Form 3

Annual Report

LaMer, Ehime University

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To Director of LaMer

Principle Investigator:
Affiliation __Kyung Hee University__
Position __Associate Professor__
Name in print ____Kim Eun-Young

Include the report on the result of the project/meeting in a separate sheet.

1. Project / Meeting title

Searching of naturally occurring and anthropognic AHR ligands in wildlife

2. Members of project / meeting

Name	Affiliation	Position	Contribution part
PI Kim Eun-Young	Kyung Hee University	Associate Professor	Data analysis
Members Hwang Ji-Hee			
Bak Su-Min	Kyung Hee University	Grad. Student	In vitro assay
Go Dong-Hee	Kyung Hee University	Grad. Student	In vitro assay
	Kyung Hee University	Grad. Student	In silico analysis
LaMer Faculty member in charge Hisato Iwata	CMES	Professor	Data analysis

3. Contents

We participated in the 19th Annual Meeting of Japan Society of Endocrine Disrupters Research and made oral and poster presentations on December 8-9, 2016.

The followings are the titles and abstracts of presentations.

Structural characteristics of avian aryl hydrocarbon receptors to decipher dioxin susceptibility and ecological factors related to their genotypes

Dioxin-related chemicals (DRCs) are highly accumulated in some population of birds. One of the molecular targets of DRCs is the aryl hydrocarbon receptor (AHR), a ligand-dependent transcription factor that mediates toxic action of DRCs and physiological responses including immune system. Avian species possess multiple AHR isoforms (AHR1, AHR1β, and AHR2) that exhibit species- and isoform-specific responses to ligands. To account for the ligand preference in terms of the structural features of avian AHRs, we generated *in silico* homology models of the ligand-binding domain of avian AHRs based on HIF2 structural template (PDB code 3H7W). The molecular dynamics simulation showed that the values of mean square displacements in Ile324 and Ser380 of TCDD-bound AHR1 of the chicken, the most sensitive species to TCDD, were smaller than those in other avian AHR1s, suggesting that the dynamic stability of these amino acid residues contributes to TCDD preference. Analysis of the 3D reference interaction site model showed that the stabilization of TCDD binding to avian AHRs may be due to the solvation effect depending on the characteristics of two amino acids corresponding to Ile324 and Ser380 in chicken AHR1. In addition to the *in silico* structural analysis, we investigated the ecological factors in avian species that may have driven natural selection pressure of AHR genotypes related to dioxin sensitivity. Cluster analyses based on the association between ecological factors and AHR1 genotypes of 113 avian species were conducted. The results showed that 2 major clusters and sub-clusters of the cluster 3 were associated with specific AHR1 genotypes depending on ecological factors including the food and habitat. The majority of the species with Ile_Ala type were the Passeriformes, which are omnivorous or herbivorous feeders in the terrestrial environment. The species with Val_Ala type was primarily composed of raptors and waterbirds, which have been exposed to naturally occurring dioxins like 1,3,7-tribromodibenzo-*p*-dioxin. These results suggest that ecological factors related to the exposure of natural dioxins contribute to natural selection of the avian AHR1 genotype, which consequently leads to different sensitivity to man-made dioxins.

Auto-induction mechanism of aryl hydrocarbon receptor 2 (AHR2) gene by AHR1 and AHR2 in the red seabream

The toxic effects of dioxin-like compounds are mediated by the aryl hydrocarbon receptor (AHR). Our previous study identified two AHR isoforms (AHR1 and AHR2) from the red seabream (*Pagrus major*). Moreover, we found that AHR2 mRNA levels were remarkably induced by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) exposure in the early life stage of red seabream embryos, while AHR1 mRNA level was not altered. In this study, to investigate this isoform-specific regulatory mechanism of AHR transcripts, we cloned and characterized 5′-upstream regions of AHR1 and AHR2 genes from the red seabream. Both of the 5′-upstream regions in AHR1 and AHR2 genes contained three potential xenobiotic-responsive elements (XREs). *In vitro* assays using reporter plasmids containing the 5′-upstream regions of each AHR gene demonstrated that only the 5′-upstream region of AHR2 gene was activated by AHR1 and AHR2 proteins in a TCDD dose-dependent manner. Electrophoretic mobility shift assay using mutations in each XRE of AHR2 gene showed that XRE1, the closest XRE from the start codon in AHR2 gene, was mainly responsible for the binding with TCDD-activated AHR protein. This suggests that TCDD-activated AHR1 and AHR2 proteins up-regulate the mRNA levels via the functional XRE1 in AHR2 gene. This auto-induced AHR2 may amplify the signal transduction of its downstream targets including CYP1A in the red seabream.

***In silicolin vitro* analyses of the interaction of avian AHR and natural ligands**

The aryl hydrocarbon receptor (AHR) is a transcription factor activated by various xenobiotics such as dioxin-like chemicals (DLCs) and polycyclic aromatic hydrocarbons. Recent studies have shown that some endogenous and naturally occurring chemicals also activate AHR as ligands. However, little is known about the potency of natural ligands to transactivate avian AHR. To screen potential natural ligands of avian AHR, we applied an *in vitro* reporter gene assay system in which chicken CYP1A5-XREs reporter plasmid was transfected into a chicken hepatocellular carcinoma cell line, LMH. The *in vitro* assays demonstrated more than 2-fold induction of the reporter signal at the maximum concentration of 6-formylindolo[3,2-b]carbazole (FICZ), indoxylsulfate, 1,3,7-tribromodibenzo-*p*-dioxin, and pyocyanin. FICZ showed the highest potency and efficacy among the tested chemicals, suggesting the preferable structure of FICZ for binding with chicken AHR. Moreover, we carried out *in silico* analysis which predicts ligand-AHR docking energy to develop high-throughput screening method for AHR ligand candidate compounds. To select a proper AHR model for the *in silico* analysis, avian AHR1 PAS-B homology models using the amino acid sequences of chicken AHR1, great cormorant AHR1, and black-footed albatross AHR1 were constructed from four crystal structures of HIF2α as templates; PDB code 3H7W, 3H82, 4GHI, and 4ZQD. The relationships between

transactivation potencies (e.g. LOEL and IEF) of ligands (DLCs and natural ligands) via avian AHR1s and ligand docking energies of the corresponding AHR1 homology models were evaluated by coefficient of determination (R^2) of logarithmic linear best-fit line. The evaluation demonstrated that the relationships were largely changed, depending on the AHR1 homology models constructed from four HIF2α templates. The results suggest that there is a limit of *in silico* ligand screening by using avian AHR1 homology models. Further investigation of the selection of proper AHR homology models according to animal species and structural characteristics of ligand candidate compounds is necessary to develop *in silico* high throughput AHR ligand screening method.

Molecular characterization of aryl hydrocarbon receptor (AHR) in Polar bears (*Ursus maritimus*)

The aryl hydrocarbon receptor (AHR) is a key transcription factor which mediates the toxicity of dioxin-related chemicals (DRCs) by regulation of a battery of target genes including cytochrome P450 1A1. The apex predator in the Arctic, the polar bear (*Ursus maritimus*), is a vulnerable species to exposure to DRCs via the food web. Thus the functional characterization of polar bear AHR (pbAHR) contributes to assessment of the susceptibility to and risk of DRCs. We cloned pbAHR which encodes 793 amino acids and investigated the transactivation potency of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in the *in vitro* reporter-gene assay in which pbAHR expression plasmid was transfected into U-2 OS cells. The *in vitro* assay showed that pbAHR had an insufficient induction of the reporter signal even at the highest TCDD concentration. This result was due to a high basal level of pbAHR-mediated transactivation in vehicle-treated cells. In contrast, C3H/*lpr* mouse AHR (C3H AHR) bore a low basal level in vehicle control and sufficient induction potencies in a TCDD dose dependent manner. To unravel the high basal level of pbAHR with no ligand, the expression levels of pbAHR and C3H AHR proteins expressed in U-2 OS cells were compared by a western blot assay. The result indicated no difference in the expression levels between the two AHR proteins; the difference in the protein expression level was not the cause of high basal level of pbAHR. To verify the hypothesis that the unique structure of pbAHR protein is responsible for the high basal level, we constructed two types of chimeric AHR plasmids which switched the N-terminus and C-terminus of pbAHR with the corresponding sites of C3H AHR and performed the *in vitro* assay using the chimeric AHR plasmids. The results revealed that the chimeric AHR, which contains the N-terminal domains of pbAHR for DNA binding, dimerization with ARNT, and ligand binding, had a high basal level similar to wild type pbAHR. This suggests that the high basal level of pbAHR is caused by the structural characteristics in the N-terminus.