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Molecular changes in skin pigmented lesions of the coral trout *Plectropomus leopardus*

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ABSTRACT

A high prevalence of skin pigmented lesions of 15% was recently reported in coral trout *Plectropomus leopardus*, a commercially important marine fish, inhabiting the Great Barrier Reef. Herein, fish were sampled at two offshore sites, characterised by high and low lesion prevalence. A transcriptomic approach using the suppressive subtractive hybridisation (SSH) method was used to analyse the differentially expressed genes between lesion and normal skin samples. Transcriptional changes of 14 genes were observed in lesion samples relative to normal skin samples. These targeted genes encoded for specific proteins which are involved in general cell function but also in different stages disrupted during the tumourigenesis process of other organisms, such as cell cycling, cell proliferation, skeletal organisation and cell migration. The results highlight transcripts that are associated with the lesion occurrence, contributing to a better understanding of the molecular aetiology of this coral trout skin disease.

1. Introduction

Fish tumours have been monitored for many years in order to assess the impact of exposure to anthropogenic stressors on the health of marine ecosystems (Malins et al., 1984). While the molecular aetiology and histological characteristics of tumours in bottom dwelling fish living in temperate marine ecosystems are well documented (Mix, 1986; Feist et al., 2015), relatively less is known on tumours of fish species from tropical regions, with examples limited to neurogenic tumours in damselfish (Schmale et al., 2002) and isolated instances of melanomas in the butterfly fish such as *Chaetodon multicinctus* and *C. miliaris* (Okihiro, 1988), and the surgeonfish *Ctenochaetus strigosus* (Work and Aeby, 2014). In contrast, in controlled aquaria settings, several model species of tropical fish are routinely used in mechanistic studies, (induced via UV- and hereditary routes), associated with human melanoma development (Patton et al., 2010; Regneri and Schartl, 2012; Schartl et al., 2012).

Melanomas are a type of skin tumour that derives from the malignant transformation of cutaneous melanocytes, the pigment-producing cells that reside in the basal layer of the epidermis in skin. In fish, melanophores are the specialized cells containing melanosomes, vesicles storing melanin, which are black or dark-brown in colour (Okihiro, 1988). To date, various aetiologies of wild fish melanoma have been suggested, including exposure to waterborne chemicals (Kimura et al., 1984), UV radiation (Setlow et al., 1989; Sweet et al., 2012), oncogenic viruses (Ramos et al., 2013) or genetic predisposition (Patton et al., 2010), however, as yet no cause-effect relationship at the underpinning molecular mechanistic level has been established.

A high prevalence of skin lesions, upwards of 15%, was recently reported in coral trout (*Plectropomus leopardus*) populations from the southern Great Barrier Reef (GBR) (Sweet et al., 2012). In the absence of microbial pathogens, and given the strong histopathological similarities of UV-induced melanomas in *Xiphophorus*, Sweet et al. (2012) have previously suggested that these lesions in

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coral trout may be examples of environmentally-induced melanomas. This wild fish species, of high economic value, has been overfished and is now considered under threat by the International Union for Conservation of Nature (IUCN). Previous studies on coral trout have focused on conservation ecology (Morris et al., 2000), reproduction (Carter et al., 2014), larval behaviour, mitochondrial genomes (Zhuang et al., 2013; Xie et al., 2016) and more recently on transcriptomic analyses of two colour morphs (Wang et al., 2015). Meanwhile, further studies on the skin pigmented lesions in this species have yet to be conducted. In this study we therefore aimed to better assess the aetiology of the lesions reported by Sweet et al. (2012) by isolating key genes associated with the skin lesion development in coral trout.

2. Materials and methods

2.1. Sample collection

Coral trout were sampled during 2013 at two locations on the Great Barrier Reef, Australia; Heron Island and Townsville (Table 1). All individuals were captured by rod and reel, or hand line fishing with a barbless 8/0 hook. Upon capture each individual was measured (cm total length), photographed and the percentage body cover of the lesions noted (Table 1). Individuals were sacrificed and immediately placed on ice for dissection and skin sampling. Samples were collected from individuals with lesions and without lesions (52.3 ± 5.0 cm, mean ± SD, $n = 8$, 41.3 ± 8.3 cm, mean ± SD, $n = 8$, respectively). Samples included skin and attached musculature and were stored at −80 °C prior to analysis.

2.2. Suppression subtractive hybridisation (SSH)

The SSH method was performed to enable the identification of genes which were differentially expressed between normal skin samples and lesion samples from coral trout. For each skin tissue sample from individual fish, total RNAs were extracted using the High Pure RNA Tissue kit (Roche Diagnostics Ltd, West Sussex, UK) according to the supplier's instructions. RNA quality of the 16 samples was evaluated by electrophoresis on a 1% agarose-formaldehyde gel. For the SSH procedure, 6 samples from each treatment group (normal and lesion) were used to create a pooled sample from each treatment, each represented at an equal concentration (150 ng/μL) (Table 1). SMARTer PCR cDNA Synthesis Kit reagents (Clontech, Saint-Germain-en-Laye, France) were used to create cDNA and the Advantage 2 PCR Kit (Clontech, France) reagents were used for PCR reactions. The SSH procedure was performed using PCR-Select cDNA Subtraction Kit reagents (Clontech, France) with normal skin tissue as the driver and lesion skin tissue samples as the tester. The protocol was carried out according to the manufacturer's guidelines.

Table 1
Sampling site location coordinates (latitude and longitude), lesion body cover (%) and lentgh (mm) of the fish collected at Heron Island and Townsville Reefs, Australia.

Sample name	Latitude	Longitude	Body cover (%)	Length (mm)
Heron Island Reefs				
Lesion MS3	−23.439	151.901	85	554
Lesion MCCTA1	−23.447	151.912	20	540
Lesion MCCTA2	−23.433	151.927	95	540
Lesion MCCTA3	−23.435	151.909	20	560
Lesion MCCTA4	−23.448	151.913	30	592
Lesion MCCTA5	−23.448	151.913	80	460
Lesion MS4[b]	−23.433	151.928	75	476
Lesion MCCTA5[b]	−23.448	151.913	80	460
Townsville Reefs[a]				
Normal MC1	−18.746	147.258	0	364
Normal MC2	−18.746	147.258	0	366
Normal MC3	−18.687	147.093	0	405
Normal MC5	−18.687	147.093	0	334
Normal MC6	−18.687	147.093	0	443
Normal MC7	−18.687	147.093	0	384
Normal MC4[b]	−18.687	147.093	0	410
Normal MC8[b]	−18.620	147.301	0	600

[a] No diseased individuals have been collected in this region so prevalence is low, possibly 0.
[b] Additional samples used for qPCR analysis which were not included in the original SSH experiment.

2.3. Subcloning and sequence identification

Two approaches were used to purify the final PCR products from the SSH reaction, prior to ligation and sub-cloning, in order to obtain clones containing variously sized gene-inserts. In the first approach, the PCR products were purified using the NucleoSpin® Extract II Kit (Macherey Nagel, UK), followed by ethanol precipitation to concentrate the samples. For the second approach, PCR products were run on a 1.5% TBE agarose gel post-stained with ethidium bromide (Invitrogen, Paisley, UK) and each lane of the gel was cut into four sections which were purified from the gel with the NucleoSpin® Extract II Kit (Macherey Nagel, UK), in order to reduce the effect of any potential size-bias the cloning procedure may exhibit.

Sub-cloning with blue/white screening was carried out with both the purified PCR products and the purified gel-excised PCR products. These were conducted using the Original TA Cloning Kit with the pCR2.1 vector (Life Technologies, UK) or the TOPO TA Cloning Kit For Sequencing with the pCR4-TOPO vector (Life Technologies, UK) as per the manufacturer's instructions, with the exception of the heat shock stage extension to 75 s. The chemically competent cells used were MAX Efficiency *DH10B E.coli* (Life Technologies, UK) and TOP10 *E. coli* (Life Technologies, UK). Following transformation, cells were grown overnight on LB miller agar plates containing kanamycin (50 μg/mL), white colonies were used to inoculate LB miller liquid cultures, which were then incubated overnight at 37 °C and 200 rpm. Overnight cultures were used directly in a PCR reaction, using M13 primers, to identify plasmids requiring purification with NucleoSpin® Extract II Kit reagents (Macherey Nagel, UK). Plasmids were sequenced by a commercial company (EZ Seq Service, Macrogen Europe, The Netherlands).

Sequences were identified by nucleotide (Blastn) and protein (Blastx) BLAST searches on the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) with results showing an E value of less than 10^{-5} excluded.

2.4. Quantitative real-time PCR validation of SSH results

In order to validate the results of the SSH experiment, 4 genes were selected for qPCR analysis. Two up-regulated transcripts were associated with lesion samples: *amyloid-like protein 2* (*APLP2*) and *Kelch repeat and BTB domain-containing protein 8* (*KBTBD8*), along with two down-regulated transcripts: *creatine kinase M-type* (*CKM*) and *strawberry notch homolog 2* (*SNO*). RNA was prepared from skin samples of the 12 individuals used for the SSH analysis ($n = 6$ for normal fish and $n = 6$ for lesion fish samples) with the addition to two further samples obtained for each sample type (Table 1). RNA extraction was performed using the High Pure RNA Tissue Kit reagents (Roche, UK). In order to increase the RNA yield, an additional step in the extraction protocol involving the addition of 10 U proteinase K (8 U/mL final concentration) (Thermo Scientific, UK) and 1 μL (28 mM final concentration) beta-mercaptoethanol (Agilent Technologies, UK), followed by a 1 h incubation at room