1 were concentrated on Danio rerio (Reimers et al., 2006), Ictalurus punctatus (Evans et al., 1996), Oncorhynchus mykiss (Fischer et al., 2013), Cyprinus carpio L. (Seppola et al., 2007) and Stegastes partitus (McKinney and Schmale, 1997). NCCR-1 is considered to be able to recognize and bind various target cells to induce cytotoxic reactions.

Tilapia is an important aquatic economic animal, which can provide high quality animal protein. Because of its delicious flavor with less thorn, more meat and strong adaptability, tilapia has been widely cultured in southern China and has brought considerable output value (Li, 2006). In recent years, environmental pollution and poor management patterns had led to large-scale outbreaks of parasites, bacteria and viruses, resulting in serious economic losses (Gan et al., 2014). Immunohistochemistry is a basic experimental method in human clinical tumor research (Li et al., 2014). Furthermore, this experimental method also plays a certain role in fish research (Yang et al., 2017), and specific substances are shown through nonspecific antibody binding and staining.
Nonspecific immunity is an extremely important immune response in teleost fish. In this study, we located and stained NCCRP-1 receptor in Oreochromis niloticus by immunohistochemical method to determine the distribution of NCC in various tissues, and would lay a foundation for the study of non-specific immunity related to tilapia in subsequent experiments.

1 Results and Analysis
1.1 Distribution of NCCRP-1 in tissues
In this study, the distribution of NCCRP-1 in the tissues was determined by antibody incubation staining and four tissues including brain, head kidney, liver and spleen were selected. The nuclei of most tissue cells were stained blue by hematoxylin, and the positive result of DAB staining was brownish yellow. The results indicated that NCCRP-1 was distributed in all four selected tissues. It was the least distributed in the brain, showing dispersion or aggregation. In other three tissues, the distribution of NCCRP-1 was more than that in brain, and was also distributed in dispersion, occasionally aggregated, or concentrated on the surface of the mucosa (Figure 1).

![Figure 1 Four tissues sections of Nile tilapia]
Note: A: Brain control; B: Brain; C: Head Kidney control; D: Head Kidney; E: Liver control; F: Liver; G: Spleen control; H: Spleen; Red arrow: NCC; Magnification: 200×

1.2 Isolation, purification and identification of NCC from head kidney
Cell suspension containing NCC from Oreochromis niloticus head-kidney was obtained by two layers of discontinuous gradient Percoll separation method. Trypan blue staining and counting showed that the cell survival rate was 85%. Through antibody incubation and HRP staining, NCC was found to be brownish yellow under light microscope. In addition, NCC was found to be yellow-green fluorescence under fluorescence inversion microscope by combining with FITC fluorescent antibody. Moreover, when high purity NCC was incubated and stained with antibodies, we found that compared with normal tissue cells, NCC individuals were smaller, and cell shapes were diverse. Nuclei occupied most of the space of the whole cell with less cytoplasm, and some cells could be observed a split in the nucleus (Figure 2).

![Figure 2 Staining of NCC in head kidney of Nile Tilapia]
Note: A: HRP control; B: HRP staining; C: FITC control; D: FITC staining; Magnification: 400×

2 Discussion
NCC is an important lymphocyte in teleost fishes, and plays an important role in non-specific immunity (Jaso-Friedmann et al., 2001). Therefore, studying the amount and distribution of NCC in fish will help us better understand the specific functions of various tissues and organs and the mechanism of NCC action. After exogenous stimulation of teleost fish, the extracellular structure of NCCRP-1 is responsible for binding antigens, while the intracellular structure is responsible for activating and regulating the killing function of NCC.
Previous studies have shown that NCCR-1 is a marker receptor of NCC (Evans et al., 1996), but there are very few reports on the study of fish NCC by immunohistochemical technique. Most of the previous studies have obtained the expression of the receptor among different tissues and organs by qPCR method (Cai et al., 2013), or verified its existence by flow cytometry after Percoll (Zhou, 2012) or Ficoll (Evans et al., 1984) separation. The relative expression of NCC from various organs and the percentage of NCC in the whole tissue of cells can be obtained by the above methods. However, the existence and distribution of NCC in tissues cannot be directly observed.

In this study, brain, head-kidney, liver and spleen were selected to carry out immunohistochemical experiments. By observing slices, it was found that NCC was distributed in all the above organs, which was consistent with the reported results. The brain plays an irreplaceable role in the process of life as central nervous organs of animals (Robinson and Meyer, 1966). Therefore, safety and steady state play important roles in the exercise of its function. However, we made continuous slices of the brain and the results showed that the number of NCC in the brain was significantly lower than that in immune organs, which was not consistent with the expectation of experiment. A large number of studies have confirmed that there is indeed a blood-brain barrier in fish, although fish belong to lower vertebrates (Stratman et al., 2017), which makes it difficult for foreign microorganisms to enter the brain. In the results of our experiment, there was still a part of NCC in the brain, because in addition to non-specific killing of foreign antigens, NCC could also kill endogenous and exogenous tumor cells (Graves et al., 1985). This part of NCC in the brain might be related to its timely killing of own tumor cells and the prevention of canceration of central nervous cells. Moreover, some studies have shown that some bacteria, such as Streptococcus agalactiae, can enter the cranial cavity with blood after being swallowed by macrophages, thus causing diseases (Schuchat, 1998). In the following experiments, we can use pathogens to stimulate fish, select a time point to sample the brain, and observe the number and distribution of NCC in the brain stimulated under the pathogens to further explore the characteristics of NCC.

The widespread distribution of NCC in head-kidney and spleen, which are important immune organs of fish (Wood and Cheers, 1985; Mulero et al., 1994), once again highlights its importance. The head kidney and liver not only bear the specific immunity of teleost fishes, but also play an extremely important role in nonspecific immunity at the early stage of pathogen stimulation. It could be observed from the slices that NCC was aggregated and distributed on the mucosal surface of the internal lumen of the organ, which indicated that NCC would carry out killing on the mucosal surface immediately when the fish was infected and the pathogen spread with body fluids. A large number of reports have shown that the mucosa of teleost fish also plays an important role in host immunity (Rombout et al., 2014), and the results of this study were consistent with the existing literature. In addition to the intraluminal mucosa inside the organ, the gills (Somamoto et al., 2015) and intestines (Georgopoulou and Vernier, 1986) of fish are also responsible for mucosal immunity. Next, the above tissues would be sectioned to observe the number and distribution of NCC. Meanwhile, NCCR-1 is also distributed in the liver of Oreochromis niloticus. Interestingly, although the liver is not the main immune organ of teleost fishes, it was also confirmed that NCCR-1 receptor existed in the liver of Oreochromis niloticus, Lutjanus sanguineus, Onchorhynchus mykiss, Cyprinus carpio and other species (Evans et al., 1996; Seppola et al., 2007; Fischer et al., 2013). It was speculated that the liver, as the detoxifying organ of fish (Cao, 2008), might be often stimulated by exogenous, and the probability of forming cancer cells might be higher than that in other organs. Cancer cell is just one of the target cells of NCC. Timely and effective phagocytosis of cancer cells would play an important role in maintaining the homeostasis of the environment in fish.

In this study, high purity NCC in the head-kidney tissue of Oreochromis niloticus was obtained by Percoll separation method. NCC positive signals were obtained through HRP and FITC antibody markers. Compared with other published fish NCC, the NCC of Oreochromis niloticus also had the marker receptor protein, which proved that NCCR-1 was a kind of marker receptor protein of NCC once again. Compared with immunohistochemical staining in tissues, this experiment excluded nonspecific antibody markers of other kinds of cells in tissues to further determine the experimental results.
3 Materials and Methods

3.1 Research materials

*Oreochromis niloticus* was purchased in Zhanjiang Seafood Market (about (300 ± 10) g/tail). PBS buffer solution was purchased from BBI Life Sciences Company of China. RPMI-1640 medium and fetal bovine serum were purchased from Gibco Company of America. Primary antibody NCCRP-1 Antibody (5C.6) was purchased from Novus Company of America. Fluorescent-labeled secondary antibody Goat Anti-Mouse IgM-FITC was purchased from American Southern Biotech Company. HRP-anti-mouse IgM was purchased from Wuhan Boster Biological Company. Desktop high-speed freezing centrifuge Centrifuge 5810R was purchased from German Eppendorf Company. Flow cytometry BDFACS Verse flow cytometer was purchased from American BD Company.

3.2 Immunohistochemical analysis

After the fish was anesthetized, 75% alcohol was used to wipe the fish body, and the corresponding tissues were obtained by dissecting the fish body. After that, the tissues were immediately put into 10% formalin and fixed at 4°C for 24 hours. Then, the tissues were embedded in pre-heated paraffin wax and sliced by a continuous slicing machine after fixation. During dewaxing, the slices were put into xylene for 15 minutes, and then taken out and put into new xylene for 15 minutes, after which the slices were taken out and put into anhydrous ethanol for 5 minutes, and then taken out and put into new anhydrous ethanol for 5 minutes. After that, the slices were continuously replaced and soaked in 85% alcohol and 75% alcohol for 5 minutes, and finally the slices were cleaned with single distilled water. Next, we put the tissue slices into an antigen repair box and completely immersed them in the EDTA antigen repair buffer with pH = 8.0. In the case of non-drying slices, we first heated and boiled the slices in a microwave oven, then stopped heating and kept the temperature for 8 minutes, and later heated for 7 minutes with a small fire. After cooling at room temperature, the slices were washed 3 times with pH = 7.4 PBS for 5 minutes each time. The slices were incubated in 3% hydrogen peroxide solution for 25 minutes at room temperature in dark environment, then the slices were washed 3 times with pH = 7.4 PBS again for 5 minutes each time to block endogenous peroxidase. PBS on the surface of the slice was gently sucked dry with absorbent paper. In order to prevent the antibody from flowing away, we coated the sample tissues with Pap Pen, and added 10% normal rabbit serum in the tissues at room temperature to seal the tissues evenly for 30 minutes. Then, the blocking liquid was gently thrown away. After that, we added the primary antibody to the slices which were incubated overnight at 4°C in a wet box. Next, the slices were washed 3 times with pH = 7.4 PBS for 5 minutes each time. Using absorbent paper to dry the PBS on the surface gently, HRP-labeled secondary antibody was dropped onto the slices, making sure that the secondary antibody could cover the tissue evenly and then the tissue was incubated at room temperature for 50 minutes. Then, the slices were washed 3 times with pH = 7.4 PBS for 5 minutes each time. We used absorbent paper to dry the PBS on the surface gently, and freshly prepared DAB coloring solution was dropped onto the slices. The positive stain was claybank. When positive signals were found under the microscope, the stain was terminated and the slices were washed with distilled water. We stained the slices with hematoxylin for about 3 minutes, and rinsed them clean with distilled water. We differentiated them with 1% hydrochloric acid alcohol for several seconds, and then washed them under clean water. The slices were returned to blue with ammonia water, and then they were rinsed with clean water. The slices were put into 75% alcohol for 6 minutes, 85% alcohol for 6 minutes, and absolute alcohol for 6 minutes successively. After that, the slices were soaked for 6 minutes in the new anhydrous ethanol and 5 minutes in xylene. Then, dehydration treatment was carried out. The slices were taken out, dried and later they were sealed with neutral gum. Finally, we put the slices under a microscope to observe and take photos.

3.3 Isolation, purification and identification of head-kidney NCC

*Oreochromis niloticus* in good condition was selected for anatomy. After dissection, the internal organs of tilapia were normal in shape and color. A total of 2 g kidney tissues were obtained and placed in 400 mesh sieve, as well as cut with a small dissecting shear and washed with a rubber-tipped dropper at the same time. The head-kidney tissues were fragmented and dispersed in 60 mL RPMI-1640 medium. We used two layers of Percoll with volume fraction of 34% and 51% to separate *Oreochromis niloticus* whole lymphocytes, and two layers of Percoll with different concentration were added to each centrifugal tube. 0.85% NaCl solution was added to the Percoll.
solution to prepare Percoll working fluid. The Percoll working fluid was formulated into 34% and 51% concentration. We took a 15 mL centrifuge tube with 51% lower layer Percoll concentration and 34% upper layer Percoll concentration. Upper layer Percoll was added gently and it was better to have clear liquid level without fluctuation. Then the cell suspension prepared in 1.2.1 was added to the upper layer Percoll, it was better to have clear liquid level without fluctuation. The volume of the two-layer Percoll was 2 mL, and the volume of the added cell suspension was 4 mL. The Percoll was centrifuged and the time was set to 30 minutes. The rotation speed was 500 g with the temperature of 4°C, and the speed of rise and fall was slow. The target cell layer was aspirated and placed in a new centrifuge tube. Then, we added PBS 2 mL and the Percoll was centrifuged for 4 minutes with the rotation speed of 300 g. The rising and falling speed was set to normal, and the supernatant was discarded. 2 mL PBS suspension and primary antibody was added to incubate on ice for 0.5 h. 2 mL PBS was added to wash out the uncombined primary antibody, and the Percoll was centrifuged for 4 minutes, setting the rotation speed to 300 g. The rising and falling speed was set to normal, and the supernatant was discarded. Next, we added the second antibody and incubated on ice without light for 0.5 h. 2 mL PBS was added, and the Percoll was centrifuged for 6 minutes with rotating speed of 300 g. The rising and falling speed was normal, and the supernatant was discarded. PBS was added to make lymphocyte suspension. Through flow cytometry analysis, we knew the distribution of lymphocyte groups and found that NCC particle size was smaller than neutrophils and monocytes. The target cells were found in the flow cytometry (Figure 2A; Figure 2C).

Authors’ contributions
ZQ was responsible for the separation of NCC from Oreochromis niloticus, immunohistochemical staining and paper writing. Professor JJC was the project manager and guided students to study. YM was responsible for the immunohistochemistry of four tissues of Oreochromis niloticus. HY was responsible for providing antibody and experimental guidance. Associate professor CJ was responsible for designing experiments. All authors read and approved the final manuscript.

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