ISOLATION AND CLONING OF ABCA1 GENE FROM RASBORA SARAWAKENSIS

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Isolation and cloning of ABCA1 gene in Rasbora sarawakensis

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DECLARATION

I hereby declare that this Final Year Project entitled “Isolation and cloning of ABCA1 gene from Rasbora sarawakensis” is based on my original work except for quotations and citation, which have been duly acknowledged. I also declare that it has not been submitted in support of an application for another degree or qualification to this or any other university or institute of higher learning.

(Annie Kuba)

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<td>APO</td>
<td>Apolipoprotein</td>
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<td>ATP</td>
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Isolation and cloning of \textit{ABCA1} gene in \textit{Rasbora sarawakensis}

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\textbf{ABSTRACT}

ATP-binding cassette (ABC) genes is a gene that encodes ABC transporter proteins that has vital functions in ATP hydrolysis. The genes also play a role in the maintenance of the plasma membrane and in the transport of fatty acids and sterols within the body. In this research, the objectives is to clone and identify \textit{ABCA1} gene from \textit{Rasbora sarawakensis}, and to establish the \textit{ABCA1} gene transcript in the \textit{Rasbora sarawakensis} for the gene expression used. The polymerase reaction chain was performed by using degenerate primer and was transform by using \textit{E.coli} XLI-Blue competent cell. Therefore, through this research it can help to improve and understand the physiological function of \textit{ABCA1} gene in \textit{R. sarawakensis}.

Keywords: ABC transporter, \textit{ABCA1} gene, Polymerase Chain Reaction, \textit{Rasbora sarawakensis}.

\textbf{ABSTRAK}


Kata kunci: ABC pengangkut, gen \textit{ABCA1}, Rantaian reaksi polimerase, \textit{Rasbora sarawakensis}.
CHAPTER 1: INTRODUCTION

A membrane proteins are proteins that interact with biological membranes. For an organisms, membrane play a very important function in the movement of ion or other molecules in or out from the cell. The movement of ions or other molecules into the cell is called influx, whereas the movement of ions or other molecules out of the cell is called efflux (Vasiliou, et al., 2009). Then, in this movement, the ATP-powered pump that will acts the role for energy released by ATP hydrolysis in order to move the substrate across the membrane in or out of cells against their electrochemical gradients. It also take part in the movement of most drugs and their metabolites through cell surface and cellular organelles membrane.

Generally, in mammals, ABC transporter are expressed mostly in the intestine, liver, blood-brain barrier, blood-testis barrier, placenta and kidney. There are 48 characterized of ABC genes in human, which divided into seven distinct subfamilies based on organization of domains and amino acid homology. The genes play a role in the maintenance of the lipid bilayer and in the transport of fatty acids and sterols within the body. At the same time, ABC genes are also beneficial in emphasized by the fact that mutations in at least 11 of these genes are already known to cause severe inherited disease (Vasiliou, et al., 2009). Then, the mutated of this gene may cause the Tangier Disease T1 because of the defective apolipoprotein-I-induced lipid outflow (Walter et al., 2004).

This research is about to clone the ABCA1 gene and to analyse the expression. ABCA1 gene is one of the type of ATP-binding cassette transporter. The ATP-binding cassette (ABC) superfamily of active transporters composed of about 50 functionally diverse prokaryotic and eukaryotic transmembrane proteins. As known, the ATP-binding cassette
function to transports the substrates into and out of the cell. It is also involved in the intracellular compartmental transport.

*Rasbora sarawakensis* is selected to be a model organisms in this research. It is a small fish that endemic to the island of Borneo. Due to this, it is easily obtain a sample in the research. *R. sarawakensis*. This species is belong to Cyprinidae’s family, which is same family with *Danio rerio*. As we know, *D. rerio* is a popular model organisms in molecular biology research for the studies of vertebrate development, disease, regulatory physiology, biological pathways and toxicological mechanisms.

As has been known, *ABCA1* gene plays an important role in an organism. It is vital in take part in the ATP system in a membrane transport protein. But the research of this gene in *R. sarawakensis* has not been further verified. Therefore, through this research it can help to improve and understand the physiological function of *ABCA1* gene in *R. sarawakensis*.

The objectives of this research are:

a. To clone and identify *ABCA1* gene from *Rasbora sarawakensis*.

b. To establish the of *ABCA1* gene transcript sequence in *Rasbora sarawakensis* for the gene expression used.
CHAPTER 2: LITERATURE REVIEW

2.1. Membrane transport protein

Membrane transport proteins is a protein that interact with biological membranes involved in the movement of ions, small molecules, or macromolecules. Transport proteins are integral transmembrane proteins which is exist permanently within the membrane across which they transport substances. Then, there are four types of membrane transport proteins, which are transporter, ion channels, ATP-powered pumps and aquaporins (Vasiliou, et al., 2009).

Transporter is type of membrane protein that will facilitate the movement of a specific substrate. This is happen either with or against its concentration gradient (Vasiliou, et al., 2009). The transporter undergoes conformational change and transport the molecule across the membrane after the substrate bound at the specific site on the transporter. Mostly, many of the transporters is belong to the solute-carrier (SLC) gene superfamily, and include passive transporters, symporters and antiporters.

Ion channels are pore-forming membrane proteins that help to create and maintain small-voltage gradients that across plasma membrane surfaces of all living cells (Vasiliou, et al., 2009). It is also acts a role to create a resting membrane potential, shaping actions potentials and other electrical by gating the flow of ion across the cell membrane, controlling the flow of ions across secretory and epithelial cells and regulating cell volume.

Then ATP-powered pumps that function by using energy released by ATP hydrolysis to move substrates across membrane in or out of cells against their electrochemical (Vasiliou, et al., 2009). There are two class of ABC transporter, which is importers and exporters. Furthermore, membrane transport proteins also divided to passive or active. The
passive transporter also known as uniporters or facilitative transporters, which is transport substrates down a concentration gradient. The active transporter has known also as cotransporters which couple the movement of a type of ion or molecules against its concentration gradients.

Figure 2.1.: The mechanism for the uniport, symport and antiport. (Adapted from https://kaiserscience.wordpress.com/biology-the-living-environment/cells/active-transport-across-cell-membranes/)

From the figure above, the transported molecule and cotransported ion move in the same direction across a membrane, the transporter is called a symporter. Then, when they move in opposite directions, the transporter is called an antiporter.

For the aquaporins is a protein that bi-directional membrane channels which transport water because water is transported as an uncharged molecule and not as an ion (Vasiliou, et al., 2009). It is an integral membrane proteins from a larger family of major intrinsic proteins that form pores in the membrane of biology cells.
2.2. ABC transporter

ABC transporter refer to ATP-binding cassette transporter, which are transmembrane proteins that utilize the energy of adenosine triphosphate (ATP) binding and hydrolysis to carry out certain biological processes. It is constitute the largest transporter gene family is the ATP-binding cassette (ABC) transporter superfamily (Dean, et al., 2001). Due to this, it is worth to mention that ABC transporter is a protein that very vital in an organism for the many processes in the cell.

Until now, there are 58 members of the ABC family that have been described, which is including 49 human ABC genes and 9 genes that found in other animal species. Then, 68% from 58 genes are present in all of vertebrates’ genomes (Ferreira, et al., 2014). According to their sequence and organization, ATP-binding domains also called nucleotide binding domains (NBDs). ABC proteins has been grouped into 8 subfamilies in eukaryotes, which from A to H. Then, also has been grouped in 7 subfamilies in human genomes, which from A to G. So far, ABCH gene only found in zebrafish, which its function still cannot be identified (Ferreira, et al., 2014).

If the mutation happen, it will cause several of human genetic disorders, for example cystic fibrosis, neurological disease, retinal degeneration, cholesterol and bile transports defects, anaemia and drug response (Dean, et al., 2001).

Generally, this type of gene is utility in the maintenance of the lipid bilayer and in the transport of fatty acids and sterols within the body. But, specifically ABC system function can be distributed into three different main classes. One of the functions of the ABC system is acts as importer, which is mediate the uptake nutrients in a prokaryotes (Davidson, et al., 2008). If there is importers, then must have the exporters, which are involved in the secretion of various molecules, such as peptides, lipids, hydrophobic drugs, polysaccharides, and
proteins, including toxins such as hemolysin (Davidson, et al., 2008). The third category of system is apparently not involved in transport, with some members being involved in translation of mRNA and in DNA repair (Davidson, et al., 2008).

2.3. *ABCAI* gene

*ABCAI* refers to ATP-binding cassette sub-family A member 1 gene that mediated the transports of cellular cholesterol, phospholipids and other metabolites to high density lipoproteins, that are associated with no or very little lipids (Oram, 2005). *ABCAI* gene is abundantly expressed at liver and tissue macrophages. At the same time, it is also known as cholesterol efflux regulatory protein (CERP) is a protein which is in humans. For the structure of *ABCAI*, each of the halves has a transmembrane domain that containing 6 helices and a nucleotide binding domain, which contain two conserved peptides motifs that known as Walker A and Walker B. The walker A motif is a motif in associated protein with phosphate binding, whereas is motif in most P-loop protein that situated well downstream of Walker A. Both of the motifs will present proteins that will utilize the ATP. Apart from that, *ABCAI* also has been expected to have an NH₂ terminus oriented into the cytosol and two large extracellular loops that are highly glycosylated which is linked by one or more bonds of cysteine (Oram, 2005).

Generally, the function is to transports the cholesterol, phospholipids and other lipophilic molecules across the cellular membrane. It plays a major role in cholesterol homeostasis and HDL metabolism (Martinez-Beamonte, et al., 2013). It is also form a channel in the membrane that will promote flipping of lipids from the inner to outer membrane leaflet by an ATPase-dependent process (Oram, 2005). Then, *ABCAI* gene also
uses to target the specific membranes domain for the lipid secretion and to remove the cholesterol which is has potential to accumulate as cytosolic cholesterol lipid droplets.

According to Martinez-Beamonte, et al. (2013), the reconstituted high density lipoprotein (HDL) with sphingomyelin (SM) and apolipoprotein (APO) has promoted the greatest cholesterol efflux in each type of cell and was enhanced by increased expression of ABCG1. The APOA1 is binds with the ABCA1 and this translocates phospholipids and cholesterol directly or indirectly to obtain the pre-β HDL.

The down-regulation of expression of ABCA1 by interferon-gamma in murine peritoneal macrophages is because the reduced of PC and SM efflux to APOA1 (Martinez-Beamonte, et al., 2013). Then, it has been proposed that the ATPase activity of ABCA1 is stimulated by PC and SM. At the same time, the ABCA1 stability is controlled by apolipoprotein-mediated signaling requiring SM. This is because the digestion of this lipid increased ABCA1 stability (Martinez-Beamonte, et al., 2013).

In human, ABCA1 has been studied, where the genetic locus is located at 9q31.1 on chromosomes 9 (NC_000009.11 (107543282.107690527). This gene is consists of 2,261 amino acids integral membrane protein that will comprise two halves of similar structure (Oram, 2005). Apart from that, it also has 54 exon counts. This gene is significant in new therapeutic for drug development which is designed for clearing cholesterol from arterial macrophages.

Figure 2.2: The red line indicates the location of ABCA1 gene on chromosome 9 in human genome.
2.4. *Rasbora sarawakensis*

First of all, Rasboras is a species that known as schooling fish species. This is due to their behaviour that always swim together in the group. This species also can tolerant to live in the aquarium, but the water need to be well-filtered. The condition also must be suitable with the rasboras, such as the pH range of water must be around 5.5 to 7.8.

For the structure of the fish, the rasboras size is around 10 cm long. The6n, the female and male rasboras have a different size of body. Usually, the female rasboras, they will have much bigger body size than the male rasboras. This is because the female rasboras need to carry the eggs. According to the body size, the sexes of rasboras can be identified. Other than that, the male raboras have the triangular markers on their bodies which is extended further back on their lower part of abdomen compared to females.

For the fertilization process, the female rasboras usually will deposit their eggs on the underside of leaves. This is because they will rubs their belly on the selected leaf, when they prepared for spawning period. Then, this action that will attract the male rasboras in other to join the spawning. The males rasboras will join the female rasboras to spawn by inverting-positioned himself together with the females. This action will repeated for almost or more than two hours, then the females can lay egg up to 100 eggs. But all this action, usually occur naturally, because it’s hard to breed the rasboras.

In this research, the type rasboras that has been used was *Rasbora sarawakensis*. The *R. sarawakensis* is a species of small freshwater fish that endemic to the island of Borneo. They are belonging to family of *Cyprinidae*. There is also mention that rasbora had already been placed in the subfamily *Daniones* (Tang et al., 2010). Other than, *R. sarawakensis*, there is other type of *Rasbora* fish such as *Rasbora amplistriga, Rasbora baliensis, Rasbora caverii* and so on.
Commonly, this species is known as Sarawak Rasbora. This due to the location where this species can be obtained. But, although the name is sound mostly like Sarawak but this species is not only found in Sarawak state (Malaysia). It is also can be found at the area around West Kalimantan/Kalimantan Barat province (Indonesia) (SeriouslyFish, 2014). It has been recorded from numerous river system including Batang Kayan and Sungai Sarawak in Sarawak and the Mempawah and Melawi in Kalimantan Barat.

In natural environment, *R. sarawakensis* is inhabits in thick marginal vegetation with slow-moving forest streams (Seriously Fish, 2014). This is because this species do not have high ability to swim at the fast-moving stream. Then, the temperature of the water for their live is around 22-26 °C. They also live in the slow moving water with slightly acidic pH in the range 6.0-7.5 and the hardness is in the range of 2-12 °H. All of the characteristic of habitat is important in order to keep the *R. sarawakensis* in the aquarium. Besides, the species’ diet is also need to be known. Generally, for the invertebrates both aquatic and terrestrial is mostly similar. Then, in the aquarium it will feed with dried foods of a suitable size. Feed sparingly and they love to eat high quality micro pellets.
For the structure, this species have small size of body, which is around 2-5 cm. This can make they will be able to be kept in large number easily and cheaply in the laboratory. The male and female species will have different features. The sexual dimorphism of the mature females *R. sarawakensis* should be notice rounder-bellied and little large than males. As usual, the females larger belly because they need to carry the eggs.
CHAPTER 3: MATERIALS AND METHODS

3.1. List of materials

- TRI reagent (Sigma, USA)
- Chloroform
- Isopropanol
- TAE (Tris-acetate EDTA) buffer
- EasyScript® Reverse Transcriptase (TransGen, China)
- QIAquick® Gel Extraction Kit (Qiagen, Germany)
- 0.1M CaCl₂
- Glycerol solution
- pGEM®-T Easy Vector (Promega, USA)
- LAIX (LB agar/ Ampicillin/ IPTG® / X-Gal)
- QIAprep Spin Miniprep Kit (Qiagen, Germany)
3.2. Methods

3.2.1. Maintenance of sample (*Rasbora sarawakensis*)

This sample which is *Rasbora sarawakensis* was obtained from the fish facility in the Department of Molecular Biology University Malaysia Sarawak. The temperature of the water was set to 26 °C, which was suitable to the fish to live. Then, the fish was feed 4 times a day, which at 9am, 12am, 3pm and 5pm. The tank of the fish was cleaned once a week. The fish was placed under 12-hour light and 12-hour dark photoperiod.

3.2.2. RNA extraction using TRI reagent

The tissue samples from *Rasbora sarawakensis* was homogenized in TRI reagent (1 ml per 50-1000 mg of tissue) in an appropriate homogenizer. The volume of the tissue should not exceed 10% of the volume of the TRI Reagent. After homogenization, the homogenate was centrifuged at 12,000 rpm for 10 minutes at 2-8 °C in order to remove the insoluble material. The supernatant that was contained RNA and protein. There was a layer of fatty material on the surface of the aqueous phase that should be remove because the sample had a high fat content. The clear supernatant was transfer to a fresh tube. The samples was allowed to stand for 2-15 minutes at room temperature in order to ensure complete dissociation of nucleoprotein complexes. Then, 0.2 ml of chloroform per ml of TRI reagent was added. The sample was covered tightly, shake for 15 seconds vigorously and allowed to stand for 2-15 minutes at room temperature. The resulting mixture was centrifuged at 12,000 rpm for 15 minutes at 2-8 °C. The mixture into 3 phases was separate by the centrifugation. The phases are a red organic phase (containing protein), an interphase (containing DNA), and a colourless upper aqueous phase (containing RNA). Aqueous phase was transferred to a fresh tube and was added 0.5 ml of isopropanol per ml of TRI reagent used in sample preparation and mix. Next, the sample also was allowed to stand for 5 - 10 minutes at room