Bulletin of Environmental Contamination and Toxicology (2018) 100:798–802
https://doi.org/10.1007/s00128-018-2329-1

Partial mRNA Sequences of the Biomarkers CYP1A, GST, CAT, GR, SOD, GPx, VTG and p53 in Flatfish *Syacium gunteri* from Gulf of Mexico

Carlos Puch-Hau[1] · Mercedes Quintanilla-Mena[1] · Jorge Rubio-Piña[1] · Marcela Del Río-García[1] · Omar Zapata-Pérez[1]

Received: 27 October 2017 / Accepted: 20 March 2018 / Published online: 22 March 2018

Abstract
The present study reports partial sequences of a group of genes used as exposure and effect biomarkers of organic contaminants and/or heavy metals in *Syacium gunteri*. In order to isolate these sequences, cDNA was used to amplify fragments between 200 and 800 bp, which were then cloned and sequenced. The sequences presented high percentages of identity with genes involved in the metabolism of xenobiotic biotransformation (cytochrome P4501A and glutathione S-transferase), oxidative stress (catalase, glutathione reductase, glutathione peroxidase and superoxide dismutase), reproductive system (vitellogenin) and with the tumor suppressor protein p53 reported in the GenBank database. Subsequently, from the deduced sequence of amino acids of each fragment, their tridimensional structure was predicted, using homologous proteins from the Protein Data Base. This study generates an important base of molecular biomarkers for the monitoring of environmental health in the Gulf of Mexico.

Keywords Molecular biomarkers · Contaminants · Environmental monitoring · *Syacium gunteri*

The activities in the Gulf of Mexico such as tourism, industry and agriculture are responsible for introducing a range of chemical contaminants, such as oil, polycyclic aromatic hydrocarbons (PAHs), halogenated compounds, polychlorinated biphenyls (PCBs) and heavy metals, which cause grave damage to marine ecosystems (Celis-Hernández et al. 2013). The evaluation of these ecosystems through the effect of the contaminants on the fish represents an important aspect for environmental protection (Gold-Bouchot et al. 2017); therefore, different biomarker genes associated with the presence of contaminants are used as tools for studies of environmental toxicology.

Among these genes, cytochrome P450 1A (CYP1A) and glutathione S-transferase (GST), play an important role in response to contamination of aquatic ecosystems (Uno et al. 2012; Huang et al. 2014; Glisic et al. 2014). CYP1A is found to be associated with phase I of the xenobiotic biotransformation system, while GST is a key enzyme involved in phase II. These genes are induced by a diversity of toxic compounds, such as dioxins, furans PCBs, PAHs and organochlorinated pesticides (Higgins and Hayes 2011; Uno et al. 2012; Kim et al. 2013). In addition to these biomarkers, genes involved in the antioxidant responses also respond to chemical insults, therefore catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR) and glutathione peroxidase (GPx), have been included, with success, in environmental biomonitoring programs (Vidal-Liñan et al. 2010; Kumari et al. 2014).

Many of these contaminants can eventually cause both estrogenic and carcinogenic effects (Aris et al. 2014; Abdel-Shafy and Mansour 2016); therefore, vitellogenin (VTG) and p53 tumor suppressor protein are also used to evaluate the state of health of the aquatic organisms (Flick et al. 2014; Williams and Hubberstey 2014). VTG is a precursor protein of yolk in females and has been used to evaluate the estrogenicity of a diversity of chemical products (detergents, pesticides and a variety of chlorinated compounds) when its expression is identified in male fish (Gräns et al. 2010; Brock-meier et al. 2014). With respect to p53, this protein is involved in DNA repair, cell cycle arrest and the induction of apoptosis as defense mechanisms to cellular stressors such as the PAHs (Hockley et al. 2008; Meek 2009; Williams and Hubberstey 2014).

✉ Omar Zapata-Pérez
ozapata@cinvestav.mx

[1] Unidad Mérida, Departamento de Recursos del Mar, Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional, Km. 6 Antigua Carretera a Progreso, Cordemex, 97310 Mérida, Yucatán, Mexico

Among the different fish used for environmental biomonitoring, flatfish are good candidates for determining biological effects of aquatic contamination (Khan 2010; Williams et al. 2014). In that regard, *Syacium gunteri* is a fish with benthic feeding habits, resulting in a constant exposure to contaminants that accumulate on the sea bed (Vidal-Martínez et al. 2014).

Due to the ecological impact to which marine ecosystems are currently exposed, the need arises to have a battery of molecular biomarkers which will allow a timely evaluation and diagnosis of the present condition of these environments; the objective of this work, therefore, was the molecular cloning of fragments of eight genes in *S. gunteri* associated with the effect of the contaminants. These genes are part of the xenobiotic biotransformation system (CYP1A and GST), oxidative stress system (CAT, SOD, GR and GPx), reproductive system (VTG) and in response to cellular damage (p53).

Materials and Methods

Sampling was performed in October 2015 in the southern Gulf of Mexico; 71 adult shoal flounder were collected in 14 stations with 20 m shrimp nets. The size and average mass of the fish was 26.00 ± 4.80 cm and 208.41 ± 133.15 g. The liver was dissected from the fish body and stored in liquid nitrogen until further analysis in the laboratory. Total RNA was extracted from 50 mg of liver tissue using the SV Total RNA isolation System kit (Promega, USA) according to the manufacturer's instructions (Promega, USA). The concentration and integrity of total RNA were determined using a Nanodrop 1000 (Thermo Scientific, USA) and 1.5% agarose gel electrophoresis. First-strand cDNA was synthesized using Oligo d (t) (Sigma-Aldrich) and 2.5 µg of RNA in conjunction with reverse-transcriptase (Promega, USA) according to the manufacturer's protocol.

The primers were designed using the Primer-premier software (Premier Biosoft Interpairs, Palo Alto, CA) from the conserved regions of CYP1A, GST, CAT, GR, GPx, VTG and p53 genes downloaded from the GenBank database. The reverse transcriptase polymerase chain reactions (rtPCR) were performed in 11 µL volumes with the following concentrations: 1 µL sample of cDNA (2.5 µg of cDNA), 10 µM of each primer, 182 µM of the dNTPs mix (Promega, USA) and 0.3 U of Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA), according to the directions of the manufacturer. Thermal cycling conditions for CYP1A, GST and VTG were established as follows: 96°C for 1 min. (initial denaturation); followed by 40 cycles of 94°C for 1 min, with an annealing temperature of 64°C for 1 min and 72°C for 1 min; the final elongation step was set at 72°C for 10 min. The same cycling conditions were established for CAT, GPx, GR, SOD and p53 with annealing temperatures set at 60, 59°C (for both GPx and GR), 52 and 55°C, respectively. All rtPCR reactions were run in triplicate for each biological sample and recombinant plasmid were used as positive controls. rtPCR products were analyzed by electrophoresis on 1% agarose gels (Promega, Madison, Wisconsin, USA) stained with ethidium bromide, visualized by a UV transilluminator and registered with the support of DigiDocIt® Software.

The amplified fragments of expected size were excised from the agarose gel and purified using a QIAquick PCR purification kit (Qiagen, Valencia, CA, USA). The DNA obtained was cloned into the pGEM-T Easy vector plasmid (Promega, Madison, Wisconsin, USA) and transformed into *Escherichia coli* One Shot Top10 competent cells according to the manufacturer's instructions (Invitrogen). To confirm the sequence of the amplified products, five recombinant plasmids were sequenced for each molecular biomarker at the National Laboratory for Genomics of CINVESTAV-Irapuato (Mexico).

Similarity searches of the sequences were performed using the BLASTX program (Altschul et al. 1997) (http://www.ncbi.nlm.nih.gov). Homology modeling was performed with the SWISS-MODEL workspace server using the automatic mode function (http://swissmodel.expasy.org) (Bordoli et al. 2009).

Results and Discussion

Different species of flounders are currently being used to evaluate the effects of contaminants, at a molecular level (Khan 2010; Williams et al. 2014); however, in *S. gunteri*, there are no reports regarding the characterization of molecular markers associated with exposure to contaminants. The initiators designed to amplify these biomarkers (Table 1) generated a defined amplicon with the expected size for each of the genes.

The fragments corresponding to genes of xenobiotic biotransformation system (CYP1A and GST) and of oxidative stress system (CAT, GR, SOD and GPx) were found between 200 and 700 bp (Fig. 1). In the case of VTG and p53, the amplified products were approximately 670 and 350 bp, respectively.

The sequences obtained from the amplified products were verified and registered in the NCBI database. The fragments corresponding to the genes CYP1A and GST presented 95% and 79% of identity with their homologues reported in the species *Paralichthys olivaceus* and *Siniperca chuatsi* respectively. With respect to genes of the oxidative stress system, CAT and GR presented high percentages of identity (90% and 93%) with the sequences of *Fundulus heteroclitus*, while SOD and GPx both coincided with 84% of identity with the sequences of *Paralichthys olivaceus* and *Esox lucius*,

respectively. VTG and p53 presented 87% and 91% of identity with the species *Platichthys flesus* and *Siniperca chuatsi*, respectively (Table 2).

In order to determine the three dimensional protein structure predicted by each gene sequence, modeling by homology was carried out. This technique facilitates

Table 1 List of the initiators used for the amplification of the molecular biomarkers

Gene	Primer sequence (5′–3′)		Product size (bp)
CYP1A	F: GTGGTGGGCAGCGGAAAC	R: GTGCAGTGTGGGATGGTGAA	~490
GST	F: CTGCTGTGGGGCTCCGG	R: TTGATGCTGGGTCTCTCCTTCA	~590
CAT	F: CGACGAGGGCAACTGGGA	R: CGGTCTGCCTCCTCCACA	~620
GR	F: ATTATGGTGGATGAGTTTCAGA	R: GACCCACAACCTTCTCCTCT	~360
SOD	F: CTTAAAGCCGTATGCGTGC	R: CGTGGATCACCATGGTTCG	~358
GPx	F: GTCTGAAGTATGTCCGTCCTG	R: TTTCTGCTGTAGCGCTTGTA	~260
VTG	F: CCCGTGGATCCCTGCAGTA	R: AGCACTTCCAAATCCAGGCA	~670
p53	F: GAGCCACAGCGGTCTACAAGA	R: GACATGCACAGACACGCACC	~350

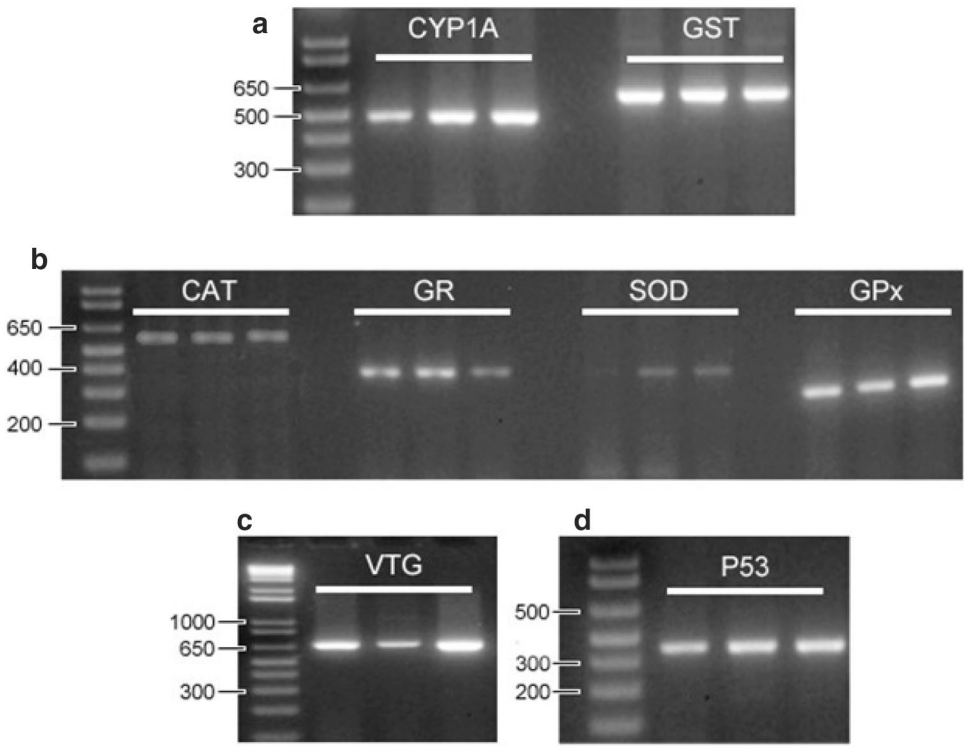

Fig. 1 Fragments amplified by rtPCR of three biological samples from *S. gunteri*. **a** Genes from the xenobiotic biotransformation system; CYP1A and GST, **b** oxidative stress genes; CAT, GR, SOD and GPx, **c** gene from the reproductive system; VTG and **d** p53 tumor suppressor gene. MM; molecular mass marker (1 kb)

Table 2 Identity percentages of the sequences amplified in *S. gunteri* with the GenBank accessions

Function	Sequence	Accession	Highest similarity with	Identity (%)
Biotransformation of xenobiotics system	CYP1A	KJ804264.1	CYP1A (*Paralichthys olivaceus*) (ABO38813.1)	95
	GST	KJ804265.1	GST (*Siniperca chuatsi*) (ACI32418.1)	79
Oxidative stress system	CAT	KJ804268.1	CAT (*Fundulus heteroclitus*) (XP_012724345.1)	90
	GR	KP994991.1	GR (*Fundulus heteroclitus*) (XP_012735466.1)	93
	SOD	KY964448	SOD (*Paralichthys olivaceus*) (XP_019952529)	84
	GPx	KP994990.1	GPx (*Esox lucius*) (XP_010879862.2)	84
Reproductive system	VTG	KJ804266.1	VTG (*Platichthys flesus*) (CAC94862.1)	87
Response to cellular damage	p53	KX537647	Tumor protein p53 (*Siniperca chuatsi*) (AMR06574)	91

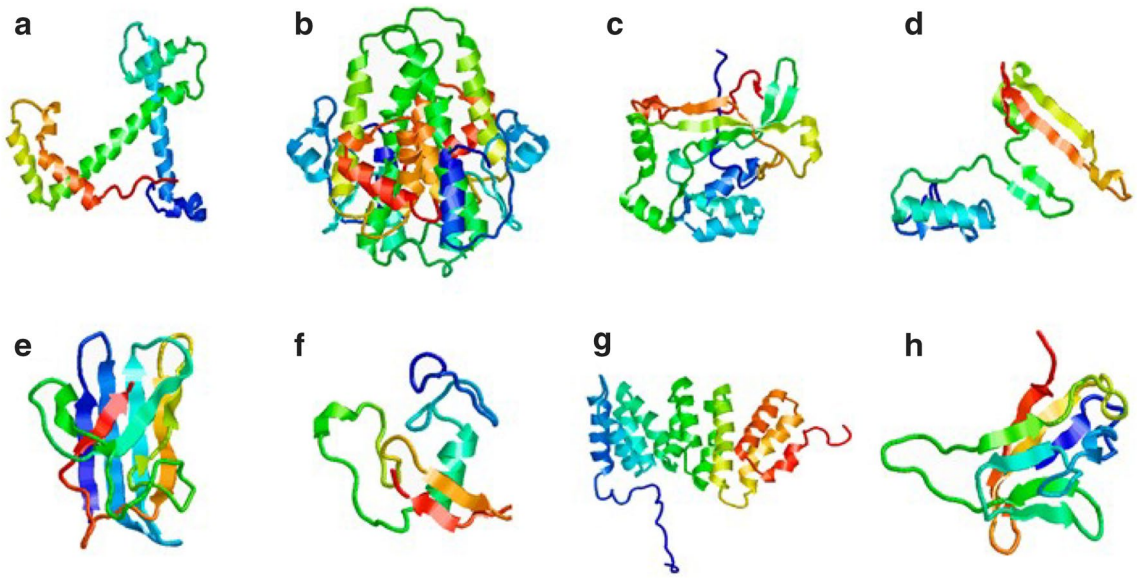

Fig. 2 Three dimensional structures predicted by homology of the sequences isolated in *S. gunteri*, **a** CYP1A, **b** GST, **c** CAT, **d** GR, **e** SOD, **f** GPx, **g** VTG and **h** p53. The three-dimensional structures were based on the templates: 4i8v.1.A, 3qav.1. A, 1gp1.1.A, 2aaq.1.A, 3gtt.1.A, 1f4j.1.A, 1gzh.1.A and 1lsh.1.A of the Protein Data Bank

the generation of 3D models of the proteins when the structures are not experimentally available. The three-dimensional structure of the CYP1A, CAT, GR and p53 aminoacid sequences were based on homologous proteins of humans (*Homo sapiens*) reported in the Protein Data Base, with identity percentages of 61.6%, 81.2%, 80.0% and 61.2%. In the case of GST, GPx, SOD and VTG, the percentages of identity were 41.2%, 72.1%, 63.9% and 38.2% with homologous proteins of different species, such as *Laternula elliptica, Bos taurus, Mus musculus* and *Ichthyomyzon unicuspis*, respectively (Fig. 2).

This study reports, for the first time, partial sequences of the genes CYP1A, GST, CAT, GR, SOD, GPx, VTG and p53 in flatfish *S. gunteri*, which can be used for the isolation and cloning of complete genes through the RACE technique (Rapid Amplification of cDNA Ends) and to evaluate the exposure and effect of the contaminants through the quantification of their levels of expression or by means of the methylation levels in the promoting regions (Mirbahai et al. 2011).

In Mexico, *S. gunteri* can be used to evaluate the toxic effects of the contaminants at a molecular level, in the coastal areas and oceans of the Gulf of Mexico and provides an important tool for the correct management of marine resources, particularly in a country with large hydrocarbon reserves. The present study generates a base of molecular biomarkers which can be used to diagnose, in a timely manner the effects of the contaminants during possible environmental contingencies, such as the occurrences in 1979 in the IXTOC-I oil (Beltrán et al. 2005; Ponce Vélez and Botello 2005) and more recently, the sinking of the "Deepwater Horizon" oil rig in 2010 (Brown-Peterson et al. 2015).

Acknowledgements We thank PEMEX Exploracion y Produccion (PEP), for the financial support granted during the environmental monitoring services of the Gulf of Mexico carried out by the CINVESTAV-IPN Campus Merida and the Sector Fund CONACyT - SENER - Hydrocarbons, project 201441. This is a contribution from the Gulf of Mexico Research Consortium (CIGoM).

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