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Expression of galaxin and oncogene homologs in growth anomaly in the coral *Montipora capitata*

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ABSTRACT: Growth anomaly (GA) is a coral disease characterized by enlarged skeletal lesions. Although negative effects of GA on several of coral's biological functions have been determined, the etiology and molecular pathology of this disease is very poorly understood. We studied the expression of 5 genes suspected to play a role in pathological development of GA in the endemic Hawaiian coral *Montipora capitata,* which is particularly susceptible to this disease. Transcript abundances of the 5 target genes in healthy tissue, GA-affected tissue, and unaffected tissue (apparently healthy tissue adjacent to GA) relative to 3 internal control genes (*actin*, *NADH*, and *rpS3*) were compared using quantitative reverse transcriptase PCR. *Galaxin*, which codes for a protein suspected to be involved in calcification and thus hypothesized to be differentially expressed in GA, was up-regulated in unaffected tissue but remained at baseline levels in GA tissue. The gene expressions of murine double minute 2 (*MDM2*) and tumor necrosis factor (*TNF*) remained unchanged in GA tissue. The expression of tyrosine protein kinase (*TPK*) and βγ-crystallin (*BGC*) were both down-regulated. These expression patterns were all inconsistent with the expression patterns of homologous genes in neoplastic diseases featuring similar morphological symptoms in humans. These expression data therefore suggest that the calcification mechanism is likely not enhanced in coral GA and that coral GA is not a malignant neoplasia.

KEY WORDS: Coral · *Montipora capitata* · Growth anomaly · Neoplasia

INTRODUCTION

Coral diseases have increased in both severity and incidence in recent years and have become a serious threat to the marine ecosystem. There are >30 known types of coral disease that affect a vast number of coral species (Willis et al. 2004). Of these, growth anomaly (GA) is a disease that affects >40 species of scleractinian corals in the Caribbean and Indo-Pacific Oceans (Aeby et al. 2011). GA is prominently visible by its primary sign of protuberant skeletal masses extending from the surface of coral colonies (Work et al. 2008). These skeletal malformations are generally circular and extend above the coral colony surface (Work & Aeby 2006). These lesions are the result of accelerations of tissue growth that have been associated with deterioration in tissue structure and decreases in fecundity, nematocyst density, symbiotic dinoflagellate density, and colony growth (Loya et al. 1984, Hunter 1999, Burns & Takabayashi 2011, Williams et al. 2011). GAs have increased in prevalence since first reported >50 yr ago. (Domart-Coulon et al. 2006, Kaczmarsky & Richardson 2007, Work et al. 2008, Takabayashi et al. 2010, Williams et al. 2011). GAs are generally not considered to be a transmittable disease (Domart-Coulon et al. 2006), but a single instance of coral-to-coral transmission of GA has been reported (Kaczmarsky & Richardson 2007).

GAs are often referred to in the literature as 'tumors' or 'neoplasms' without a detailed molecular pathological examination to determine that the

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lesions qualify as such (Yamashiro et al. 2000). Malignant neoplasms are abnormal cells that proliferate and sustain continued growth after an initial stimulus, yet that does not seem to be the case with the vast majority of GAs (Yamashiro et al. 2000, Domart-Coulon et al. 2006, Kaczmarsky & Richardson 2007). Increased polyp growth rate, yet the ability to discern individual polyps, as well as the retention of pigment in the abnormal growths of some coral species has lead to the use of the term 'hyperplasia' in contrast to the earlier use of the word 'neoplasia' (Willis et al. 2004). Hyperplasia is a proliferation in cells, which remain subject to normal cellular regulatory mechanisms, in response to a particular stimulus, and the cells revert to normal morphology once the stimulus is removed (Robbins et al. 1999).

While recent histological evaluations suggest that the GAs, initially determined to be neoplastic, behave more like hyperplasia, there has been no molecular assessment of this claim. In fact, only a few molecular investigations of coral GAs have occurred worldwide, and none have been performed on the endemic Hawaiian coral *Montipora capitata* (Yamashiro et al. 2001, Domart-Coulon et al. 2006). The present study examines the molecular pathology of GAs in *M. capitata* by examining the expression of 4 genes whose expressions are altered in other calcifying organisms with neoplasia (Naylor et al. 1993, Ray et al. 1997, Weiner et al. 1999, Iwakuma & Lozano 2003). We also examined the expression of the organic matrix gene *galaxin*, whose genetic homolog, collagen, has an altered expression under neoplastic conditions (Kubista et al. 2011).

Little is known about the coral genome, but recent studies in other cnidarians demonstrated that the cnidarian genome shares many of the same disease genes that are found within the human genome. The coral skeleton is surprisingly similar in mineral composition and structure to human bone. Sullivan & Finnerty (2007) examined >500 human disease gene homologs in the cnidarian genome of *Nematostella vectensis*, the sea starlet anemone. *Nematostella* showed homology to a number of human diseases, and the authors found 61 cancer genes that are shared between humans and *Nematostella.* The Wnt signal pathway has been extensively studied for its involvement in the development of cancer. Sullivan & Finnerty (2007) noted that *Nematostella* shares 11 of the 12 Wnt genes found in humans, while *Drosophila* only has 6 of the 12 genes (Kusserow et al. 2005). Human disease models are often the most complete reference that we have for describing invertebrate diseases, and an investigation of the same pathways involved in human neoplasia may help to provide answers about coral GA, given the high level of homology between genomes of the 2 species. A number of pathways can lead to the formation of neoplasia, and the genes known to be affected by neoplasia are good candidates for assessing whether or not GAs are malignant neoplasia. We chose genes that are involved in some of the major signaling pathways involved in cancer. These pathways are among the best understood in cancer development and have been heavily researched. There are also areas of overlap where these molecules are involved in multiple pathways. Tumor necrosis factor (TNF)α is an initiating molecule in the cellular apoptosis pathway, but murine double minute 2 (MDM2) is involved downstream in the same pathway. The p53 pathway is a second major pathway involved in cancer, and MDM2 is a direct regulator of p53 (Ogawara et al. 2002). A third well-studied cancer pathway is the AKT signaling pathway. AKT has been shown to directly induce the phosphorylation of MDM2. Lastly, we looked at tyrosine protein kinase (TPK), which showed high homology to the Src family of kinases involved in the epidermal growth factor (EGF) cancer pathway. The 4 pathways have been shown to be involved in a wide variety of cancer types. Cancer diagnoses and prognoses are often given by measuring levels of p53/MDM2 as well as antibodies for the MDM2 protein. The same can be shown for TNFα as well as Src kinases.

MATERIALS AND METHODS

Sample collection

Montipora capitata samples were collected from the Wai'ōpae tide pools, Hawaii, USA, where GAs are prevalent (Burns et al. 2011). Samples were collected from tide pools outside of the marine life conservation district. Coral tissue samples from 3 separate healthy colonies ('healthy'), GA lesion tissue from 3 separate diseased colonies ('affected'), and apparently healthy tissue adjacent to the GA lesion in the same colonies ('unaffected') were harvested using a clean hammer and metal corer with gloved hands (Fig. 1). Three different diseased coral colonies were studied as well as 3 additional healthy colonies. Coral samples were placed into RNAlater (Ambion) and stored on ice until processing in the lab. Tissue was removed from each coral skeleton using a SCUBA tank attached to an airbrush gun with a sterile unfiltered 1000 µl pipette tip. Each coral

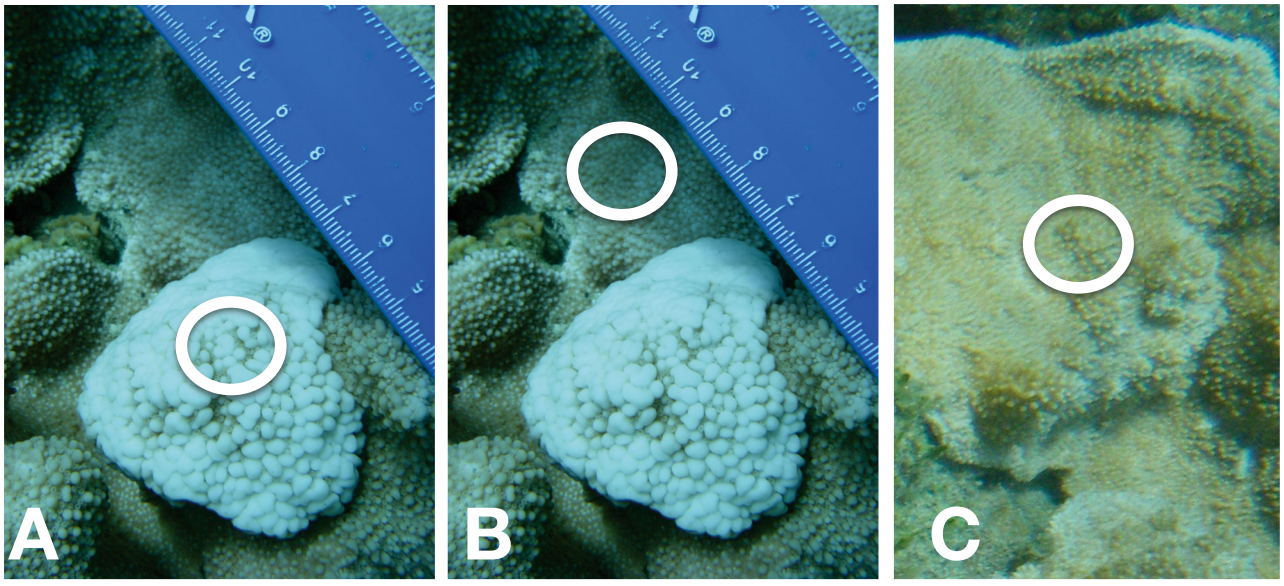

Fig 1. Types of *Montipora capitata* tissue: (A) affected tissue, (B) unaffected tissue, (C) healthy tissue. Ruler is in cm. White circles indicate area of tissue extraction

piece was placed into a sterile Whirl-pak (Nasco) bag, and tissue was blown off the coral skeleton. Each coral tissue type was removed by pipette and placed into sterile RNase-free tubes. Additional RNAlater was added to each tube and placed in the refrigerator overnight before storing the samples long term at −20°C.

RNA extraction and reverse transcription

RNA was extracted using a modified version of the TRIzol (Invitrogen)/RNeasy (Qiagen) Hybrid Protocol (Rodriguez-Lanetty et al. 2006). DNase treatment (RNase-free DNase set, Qiagen) was performed as part of the extraction, and RNA samples were treated for 25 min. An aliquot of 2 µl of RNAsin (Promega) was added to each sample before reverse transcription. The RNA was reverse transcribed (RT) using a High-Capacity RNA-to-cDNA Kit (ABI Systems, Life Technologies). The majority of the RNA was reverse transcribed into cDNA according to the kit protocol, with a portion of the RNA reserved for a −RT (no reverse transcriptase enzyme included) reaction to use as a control for genomic DNA.

Selection of internal control genes

For the present study, we used quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) for quantifying transcript abundance. We used relative quantification, which measures the gene expression, or transcript abundance, of a target gene relative to stable internal control genes that maintain a constant level of expression (Hoffmann et al. 2009). To select internal control genes with constitutive expression among our tissue types, we tested 5 candidate genes: *actin* (GenBank ID: FJ858776), βγ-crystallin (*BGC*) (GenBank ID: EU022143), *NADH* (GenBank ID: DQ351257), retinitis pigmentosa GTPase regulator (*RPGR*), and *rpS3* (both from Hauck 2007). The 5 genes were run through qRT-PCR using each tissue type. The Cotton EST Database (Xie et al. 2011) at East Carolina University has an algorithm that combines multiple analysis methods and provides a comprehensive ranking based on the results of the GeNorm, Normfinder, BestKeeper, and DeltaCT methods. The triplicate cycle threshold (CT) values were used in the algorithm, and a geometric mean ranking was used as an index for gene expression stability.

While many quantitative studies use a single internal control gene for verification, others have used expression of multiple internal control genes and normalized them using their mean expression to reduce the likelihood of error that exists using a single gene (de Kok et al. 2005, Rodriguez-Lanetty et al. 2008). Expression of all 3 internal control genes can be measured, and their mean CT values can be used for normalization using the equation $\Delta CT = CT(Target) - CT(mean\ of\ gene1, gene2, gene3)$ (Hamalainen et al. 2001).

Selection of target genes

We examined the expression of 5 genes found in coral whose homolog expressions would be affected by the presence of neoplasia in the human genome. The genes we examined were *BGC*, *MDM2*, *TNF*, *TPK*, and *galaxin*. *BGC*, *MDM2*, *TNF*, and *TPK* are all oncogenes. The expression levels of oncogenes are often increased in tumor cells. Galaxin is homologous to the protein family of collagens, which is the most abundant protein in bone and has been shown to be differentially expressed in the presence of osteosarcoma, a bone cancer (Roessner et al. 1983, Kubista et al. 2011).

Quantification of target gene transcripts

Primers and TaqMan probes were designed and ordered for 5 target genes using Integrated DNA Technologies' Primer Quest software (Table 1). Each probe was tagged with a FAM fluorophore (5′) and an IBFQ quencher (3′) as well as the 'Zen' internal double quench option (Integrated DNA Technologies) in our probes to reduce background fluorescence. There were 3 different series of cDNA created from 3 different extracted RNA samples from collected coral tissues (series 1–3 cDNAs). Each series of cDNA consisted of healthy, unaffected, and affected cDNA. Three different diseased colonies were sampled as well as 3 different healthy colonies. qPCR was performed using TaqMan Gene expression mastermix (Life Technologies) as well as a StepOnePlus thermocycler (Life Technologies). A total of 2 µl of cDNA was used per qPCR reaction, and the reactions were set up in near-darkness to minimize degradation of the probes by light. The qPCR reaction was run for 2 min at 50°C, 10 minutes at 95°C, and then 40 cycles of 15 s at 95°C and 1 min at 60°C. Each gene was run in triplicate for each tissue type and included a no-RT negative control and a no-template negative control. Each gene was run along with 3 internal control genes, *NADH*, *rpS3* and *actin* (Table 1), that were determined to be the most consti-

Table 1. TaqMan primer and probe sequences. *BGC*: βγ-crystallin, *MDM2*: murine double minute 2, *TNF*: tumor necrosis factor, *TPK*: tyrosine protein kinase, *RPGR*: retinitis pigmentosa GTPase regulator

Gene	Oligonucleotide	Sequence (5′ to 3′)	Amplicon
Actin	Forward	TGT TCC CAG GAA TCG CTG ACA GAA	156 bp
	Probe	AGC TCT GGC TCC TCC AAC AAT GAA GA	
	Reverse	TCC ACA TTT GCT GGA AGG TGG ACA	
NADH	Forward	CCC TAA AGA CTC CCG GCC TTC AAT TA	106 bp
	Probe	AGT CCT CCC AGC ATA CAG TGA CTC AA	
	Reverse	ATG GAG GGT AAG TGT TGG GCC ATT	
rpS3	Forward	TTG CAG ATG GCG TAT TCA AAG CCG	109 bp
	Probe	TGA CTC GTG AGC TAG CAG AAG ATG GA	
	Reverse	ATC TCA GTT CGA ATG GGC GTC ACA	
Galaxin	Forward	GGC ACC ATT GTA ACG AAG AAC GCA	126 bp
	Probe	ACA GCG CAC TCC AAA GCA GGA TTT	
	Reverse	TAA ATG GCT CCA CCA CAA CAA GCG	
BGC	Forward	AGC ACG CCA ACT TCA ATG ACA AGC	132 bp
	Probe	CGA TCA CAA GAT CTT AAA TCC TGG AGC A	
	Reverse	TTC CTT CCG TCT GAT CCT TCA CCA	
MDM2	Forward	ATG AAG AGG GCC AGG ACT TGG ATT	125 bp
	Probe	AGA GGA TTT ATG GGC CTG TCC AGA GT	
	Reverse	TTC ATG CCT TTC AAC TCA GCA GCG	
TNF	Forward	AGG CCG TGT ATG CAA TGG TTC CTA	176 bp
	Probe	ATG ACG GCC ATA CAT AGC CCA GCT TT	
	Reverse	TTC CAA CTC CAC TGT CCA CAC CAT	
TPK	Forward	TAT GCT GTG TGT ACG CAG GAG GAA	154 bp
	Probe	TTT GCC ACA GTT GAT CTA CAT GGC CG	
	Reverse	ATG CCA TGC CTG CAG CTA TTT GAG	
RPGR	Forward	ACC CTC CTC GAT CGT TTC TTG CAT	103 bp
	Probe	TTC CTT TAC GCC TCA GAT CAG GTT TCT C	
	Reverse	AGC TCT CGA AGA AAG CGA AGC TGA	

tutively expressed in these particular tissue types (see 'Selection of internal control genes'). All PCR products were sequenced for verification of correct gene amplification (data not shown).

RESULTS

Selection of internal control genes

The CT values from the outcome of each qPCR reaction were uploaded into the Cotton EST Database (Xie et al. 2011) and the most stable internal control genes were comprehensively ranked (Fig. 2). *Actin* was the most stable gene, with *NADH* and *rpS3* also stable among tissue types. While many researchers feel it is sufficient to use 1 internal gene for their gene expression assays, we felt that using all 3 genes as a reference of control would increase the accuracy of our quantification results.

Quantification of target gene transcripts

From the ΔCT values, relative abundances of transcripts are calculated as fold differences (Fig. 3). Data were checked for normal distribution and equal variances. The ΔCT values for healthy, unaffected, and affected tissues were calculated for each cDNA series that was tested. Only 1 internal control gene (*NADH*) was included in series 1 cDNA analysis due to a low abundance of cDNA. However, all 3 internal control genes were included in analysis of the other 2 series of cDNA. The ΔCT values of each target gene were analyzed using an analysis of variance (ANOVA) as well as Tukey's multiple comparisons test. The 3 tissue types were the 3 treatment types used in analyses. There was a sample size of 3 for each of the treatment types, giving an overall sample size of n = 9. The transcript abundance of *galaxin* showed no differences between healthy and affected or between healthy and unaffected, but *galaxin* levels were significantly higher in unaffected than in affected tissue (ANOVA, $F = 9.696$, $p = 0.013$, Tukey's $p = 0.05$). *BGC* expression in both affected and unaffected tissue was significantly lower than in healthy tissue (ANOVA, $F = 220.9$, $p < 0.001$, Tukey's $p = 0.05$). The transcript abundances of *MDM2* and *TNF*

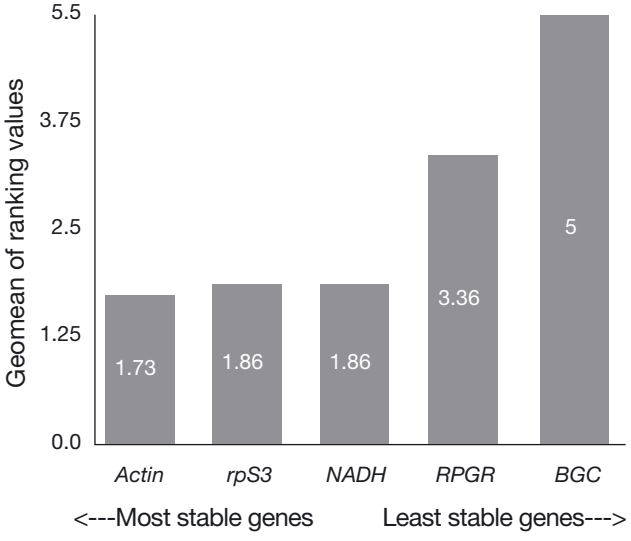

Fig. 2. Comprehensive ranking of internal control genes. See Table 1 for abbreviations

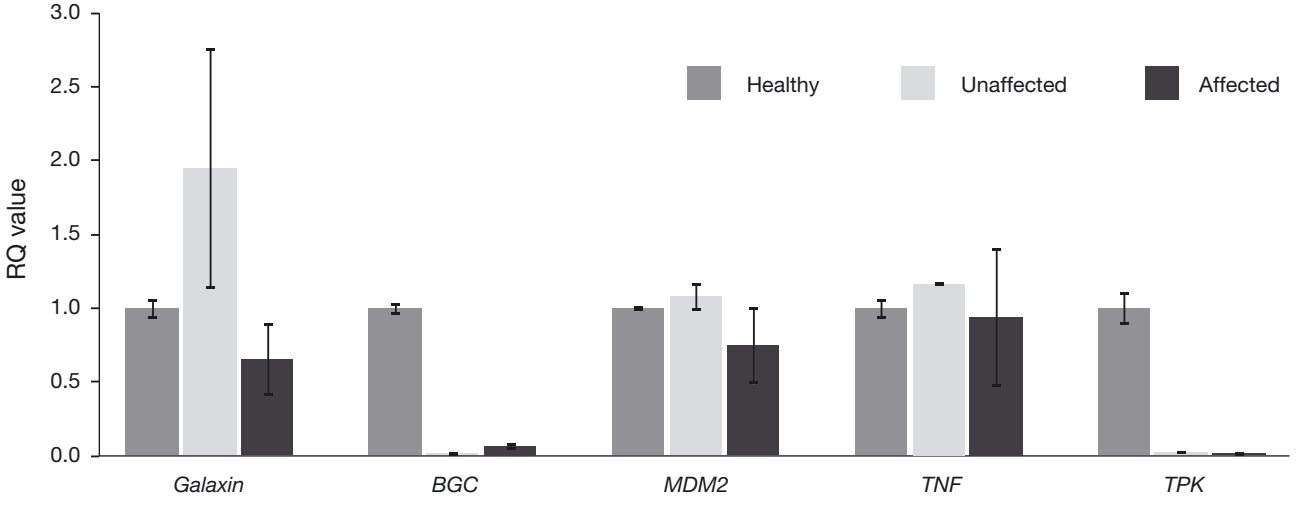

Fig. 3. Normalized relative transcript abundance of target genes in healthy, unaffected (apparently healthy tissue from colonies housing growth anomaly lesions), and affected (growth anomaly lesion) tissues of the coral *Montipora capitata.* RQ: relative quantification. See Table 1 for abbreviations

showed no difference among treatments (ANOVA, $F = 4.74$, $p = 0.06$ and $F = 0.614$, $p = 0.572$, respectively). Finally, the transcript abundance of *TPK* was significantly lower in affected and unaffected tissue compared to healthy tissue (ANOVA, $F = 8.814$, $p = 0.016$, Tukey's $p = 0.05$).

DISCUSSION

Growth anomaly is a disease that affects scleractinian corals and has a profound negative effect on biological functions of the endemic coral species of Hawaii (Takabayashi et al. 2010, Burns & Takabayashi 2011). These growths have historically been referred to as tumors or neoplasia, without a molecular pathological investigation to determine if such claims are accurate. Neoplasia by definition are a grouping of abnormal cells that proliferate and sustain continued growth after an initial stimulus, relative to normal surrounding cells. The goal of the present study was to examine the expression of genes that are expected to be altered under neoplastic conditions to see if they support the hypothesized nature of GAs as malignant, or cancerous, neoplasia.

The calcareous skeleton of coral is very similar to human bone, so much so that coral has previously been used as bone grafting material in humans (Damien et al. 1994). A human disease closely resembling GA is osteosarcoma, a malignant neoplasm of bone. Osteosarcoma is similar in structure and appearance to GA, and they share similar organic matrix structures (Cornell & Lane 1998). The most abundant protein in the organic matrix of bone is collagen. Similarly, the most abundant protein in the coral organic matrix is galaxin, though the function of galaxin in coral skeletons is still unknown. In the presence of osteosarcoma, it has been shown that collagen expression is down-regulated (Kubista et al. 2011). Our results show that the expression of *galaxin* remains relatively unchanged in GA-affected tissue. However, *galaxin* expression in unaffected tissue (apparently healthy tissue from colonies hosting GA lesions) showed nearly a 2-fold increase in expression. It is possible that this increase in *galaxin* expression is a result of metabolic activities within the GA. In mammalian models, the activation of macrophages during immune response has been shown to cause a decrease in pH, leading to an acidic pathway that causes collagen breakdown (Friess 1998). The expression of collagen has been shown to be up-regulated in tissues associated with tissue repair in response to pathogens that cause inflammation (Sandler et al. 2003). It is possible that the tissues adjacent to GA are responding to the diseased tissue and attempting to repair it.

The expression patterns of oncogene homologs in *Montipora capitata* GA in the present study were inconsistent with those expected for malignant neoplasia. *TNF* and *MDM2* expression remained constant among tissue types. The expression of TNF is up-regulated in a wide variety of human cancers (Mocellin et al. 2005); however, it was unchanged in both GA-affected and unaffected tissues compared to healthy corals. The expression of *MDM2*, which regulates the tumor suppressor gene *p53*, is often increased in the presence of neoplasia, and we observed no change in expression (Iwakuma & Lozano 2003). Similarly, *TPK* is often over-expressed under neoplastic conditions in humans (Bennasroune et al. 2004). In contrast, *TPK* and *BGC* both showed a decrease in expression level in both unaffected and affected tissue types compared to healthy tissue in *M. capitata*. *BCG* has also been shown to be overexpressed in several cancer types (Ogawa et al. 1997, Ray et al. 1997). Again, our results regarding *BCG* expression were not consistent with those expected if GAs are a malignant neoplastic condition.

It is interesting to note that the oncogene homolog expressions behaved similarly in affected and unaffected tissues. A previous study of the same coral species showed that the histologically detectible effect of GA was confined to the lesion and not visible in immediately neighboring tissue (Burns & Takabayashi 2011). In comparison, our study showed that the change in expression of *BGC* and *TPK* extended from GA tissue to neighboring tissue that was histologically unaffected. This indicates that coral tissue surrounding the GA lesion responds at molecular level before the effect manifests itself at the histological level.

In conclusion, our analysis of the transcript abundance of 4 oncogene homologs and *galaxin* does not support the possibility that GAs are malignant neoplasia. This is in agreement with previous studies that have morphologically characterized GAs in other coral species as hyperplasia instead of neoplasia (Willis et al. 2004, Domart-Coulon et al. 2006), although the possibility exists that GA may progress from a hyperplastic (pre-neoplastic) condition to a benign neoplastic condition. Transcriptomic analyses and time-series investigation of expressions of oncogenes will further our understanding of the molecular pathology of this disease that is affecting many species of corals in the Indo-Pacific and the Caribbean.

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