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Effects of hypoxia on the concentration of circulating miR-210 in serum and the expression of HIF-1α and HSP90α in tissues of olive flounder (*Paralichthys olivaceus*)

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Hypoxia is a serious problem in the marine ecosystem causing a decline in aquatic resources. MicroRNAs (miRNAs) regulate the expression of genes through binding to the corresponding sequences of their target mRNAs. Especially, miRNAs in the cytoplasm can be secreted into body fluids, which called circulating miRNAs, and the availability of circulating miRNAs as biomarkers for hypoxia has been demonstrated in mammals. However, there has been no report on the hypoxia-mediated changes in the circulating miRNAs in fish. miR-210 is known as the representative hypoxia-responsive circulating miRNA in mammals. To know whether fish miR-210 also respond to hypoxia, we analyzed the change of circulating miR-210 quantity in the serum of olive flounder (*Paralichthys olivaceus*) in response to hypoxia. The expression of hypoxia related genes, hypoxia inducible factor 1α (HIF-1α) and the heat shock protein 90α (HSP90α) was also analyzed. Similar to the reports from mammals, miR-210-5p and miR-210-3p were significantly increased in the serum of olive flounder in response to hypoxia, suggesting that circulating miR-210 levels in the serum can be used as a noninvasive prognostic biomarker for fish suffered hypoxia. The target genes of miR-210 were related to various biological processes, which explains the major regulatory role of miR-210 in response to hypoxia. The expression of HIF-1α and HSP90α in the tissues was also up-regulated by hypoxia. Considering the critical role of HIF-1α in miR-210 expression and HSP90 in miRNAs function, the present up-regulation of HIF-1α and HSP90α might be related to the increase of circulatory miR-210, and the interaction mechanism among HIF-1α, HSP90α, and hypoxia-responsive microRNAs in fish should be further studied.

Key words: Hypoxia, Circulatory microRNA, Olive flounder, Serum miR-210, HIF-1α, HSP90α.

Introduction

Organisms living in aquatic environments are often faced with hypoxia that caused by various environmental factors. Hypoxia can be disastrous especially for sessile organisms that are not able to move to other regions. The fish in aquaculture farms also can become a victim of hypoxia because of the restricted movement of culture facilities. Recently, the frequent occurrences of hypoxic water masses (< 2 mg O_2/L) have been a threat to the fish cultured in coastal areas (Rabalais et al., 2010). Fish alter their physiological responses to cope with the hypoxia, and many factors related to oxygen sensing and transport, erythropoiesis, and angiogenesis are involved in the adaptation to hypoxia Richards, 2011(Lays etal., 2009;).

MicroRNAs (miRNAs) are small noncoding RNAs

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(19-23 nucleotides in length), and regulate the expression of genes through binding to the corresponding sequences of their target mRNAs. miRNAs are initially transcribed as long precursors, pri-miRNAs, which are processed by Drosha to generate approximately 70 nucleotide stem loops (pre-miRNAs) that are transported to the cytoplasm where Dicer cleaves these pre-miRNAs into functional mature miRNAs (Bartel, 2004; Hutvagner and Zamore, 2002). As one kind of miRNA can simultaneously target several different mRNAs, the expression of a great portion of genes in eukaryotes are regulated by miRNAs. Especially, miRNAs in the cytoplasm can be secreted into body fluids, which called circulating miRNAs, through being packaged in small membranous vesicles (exosomes, shedding vesicles, and apoptotic bodies) or through being associated with RNA-binding proteins or lipoprotein complexes, which protect those extracellular miRNAs from degradation by ubiquitous ribonucleases (RNases) in the circulation (Gibbings et al., 2009; Valadi et al., 2007). In mammals, the availability of circulating miRNAs as early biomarkers for specific disease states has been demonstrated (Mitchell et al., 2008; Zhang et al., 2010). Especially, as the hypoxia is closely associated with cancers, researches on the hypoxia-responsive miRNAs in tissue and blood have been extensively conducted (Bandara et al., 2014). However, in fish, although the effect of hypoxia on the expression of miRNAs in tissues of marine medaka (*Oryzias melastigma*) was reported (Lai et al., 2016; Lau et al., 2014; Tse et al., 2015), there is no report on the hypoxia-mediated changes in the circulating miRNAs.

The hypoxia-inducible factor 1α (HIF-1α) has been noticed as the key regulator for cellular hypoxia adaptation (Semenza, 2009). Under normoxic conditions, several proline residues of HIF-1α are hydroxylated by proline hydroxylases, which mediates binding of HIF-1α to von Hippel-Lindau tumor suppressor protein (vHL) that ubiquitinates HIF-1α to be degraded in the proteasome (Ohh et al., 2000). However, under hypoxic conditions, hydroxylation of HIF-1α is inhibited, which leads to stabilization of HIF-1α. HIF-1 α accumulated in the cytoplasm translocates to the nucleus and dimerizes with HIF-1β that is constitutively expressed irrespective of oxygen tension, then, binds to hypoxia response elements and regulates transcription of HIF-dependent genes (Kallio et al., 1997; Poellinger and Johnson, 2004). Furthermore, the regulatory role of HIF-1α over multiple microRNAs including miR-210 has been reported in mammals (Huang et al., 2009). A chaperone protein heat shock protein 90 (HSP90) plays an important role in the regulation of the activity of transcription factors including HIF-1 under hypoxia (Minet et al., 1999). Moreover, the association between HSP90 and Argonaute 2 that is essential for the incorporation of microRNAs into the RNA-induced silencing complex (RISC) can be induced by hypoxia (Iwasaki et al., 2010).

miR-210 is the representative hypoxia-responsive circulating miRNA in mammals (Chan et al., 2009; Hua et al., 2006; Kulshreshtha et al., 2007). In this study, to know whether fish miR-210 also respond to hypoxia, we investigated the change of circulating miR-210 quantity in the serum of olive flounder (*Paralichthys olivaceus*), the major marine cultured fish in Korea, in response to hypoxia. Furthermore, to know whether the quantity of serum miR-210 is related to HIF-1 and HSP90, the expression of HIF-1α and HSP90α was also analyzed.

Materials and methods

Fish and experimental design

Ten olive flounder weighing approximately 100 g were obtained from a local fish farm and were maintained in a 500 L tank with aeration. The fish were acclimated at 18 ± 1°C for one week before starting the experiment. The level of dissolved oxygen (DO) was monitored using DO meter iSTEK Multi 90i (iSTEK, KOREA) through the experiment. To induce

hypoxia, DO was lowered and maintained at 2 mg/L for 6 h by bubbling nitrogen gas into the tank. Just before lowering DO, 5 fish in the tank were randomly sampled and used as the control group, and at 6 h post-exposure to hypoxia, the remained 5 fish were sampled. Blood collected from the caudal vein was stored in 4°C for 12 h, then, serum was isolated by centrifugation. The liver, brain and muscle tissue were isolated from each fish and stored at -80°C.

Extraction of RNA from serum and quantitative real time polymerase chain reaction (Q-PCR) for circulating microRNA

Total RNA was isolated from 100 μl of serum using miRNeasy serum Mini kit (Qiagen) in accordance with the manufacturer's protocol. Briefly, 500 μl of Qiazol and 100 μl of chloroform were added to 100 μl of serum, followed by centrifugation for 15 min at 12,000 g at 4°C. Next, 300 μl of the RNA-containing aqueous phase was transferred into a new tube, and RNA was precipitated with 450 μl of 100% ethanol and loaded on miRNeasy purification columns. Purified RNA was eluted from the column matrix with 14 μl of RNase free water. In order to adjust for variations of miRNA extraction efficiency among samples, 5 μl of synthetic *Caenorhabditis elegans* miR-39 (Syn-cel-miR-39, Qiagen) was spiked into each sample as the external reference after the addition of the denaturing solution and went through the entire RNA isolation process. To aid in improving the RNA yield, total RNA from bacteriophage MS2 (Roche) was added to each sample as a carrier. Each total RNA (2 μl) was converted to complementary DNA (cDNA) using a miScript II RT Kit (Qiagen). Q-PCR was performed with a miScript SYBR Green PCR Kit (Qiagen) according to the manufacture's protocol. The primers specific for miR-210 are listed in Table 1, and the Syn-cel-miR-39 was used for normalization. Cycling conditions were 95°C for 15 min followed by 45 cycles of 94°C for 15 s and 55°C for 30 s using Roche light cycler 480.

Extraction of RNA from tissues and Q-PCR for HIF-1α and HSP90α

Total RNA was extracted from each tissue (liver, brain, and muscle) using TRIzol (Invitrogen) according to the manufacturer's instruction, and 1 μg of DNaseI (Promega)-treated total RNA was incubated with 0.5 μl of random primer (0.5 μg/ml, Promega) at 80°C for 5 min and further incubated at 42°C for 60 min in reaction mixture containing 2 μl of each 10 mM dNTP mix (TaKaRa), 0.5 μl of M-MLV reverse transcriptase (Promega) and 0.25 μl of RNase inhibitor (Promega) in a final reaction volume of 10 μl. The PCR primer pairs used for amplification of the hypoxia-related genes (HIF-1α and HSP90α) and the ß-actin gene as an internal control are described in Table 1. The PCR reactions in a volume of 20 μl were run using 2× SYBR Green Premix (Enzynomics) with 5 μl of cDNA and 5 pM of each primer. Thermal cycling condition was 1 cycle of 15 min at 95°C (pre-incubation), followed by 40 cycles of 10

Table 1. Primers used for real time-PCR

Primer	Sequence (5′- 3′)
pol-miR-210-5p-F	AGCCACTGACTAACGCACATTG
pol-miR-210-3p-F	CTGTGCGTGTGACAGCGGCT
HIF1α-RT-F	GACACAGCGTGTTTGACTTTACA
HIF1α-RT-R	TGTGTTTGACTCCTTGGTCTT
HSP90α-RT-F	GGACGAAGACAAGCCAGAAA
HSP90α-RT-R	AGCTCCTCCTGGTCGATATAC
HSP90β-RT-F	CATTGTCAGTCTGGAGGGTTAG
HSP90β-RT-R	GGACTCAGGCAAAGGAATCA

s at 95°C, 10 s at 60°C, and 20 s at 72°C.

Target prediction of miR-210 and gene ontology

The target prediction of miR-210 was done using miRanda algorithm (Enright et al., 2003), TargetSpy algorithm (Sturm et al., 2010) and PITA algorithm (John et al., 2004) through sRNAtoolbox (Rueda et al., 2015). Briefly, the 3'UTR sequences of olive flounder were retrieved from NCBI database and target prediction was done using sRNAtoolbox. The target genes were blasted, annotated using Blast2Go (https://www.blast2go.com/) and the gene ontology was exhibited through Web Gene Ontology Annotation Plot (WEGO, http://wego.genomics.org.cn/cgi-bin/wego/index.pl) (level 2) (Ye et al., 2006).

Statistical analysis

Statistical analysis was performed using SPSS for Windows (Chicago, IL, USA). Differences among groups were analyzed using ANOVA followed by Tukey HSD post-hoc test, and the level of statistical significance was set at $P<0.05$.

Results

Levels of circulatory miR-210

After lowering DO to 2 mg/L for 6 hours, the circulatory miR-210 was significantly increased when compared to that of the control fish (Fig. 1). miR-210-5p and miR-210-3p were increased 4-5 folds in the serum by hypoxia.

Target genes and gene ontology

Forty four target genes of miR-210 were predicted based on the intersection of the three algorithms (Table 2), and most of the target genes were related to metabolism and immune responses. The gene ontology analysis showed that the target genes were involved in various biological processes (Fig. 2).

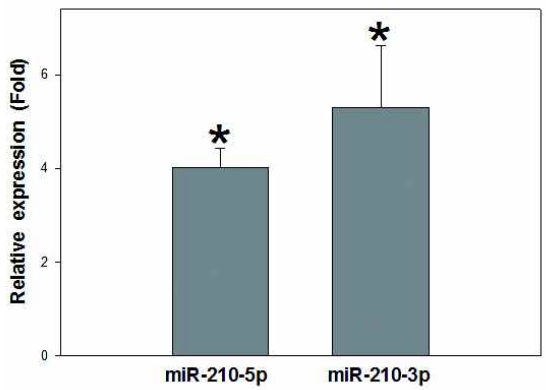

Fig. 1. The level of serum miR-210 of olive flounder (*Paralichthys olivaceus*) after exposure to hypoxia. DO was lowered and maintained at 2 mg/L for 6 h. Just before lowering DO, 5 fish in the tank were randomly sampled and used as the control group. At 6 h post-exposure to hypoxia, blood was collected from the caudal vein for serum isolation. The quantitation of miR-210 was done using Q-PCR analysis. Values are mean + standard error. The asterisk on the bar means significant ($p<0.05$) difference from the control group.

Expression of hypoxia-related genes

Although the expression of HIF-1α in the liver was significantly increased by hypoxia, the increased amplitude was not high (Fig. 3). On the other hand, the expression of HSP90α in the liver and muscle was greatly increased by hypoxia, more than 13 and 8 times, respectively, when compared to the expression values in control fish (Fig. 3).

Discussion

The significant role of microRNAs in the regulation of pathophysiologic processes has been well demonstrated in mammals. Although the role of circulating microRNAs is not fully understood, recent reports showed that circulating microRNAs also participate in biological phenomena through intercellular signaling (Katakowski et al., 2010; Kharaziha et al., 2012). Hypoxia leads to the shift of cellular metabolism from mitochondrial oxidative phosphorylation to glycolysis (Granchi et al., 2014), and microRNAs are

Table 2. Predicted target genes of pol-miR-210 based on miRanda, PITA, and TargetSpy tools

Target genes	GenBank ID
pitx2	AB050722
NPY for neuropeptide Y	AB055211
CCR3 for C-C chemokine receptor-3	AB081311
for CD40	AB081752
tlr3 for toll-like receptor 3	AB109394
fChi1 for chitinase1	AB121732
IL-11b for interleukin 11 type b	AB299205
TLR5M for toll-like receptor 5 membrane form	AB562152
CD8 beta for T-cell surface glycoprotein CD8 beta chain	AB643633
Per2 for period circadian protein homolog 2	AB729074
HSL1 for hormone-sensitive lipase1	AB828672
HSL2 for hormone-sensitive lipase2	AB828673
insulin-like growth factor I	AF061278
mitogen-activated protein kinase	AF433655
ornithine decarboxylase antizyme small isoform ORF1 and ornithine decarboxylase antizyme small isoform	AY257551
phospholipase D (PLD)	AY396567
partial for C1 inhibitor precursor (c1inh gene)	BN000290
DnaJ-like subfamily B member 6 (Hsp40B6)	DQ199620
glucose-regulated protein 78 (Grp78)	DQ662232
protein tyrosine phosphatases epsilon variant 1	DQ825344
protein tyrosine phosphatases epsilon variant 2	DQ825345
asparaginyl endopeptidase (AEP) AEP-Legumain allele	EF198070
cytochrome P450 1A (CYP1A)	EF451958
doublesex and mab-3 related transcription factor 1 (DMRT1)	EU490514
stanniocalcin 2	EU816770
phospholipase D2	EU872185
phospholipase D1B	EU872186
phospholipase C-beta 1 (PLC-beta1)	FJ198070
nephosin	FJ211409
p65 NF-kB subunit	HM771267
toll-like receptor 5 membrane form	JF266563
toll-like receptor 21	JQ411238
peptidoglycan recognition protein (PGRP)	JQ890080
phospholipase C beta 4	JX014240
scinderin-like protein	JX235336
fushi tarazu factor 1	JX999939
phospholipase C gamma 2 (PLC-gamma2)	KC978886
phospholipase C gamma 1 (PLC-gamma1)	KC978887
F-spondin extracellular matrix protein	KF793938
dynein heavy chain 9	KJ546040
isolate Po-P1 PR domain containing 1 (PRDM1)	KM624611
mitogen-activated protein kinase kinase kinase 7 (map3k7)	KM655803
DEAD (Asp-Glu-Ala-Asp) box helicase 5-1a (DDX5-1) alternatively spliced	KP899070
DEAD (Asp-Glu-Ala-Asp) box helicase 5-1b (DDX5-1) alternatively spliced	KP899071

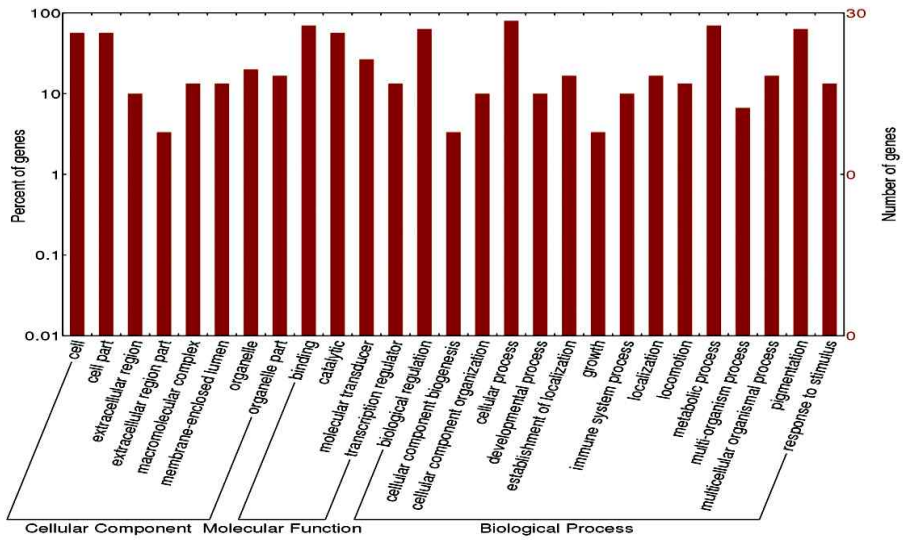

Fig. 2. Gene ontology classification of the predicted target genes of miR-210. The annotation of target genes was done using Blast2Go and the plotting was done using WEGO tool.

involved in the adaptation of organisms to hypoxia by the up- or down-regulation of hypoxia related genes (Ivan et al., 2008; Kulshreshtha et al., 2007).

miRNA-210 is the representative hypoxia-regulated miRNA (HRM), and is up-regulated by HIF that binds to HIF-response elements in the promoter region of miR-210 (Chan and Loscalzo, 2010). One of the regulatory roles of miR-210 is the repression of mitochondrial metabolism by the down-regulation of several steps for electron transport chain complexes-mediated ATP production (Chan et al., 2009). In human, the conspicuous increase of the circulating miR-210 level was reported in patients with pancreatic cancer, aortic stenosis, or low maximal oxygen uptake (Bye et al., 2013; Ho et al., 2010; Røsjø et al., 2014). In this study, similar to the reports from mammals, miR-210-5p and miR-210-3p were highly increased in the serum of olive flounder in response to hypoxia, suggesting that circulating miR-210 levels in the serum can be used as a prognostic biomarker for fish suffered hypoxia. Among the predicted target genes of miR-210, phospholipase D2 is known to promote the degradation of HIF-1α (Park et al., 2015), and the DEAD (Asp-Glu-Ala-Asp) box polypeptide 5 is known to be down-regulated during hypoxia (Guimbellot et al., 2009). Furthermore, the target genes of miR-210 were related to various biological processes, which explains the major regulator role of miR-210 in response to hypoxia (Huang et al., 2010).

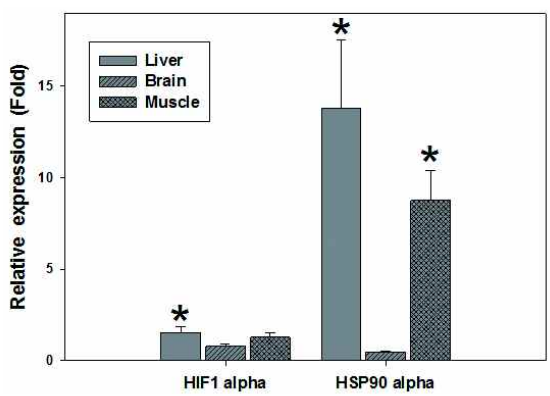

Fig. 3. Relative expression of hypoxia related genes, hypoxia inducible factor 1α (HIF-1α) and the heat shock protein 90α (HSP90α), in the liver, brain and muscle tissues of olive flounder that were exposed to hypoxia for 6 h. The quantitation of genes expression was done using real-time PCR. Values are mean + standard error. The asterisk on the bar means significant ($p<0.05$) difference from the control group.

In general, the regulation HIF-1α is dependent on the protein level rather than mRNA level, which was demonstrated in mammals and fish (Soitamo et al., 2001; Stroka et al., 2001). Similarly, in this study, although the transcriptional expression of HIF-1α in response to hypoxia was significantly increased in the liver, the increased amplitude was not high. The significant up-regulation of HSP90α in the liver and muscle by hypoxia in the preset study suggests that a large amount of HSP90 is required for the hypoxia adaptation of olive flounder as in other mammals. Considering the critical role of HIF-1α in miR-210 expression (Cicchillitti et al., 2012; Huang et al., 2009) and HSP90 (Iwasaki et al., 2010) in miRNAs function, the present up-regulation of HIF-1α and HSP90α might be related to the increase of circulatory miR-210.

In conclusion, severe hypoxia significantly up-regulated circulatory miR-210 in the serum of olive flounder, which suggests that serum miR-210 can be used as a potential noninvasive biomarker for hypoxia in fish. The expression of HIF-1α and HSP90α in the tissues was also up-regulated by hypoxia. However, to clearly uncover the relationship among serum miR-210, HIF-1α and HSP90, further studies on the regulatory interaction among HIF-1α, HSP90 and hypoxia-responsive miRNAs in fish are needed.

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