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

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Keywords: fish, skin lesion, melanosis, gene expression

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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 surgeon *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 (Okohito et al. 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. 1986; 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 yet.

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 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 (Zhang et al. 2013; Xie et al. 2014) 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.

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.

Heron Island Reef				
Sample name	**Latitude**	**Longitude**	**Body cover (%)**	**Length (mm)**
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†	-23.433	151.928	75	476
lesion MCCTA5†	-23.448	151.913	80	460
Townsville Reefs *				
Sample name	**Latitude**	**Longitude**	**Body cover (%)**	**Length (mm)**
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†	-18.687	147.093	0	410
normal MC8†	-18.620	147.301	0	600

*no diseased individuals have been collected in this region so prevalence is low, possibly 0

†Additional samples used for qPCR analysis which were not included in the original SSH experiment

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.

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 37ºC 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 of) beta-mercaptoethanol (Agilent Technologies, UK), followed by a 1 hr incubation at room temperature, was performed after rotor stator homogenisation. The total RNA concentrations were calculated using a Qubit 1.0 Fluorometer (Life Technologies, UK) and the Qubit RNA BR Assay Kit (Life Technologies, UK). cDNA synthesis was performed using 190 ng of total RNA for each sample with reagents from the SuperScript® VILO cDNA Synthesis Kit (Life Technologies, UK). cDNA samples were then treated with Ribonuclease H enzyme (at a final concentration of 125 U/mL) with the corresponding 10X RNase H Reaction Buffer (New England Biolabs, Hitchin, UK).

Primers for the qPCR reactions were designed based on the sequences obtained from the SSH experiment (Table 2) using the online bioinformatics resource Primer Designing Tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Primer sequences and their corresponding amplicon sizes are shown in Table 2. qPCR reactions were performed on a CFX96 Real-Time PCR

Detection System (BioRad, UK) and consisted of the following reagents: 10 µL FastStart Universal SYBR Green Master (Rox) (Roche, UK), 7 µL molecular-grade water (Fisher Scientific, UK), 1 µL of each primer (at a final concentration of 300 nM) and 1 µL of template cDNA. The following thermal cycling conditions were used: 95 ºC for 2 min, followed by 45 cycles of 95 ºC for 10 s, 60 ºC for 1 min, and 72 ºC for 1 min. Finally a melt curve generation step was included which heated samples from 60 ºC to 95 ºC in 0.5 ºC increments to allow melt curve generation allowing primer specificity to be confirmed. Template-negative controls were included alongside all runs to confirm lack of contamination and lack of secondary product formation such as primer dimers. Primer efficiencies were calculated for all primer pairs over a 10X dilution range of cDNA template and were all found to be within the desired 90 – 110% range.

Gene	Primer name	Sequence (5'-3')	Tm (˚C)	Amplicon size (bp)
EF1	*EF1*_F3	GTG TTG AGA CCG GTG TCC TG	57.9	111
	*EF1*_R3	CAG CCT CAG GCA GAG ATT CG	57.9	
Notch	*SNO*_F1	CCT CGG ACC TAC TCC CTC TC	58.2	129
	*SNO*_R1	TTG ATG GAG CCC GCT AAC AC	57.6	
Creatine kinase	*CKM*_F1	TAG CCG TGA CCA GAC TAT GC	56.5	169
	*CKM*_R1	CCA TCA AGA GGA CAC TCC ACA	56.5	
Kelch8	*Kelch*_F3	TTC TGA GGG CAC GGT TCA AG	57.5	110
	*Kelch*_R3	ACA TTC AGT GAG GAC GTG AGG	56.7	
Amyloid	*AP2*_F1	TGC TTA GTG CCA CAC CTT GT	57.0	136
	*AP2*_R1	AGG GTC ATG CTT TTC ACC TGT	56.8	

Table 2. Primer pairs used for qPCR validation of SSH results.

The delta Ct method ($2^{-\Delta Ct}$) was used to normalise the gene expression data of each of the genes of interest to that of the reference gene (Livak and Schmittgen, 2001). The GraphPad InStat

v3 (GraphPad Software Inc., La Jolla, USA) program was used to perform the statistical analyses, which consisted of unpaired t-tests to assess the suitability of *EF1* as a stably expressed reference gene and to detect differences in relative gene expression levels for each of the different genes of interest between normal and lesion samples. Values of $p < 0.05$ were considered significant.

3. Results

3.1. Skin lesion incidence

Fifteen percent of the fish caught at Heron Island Reef displayed dark skin lesions covering 20 to 95 % of the body surface (Table 1, Figure 1). No skin lesions were observed in fish caught at Townsville reefs.

Figure 1. Coral trout displaying (**A**) normal skin, and increasing percent lesion coverage of (**B**) 20%, (**C**) 30%, (**D**) 75%, (**E**) 85%.

A

B

C

D

E

3.2. SSH analysis

A total of 14 genes were identified as differentially expressed between normal and lesion skin samples, 6 up-regulated and 8 down-regulated genes were present in lesion samples (Table 3). These were identified based on sequence similarity to NCBI database sequences and in all cases the greatest degree of sequence similarity was shared with other fish species. All coral trout sequences generated here were submitted to the NCBI database and awarded accession numbers (Table 3).

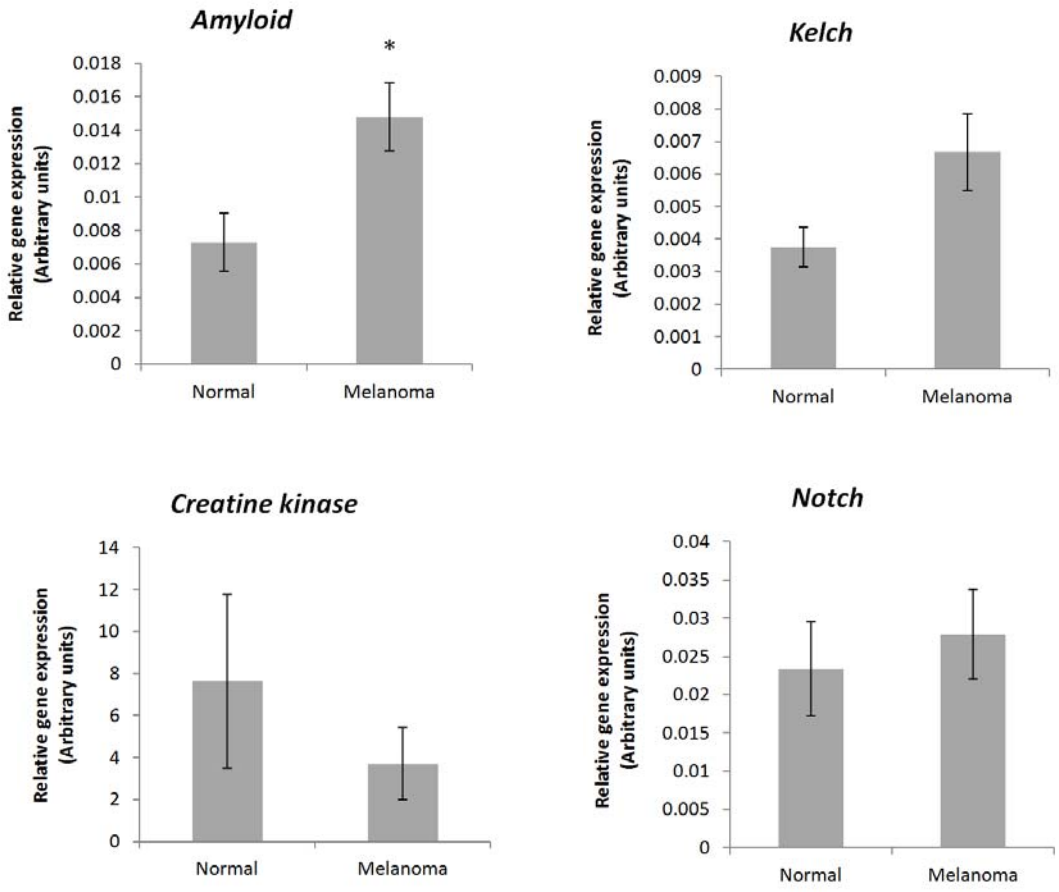
3.3. Validation of differentially expressed transcripts

No statistically significant difference was detected between the *EF1*expression levels of healthy samples compared with lesion samples (unpaired t-test, $p = 0.7507$), a result indicating that this transcript shows stable expression levels between treatments and is suitable for use as a reference gene with which samples can be normalised. The relative expression levels of the 4 gene transcripts selected for qPCR validation are shown in Figure 2. For the genes identified by SSH as up-regulated in lesion samples, a statistically significant difference was found for *amyloid APLP2* ($p = 0.02$) and, although the expected trend was shown by *Kelch* (*KBTBD8)*, the difference was not quite pronounced enough to be considered statistically significant ($p = 0.07$). For the genes for which SSH revealed down-regulation in lesion samples, *creatine kinase* (*CKM)* and *Notch* (*SNO)*, no statistically significant differences were detected ($p = 0.3986$ and $p = 0.6044$ respectively), however *CKM* showed the expected trend (Figure 2).

Table 3. Coral trout sequences obtained from SSH which are either up-regulated or down-regulated in skin pigmented lesion samples, and their identifications based on sequence similarity obtained by NCBI database BLAST searches. Asterisks denote genes selected for qPCR validation.

Clone accession number	Gene identity	Amplicon size (bp)	Species match	Accession number of match	E value
Up-regulated in lesion samples					
JZ693893	Amyloid-like protein 2-like*	332	*Haplochromis burtoni*	XM_005943131.1	3.00E-91
JZ693894	Guanine nucleotide-binding protein G(i) subunit alpha-2-like	368	*Oreochromis niloticus*	XM_003441449.2	3.00E-77
JZ693895	Kelch repeat and BTB domain-containing protein 8-like*	297	*O. niloticus*	XM_003442681.2	2.00E-57
JZ693896	Rap1 GTPase-GDP dissociation stimulator 1-like	187	*O. niloticus*	XM_005465283.1	3.00E-33
JZ693897	Iroquois-class homeodomain protein IRX-5-like	398	*O. niloticus*	XM_003437536.2	5.00E-95
JZ693898	Importin subunit alpha-4-like	175	*O. niloticus*	XM_003451617.2	4.00E-06
Down-regulated in lesion samples					
JZ693899	NudC domain-containing protein 2-like protein (NUDC2) and cyclin G1 (CCNG1) genes	162	*Perca flavescens*	JX629441.1	3.00E-53
JZ693900	Ubiquitin carboxyl-terminal hydrolase 7-like	69	*H. burtoni*	XM_005943595.1	3.00E-24
JZ693901	60S ribosomal protein L4-A-like	252	*Maylandia zebra*	XM_004553076.1	5.00E-78
JZ693902	Fructose-bisphosphate aldolase A-like	58	*Neolamprologus brichardi*	XM_006805719.1	2.00E-14
JZ693903	Calcium/calmodulin-dependent protein kinase type II subunit gamma-like	358	*Poecilia formosa*	XM_007562456.1	2.00E-23
JZ693904	Myocyte-specific enhancer factor 2A-like	129	*N. brichardi*	XM_006789383.1	3.00E-32
JZ693905	Creatine kinase M-type-like*	436	*O. niloticus*	XM_003456381.2	2.00E-27
JZ693906	Protein strawberry notch homolog 2-like*	288	*M. zebra*	XM_004554876.1	8.00E-72

Figure 2. Bar charts showing relative gene expression of healthy and lesion-containing coral trout skin samples for *SNO*,*CKM*, *KBTBD8*, and *APLP2* with mean data plotted ± SEM; $n = 6$ to 8.



4. Discussion

Dark skin lesions, due to the overproduction of melanin, have been previously identified in fish as melanoma based on histological observations (Okihiro et al. 1993; Sweet et al. 2012; Work and Aedy, 2014). Sampling for this study revealed that 15% of coral trout sampled at Heron Island Reef displayed a dark skin lesions covering 20 to 95 % of their body surface, reflecting the numbers initially reported by Sweet et al. (2012). Furthermore, this coverage percentage is in the range of what has been previously reported for other fish species including the goldring surgeon fish (Work and Aedy, 2014), and the pacific rockfish (Okihiro et al. 1993). Interestingly, although the same sample effort was conducted in the northern reaches of the GBR, no coral trout were found to be suffering from the disease at this location. Such variance in prevalence between locations (separated by 700 km) could have important repercussions to commercial and recreational fisheries, especially since the aetiology of the disease remains unknown.

In this study, two subtracted libraries, enriched with transcripts that differ between normal skin and lesions, have been constructed. Transcriptional changes of several transcripts, up- or down- regulated, relative to normal skin samples, were found in fish skin lesions (Table 3). These transcripts variously encode for proteins involved in general cell function (*calcium/calmodulin-dependent protein kinase*, *60S ribosomal protein L4-A-like*), in addition to those associated with different stages of disrupted cells which occur during the tumourigenesis process in other organisms, such as cell cycling (*iroquois-class homeodomain protein, microtubule motor associated protein*), cell proliferation (*amyloid-like protein 2-like, importin subunit alpha-4-like, ubiquitin carboxyl-terminal hydrolase 7-like*), skeletal organisation (*kelch-BTB protein*) and cell migration (*ras-associated protein-1, fructose-biphosphate aldolase A-like*) which will be discussed in turn.

Several transcripts involved in the control of the cell cycle were diffentially regulated as follows. The transcriptional response of the *iroquois homeobox protein5* (*Irx5*) gene was increased in the skin lesion samples. *Irx5* is a member of the iroquois homeobox gene family and is involved in the regulation of proliferation through their interaction with several cell cycle regulators (Myrthue et al. 2008). Aberrant expression of such homeobox genes deregulates cell cycle control contributing to carcinogenesis (Abate-Shen, 2002; Myrthue et al. 2008). The *amyloid precursor-like protein 2-like* (*APLP2*) gene encodes an amyloid precursor protein (APP) involved in cell progression (O'Brien and Wong, 2011), and was also up-regulated in the skin lesion samples. Several studies have reported a similar up-regulation of APP as seen in this study in various cancers associated with a variety of organisms including melanomas (Siemens et al. 2006; Bothelo et al. 2010; Russell et al. 2015). In contrast, the *microtubule motor associated protein* (*NudC*) and the *cyclin G1* genes were both down-regulated in the skin lesion samples. Microtubules play a central role in coordinating several cellular functions of the cell cycle, during which overexpression of *NudC* has been found to inhibit the proliferation of prostate cancer cells in a potential tumour suppressive manner (Lin et al. 2004). *Cyclin G1* is a transcriptional target of p53 and has also been shown induced by DNA damage in a p53 dependent manner (Kimura and Nojima 2002).

Another potential cellular proliferation cue includes the *importin subunit alpha-4-like* gene (also known as *karyopherin*, *KPNA*) that was identified as up-regulated in coral trout skin lesion samples relative to normal skin samples in this study. The importin alpha/beta heterodimer mediates the transport of proteins into the nucleus, modulating signal transduction processes (Kolher et al. 1997) and controlling the migration and viability of cancerous cells in certain human cancers (Wang et al. 2010). KPNA2 is considered to play a major role in the signal transduction pathways that regulate epidermal cell proliferation and differentiation (Umegaki et al. 2007). Epidermal keratinocytes regulate their proliferation and differentiation

by transducing signals from outside the cell membrane to the nucleus through nuclear pores, thereby regulating the expression of epidermal proliferation and differentiation of specific genes (Umegaki et al. 2007).

The *ubiquitin carboxyl-terminal hydrolase 7-like* (*UCHL*) gene is down-regulated in skin lesion tissues. Ubiquination plays a key role in the post-translational modification of proteins and regulates a number of cellular processes such as proliferation, apoptosis and neoplastic transformation. UCHL1 is an enzyme that protects ubiquinated proteins from degradation and recycles ubiquitin moieties (Wulfanger et al. 2013). Wulfanger et al. (2013) found that down-regulation of *UCHL1* was evident in melanoma cells and that it correlated with promoter DNA hypermethylation (Wulfanger et al. 2013). Other key genes, such as egfrb and xmrk involved in melanoma progression have been found deregulated by epigenetic mechanisms (Montero et al. 2006; Altschmied et al. 2007; Regneri et al. 2015).

Up-regulation of the *kelch-BTB protein* gene, involved in cytoskeletal organisation, was observed in the skin lesion samples. Changes in actin skeleton organization, adhesiveness and motility are important for tumour development and progression. Selected kelch-BTB proteins have been found to play important roles in invasion (Ohta et al. 2010; Brunner et al. 2013) and metastasis of cancer cells by regulating the actin cytoskeleton and Rho family proteins (Ohta et al. 2010) and are also considered as predictive markers of melanoma (Brunner et al. 2013).

Up-regulation of a gene potentially involved in cell invasion, *Ras-associated protein-1* (*Rap1*), was also observed in the skin lesion samples. Rap1, a close member of Ras in the small GTPase family, regulates two important cellular processes: Ras/BRAF/ERK activation and integrin-mediated cell adhesion/migration (Stork 2003; Bos et al. 2003). Rap1 is involved in the activation of MAPK pathway and integrin activation in human melanoma and may play a role in melanoma tumourigenesis and metastasis (Gao et al. 2006). Finally, the *fructose-biphosphate aldolase A-like* (*ALDOA*) gene was identified as down-regulated in skin lesion

samples. ALDOA is involved in glycolysis and its decrease has been found in several human malignant cancers (Kinoshita and Miyata, 2002; Kuramitsu and Nakamura, 2006; Du et al. 2014).

In conclusion, we have identified differentially regulated transcripts associated with the development of skin pigmented lesions in coral trout. The results contribute to a better understanding of the molecular aetiology of the disease, developing on the study by Sweet et al. (2012). These findings reported in this study are also of potential significance for both fisheries and marine park management in general.

Acknowledgements

The work was funded by a Fisheries Society of the British Isles (UK) small research grant (2013). Fish collection was conducted under research permits from the Great Barrier Reef Marine Park Authority (G10/33754.1 and G10/33758.1). Funding was provided to MRH as part of a Future Fellowship (FT100101004) from the Australian Reseach Council; additional funding was provided by the Australian Institute of Marine Science. Treatment of all animals was conducted under James Cook University animal ethics number A1566.

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Supplemental Information

Table S1. Raw dataset for qPCR experiments

NORMAL	EF1			SNO					KBTBD8				
Trout ID Number	Ct	Duplicate	Mean	Ct	Duplicate	Mean	dCT	RQ	Ct	Duplicate	Mean	dCT	RQ
MC7	20.75	20.88	20.815	27.35	27.22	27.285	6.47	0.0112807	29.62	29.73	29.675	8.86	0.002152
MC5	22.54	22.29	22.415	26.74	27.48	27.11	4.695	0.03860683					
MC2	21.21	21.12	21.165	27.93	28.61	28.27	7.105	0.0072641	29.98	29.93	29.955	8.79	0.002259
MC6	23.07	23.26	23.165	27.75	27.01	27.38	4.215	0.05384663	31.11	30.67	30.89	7.725	0.004727
MC4	22.04	22.27	22.155	28.67	29.34	29.005	6.85	0.00866851	30.72	30.53	30.625	8.47	0.00282
MC8	26.73	26.84	26.785	31.3	31.84	31.57	4.785	0.036272	34.28	34.53	34.405	7.62	0.005083
MC3	22.25	22.6	22.425	28	28.23	28.115	5.69	0.01937043					
MC1	23.75	23.63	23.69	30.09	30.06	30.075	6.385	0.0119653	31.08	31.32	31.2	7.51	0.005486
							Average	**0.02340931**				**Average**	**0.003755**
							SE	**0.0061167**				**SE**	**0.000616**

LESION	EF1			SNO					KBTBD8				
Trout ID Number	Ct	Duplicate	Mean	Ct	Duplicate	Mean	dCT	RQ	Ct	Duplicate	Mean	dCT	RQ
MCCTA3	24.66	24.66	24.66	30.22	29.99	30.105	5.445	0.02295576	31.47	31.83	31.65	6.99	0.007867
MS4	21.5	21.33	21.415	28	28.01	28.005	6.59	0.01038036	29.19	28.61	28.9	7.485	0.005582
MS3	22.22	22.28	22.25	28.17	28.36	28.265	6.015	0.01546339	30.4	30.49	30.445	8.195	0.003412
MCCTA5	22.16	22.16	22.16	26.59	27.15	26.87	4.71	0.03820751	29.63	29.93	29.78	7.62	0.005083
MS5	21.75	21.85	21.8	26.48	26.9	26.69	4.89	0.03372588	29.42	29.2	29.31	7.51	0.005486
MCCTA4	21.36	21.46	21.41	27.82	28.06	27.94	6.53	0.01082117	29.45	30.17	29.81	8.4	0.00296
MCCTA1	23.56	23.73	23.645	28.03	27.42	27.725	4.08	0.0591286	30.12	30	30.06	6.415	0.011719
MCCTA2	23.27	23.26	23.265	28.12	28.3	28.21	4.945	0.03246435	29.83	29.62	29.725	6.46	0.011359
							Average	**0.02789338**				**Average**	**0.006684**
							SE	**0.00584622**				**SE**	**0.001183**

NORMAL	CKM					APLP2				
Trout ID Number	Ct	Duplicate	Mean	dCT	RQ	Ct	Duplicate	Mean	dCT	RQ
MC7	17.65	17.46	17.555	-3.26	9.57983	28.85	28.44	28.645	7.83	0.004395
MC5	21.36	22.3	21.83	-0.585	1.500039					
MC2						28.47	29.03	28.75	7.585	0.005208
MC6	23.4	22.89	23.145	-0.02	1.013959	29.6	29.11	29.355	6.19	0.013697
MC4	20.1	19.37	19.735	-2.42	5.35171	29.4	29.26	29.33	7.175	0.00692
MC8	22.01	21.6	21.805	-4.98	31.55945	33.35	33.25	33.3	6.515	0.010934
MC3	21.67		21.67	-0.755	1.687632					
MC1	22.41	22.06	22.235	-1.455	2.741566	33.29	31.26	32.275	8.585	0.002604
				Average	**7.633455**				**Average**	**0.007293**
				SE	**4.146621**				**SE**	**0.001724**

LESION	CKM					APLP2				
Trout ID Number	Ct	Duplicate	Mean	dCT	RQ	Ct	Duplicate	Mean	dCT	RQ
MCCTA3	21.15	21.31	21.23	-3.43	10.77787	30.54	31.38	30.96	6.3	0.012691
MS4	21.73	21.47	21.6	0.185	0.879649	28.17	28.41	28.29	6.875	0.00852
MS3	24.41	23.89	24.15	1.9	0.267943	28.86	28.94	28.9	6.65	0.009958
MCCTA5	25.4	25.3	25.35	3.19	0.109576	28.12	28.09	28.105	5.945	0.016232
MS5	21.85	22.08	21.965	0.165	0.891929	27.65	27.92	27.785	5.985	0.015788
MCCTA4						27.9	28.33	28.115	6.705	0.009585
MCCTA1	20.39	20.44	20.415	-3.23	9.38268	29	28.96	28.98	5.335	0.024775
MCCTA2	21.19	21.61	21.4	-1.865	3.642679	28.76	28.95	28.855	5.59	0.020761
				Average	**3.707475**				**Average**	**0.014789**
				SE	**1.710623**				**SE**	**0.002038**

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