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Note

The chronicles of the contaminated Mediterranean seas: a story told by the cetaceans' skin genes

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ABSTRACT

Wild animals in their natural environment could provide a big source of information, but sampling can be very challenging, above all for protected species, like marine mammals. Nevertheless, significant data can be obtained sampling stranded animals right after their death, taking into account proper sampling time and methodology.

RNA samples from the skin of 12 individuals including the species *Stenella coeruleoalba*, *Tursiops truncatus*, and *Grampus griseus* were used to test 4 potential gene markers of anthropogenic contaminants exposure. The individuals were sampled in 3 geographic areas: the Adriatic, Ionian and Tyrrhenian seas. Three out of the 4 genes tested showed higher expression in the samples collected from the Adriatic Sea. Minute skin samples tell the story of the specific geographic location where the marine mammal spent its life, thanks to the different impact on gene expression exerted by different contamination levels.

1. Introduction

Marine mammals are top predators that are essential for the health and function of the oceans, too often affected by various detrimental factors. Forty-four percent of stranded marine mammals die from unknown causes (Gulland and Hall, 2007), while disease is a major cause of population decline, enhanced by the immune suppression and dysfunction caused by anthropogenic compounds released in the environment (Gulland and Hall, 2007; Van Bressem et al., 2009). Contaminants of emerging concern (CEC), *e.g.* chemicals, pharmaceuticals, personal care products, nanomaterials and plastics, are increasingly being detected in surface water. Several are classified as endocrine disruptors compounds (EDCs), for they can alter the normal hormone functions affecting reproduction, development and metabolism (Casals-Casas and Desvergne, 2011). EDCs pose the entire marine ecosystem at risk, at every trophic food levels, with the top predators, such as cetaceans, being the most vulnerable.

This study aimed at identifying gene markers specific for different CEC exposure in the skin of cetaceans. Accordingly, we analyzed skin biopsies of stranded specimens from geographic locations known to be differentially contaminated. Few skin samples from dart biopsies of free-ranging animals were included in the study to support the findings. Samples were collected from three principal basins (Ionian, Adriatic and Tyrrhenian) in the Mediterranean Sea, for a total of 33 skin biopsies of about 1 cm^3 collected from the side of the animal, close to the dorsal fin. Thirty-two biopsies were from toothed whales (Odontocetes) and precisely 2 from the *Tursiops truncatus* species, 29 from *Stenella coeruleoalba* and 1 from *Grampus griseus*; one sample was from the baleen whale (Mysticetes) *Balaenoptera physalus*. Timing and methods of sampling are crucial to obtain good quality RNA, therefore we considered only a subset of samples (N = 12) suitable for quantitative gene expression analysis.

2. Methods

A total of 30 skin samples were collected between 2014 and 2016 from stranded *T. truncatus* and *S. coeruleoalba* along the Italian coasts. Three more skin biopsies from free-ranging cetaceans, *S. coeruleoalba*, *G. griseus* and *B. physalus*, were collected by dart biopsy in the Italian marine protected area Santuario Pelagos for Mediterranean Marine Mammals located in the Ligurian basin of the Mediterranean Sea (Prot. Num: 0017889/PM). Skin samples were dissected using sterile surgical tools and preserved in RNA-later (Qiagen, Hilden, Germany).

For all skin biopsies, total RNA was extracted using RNeasy Fibrous Tissue Mini Kit (Qiagen) and cDNA was obtained using iScript Select cDNA Synthesis Kit (Bio−Rad, Hercules, CA, USA) both according to manufacturer's instructions. Relative RNA expression levels of 4 genes were determined through quantitative real time PCR (qPCR) analysis on CFX Connect (Bio−Rad) with species-specific primers designed and optimized for efficiency and specificity by running standard curves. qPCR efficiencies were calculated using the equation from Dhar et al. (2009). Optimized qPCR parameters for each gene were determined

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Table 1
Sequence of primers used in quantitative real time PCR.

Primers were designed using Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/). Each gene primer set was optimized for efficiency and specificity by running standard curves on pooled skin cDNA samples resulting in a correlation coefficient R2 > 98.4% and efficiency = 103–109%. Quantitative RT-PCR efficiencies were calculated as previously described (Dhar et al., 2009). The range of the amplicon size was [90–150]. *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase; *YWHAZ*, tyrosine 3-monooxygenase/tryptophan 5- monooxygenase activation protein zeta; *BCAP31*, B-cell receptor 31; *CDC42*, cell division control protein 42 homolog; *MTSS1*, metastasis suppressor 1; *AHR*, aryl hydrocarbon receptor. F, forward primer; R, reverse primer.

ID	Sequence (5′ − > 3′)	Tm	Amplicon size (bp)	Efficiency (%)	R^2 (%)	Slope
GAPDH-F	CGACCACTTTGTCAAGCTCA	60.02	153	109	99.5	3.137
GAPDH-R	CGGAGGACCTCTCTCTTCCT	59.95				
YWHAZ-F	AGACGGAAGGTGCTGAGAAA	59.99	215	109.9	98.7	3.106
YWAHZ-R	TTTCTTGTCGTCACCAGCAG	60.02				
BCAP31-F	GGTCGTACTCCTTGGTCAGG	60.3	88	108.4	99.4	3.137
BCAP31-R	GCCGTCAACAAGCAAAAACT.	60.2				
CDC42-F	AGTTGCTGGCCTTCTGAATC	59.4	77	104.1	98.4	3.228
CDC42-R	TCCAAGAGAGAAGGAATACATGC	59.7				
MTSS1-F	AACCCACTTCAAGAGCAGATG	59.3	50	107.9	98.9	3.146
MTSS1-R	AGCTGGTTGGCCACTTTCT	59.9				
AHR-F	AAGTCCATCCCAGGTGACAG	59.01	72	103.9	99	3.231
AHR-R	GCAAGTTCAGGCCTTCTCTG	58.84				

using diluted (1:10) cDNA reverse transcribed from 1 μg of total RNA using SsoFast™ EvaGreen® Supermix (Bio−Rad) in a total volume of 10 μl of a reaction mix containing 10 ng cDNA, 0.3 μM of each primer (Table 1), 2 × Evagreen enzyme and DNase-free sterile water. qPCR reactions were run as follows: 1 cycle of 98 °C for 30 min, 49 cycles of 95 °C for 5 s, 60 °C for 10 s; melting curve 65 °C–95 °C: increment 0.5 °C every 5 min. Each reaction was run in triplicate, together with a triplicate of no-template controls. The average Ct values were normalized to the values of the housekeeping genes *GAPDH* and *YWHAZ*. Comparative Ct method of analysis ($2^{-\Delta\Delta ct}$) was used to determined changes of expression between control and treated samples on CFX connect manager software 3.1 (Bio−Rad). Two-tailed, unpaired one-way ANOVA and Tukey *post hoc* test were performed using GraphPad Prism 5 (https://www.graphpad.com/scientific-software/prism/).

3. Results

The RNA samples used to test the 4 potential gene markers were extracted from 12 individuals. Although the skin RNA recovery was successful from 14 individuals (Fig. 1, Table 2), 2 samples were excluded from further analysis, based on RNA contamination (sample Tt02IZS) or pronounced differences in gene expression compared with the other cetacean species and within different suborders (sample Bp01PB).

Three out of the 4 genes analyzed were selected within those differentially expressed after exposition of dolphin skin cultures to environmentally relevant concentrations of two CEC (Lunardi et al., 2016). Cell division control protein 42 homolog (*CDC42*) is differentially regulated by perfluorooctanoic acid (PFOA), while metastasis suppressor 1 (*MTSS1*) and B-cell receptor 31 (*BCAP31*) are differentially regulated by bisphenol A (BPA).

CDC42 is a small GTPase of the Rho-subfamily, regulating signaling pathways that control diverse cellular functions including morphology, migration, endocytosis and cycle progression. The role of CDC42 is important for normal cell function and its failure has been associated with a number of pathological conditions associated with several human disease states and/or developmental disorders (Melendez et al., 2011). MTSS1 was first identified as a suppressor of metastasis while accumulating evidences support the concept that as a scaffold protein would interact with multiple partners to regulate actin dynamics and be involved in the Shh signaling pathway in the developing hair follicle and in basal cell carcinomas of the skin. MTSS1 is a multiple functional molecular player and has an important role in development, carcinogenesis and metastasis (Xie et al., 2011). BCAP31 is a multi-pass transmembrane protein of the endoplasmic reticulum involved in the anterograde transport and also in caspases mediated apoptosis. The increased expression of BCAP31 protein activated by DNA demethylation is associated to psoriasis, a human disease of abnormal keratinocyte differentiation and apoptosis (Ruchusatsawat et al., 2017). Moreover, recent findings showed a protective role of BCAP31 in keratinocytes during UVB-induced skin cancer development through the induction of caspase-1 apoptosis (Sollberger et al., 2015). It has been recently shown that BCAP31 may play an important role in T cell activation by regulating TCR signaling (Niu et al., 2017).

An additional fourth gene, aryl hydrocarbon receptor (*AHR*), was also analyzed in this study due to its well-known role in the xenobiotic metabolism of mammals (Hahn, 1998). AHR is a ligand-activated transcription factor abundantly expressed in epidermal keratinocytes with the function of chemical sensor mediating the production of reactive oxygen species. AHR interacts with polycyclic and planar halogenated aromatic hydrocarbons (PAH or PHAH) and dioxins, activating the cytochrome P4501A, a member of the superfamily of enzymes involved in Phase I of the oxidative metabolism of exogenous compounds, with a key role in the biotransformation of contaminants (Hahn, 1998). EDCs, such as BPA, have diverse endocrine effects on mammalian and non-mammalian systems because they can bind the AHR, promoting both antagonist and agonist activities (Kharrazian, 2014).

The expression levels of the four selected genes were quantified and compared across geographic areas of sampling and genders.

Different results in the three different Mediterranean Sea basins are reported. The expression of the genes by area of sampling seems to follow a trend in the samples analyzed. With such a small sample set it is not possible to make such a strong statement but the samples set used represents a good group for a comparison study, strengthening our observation. In fact, 83% of the samples analyzed were from skin biopsy of stranded *S. coeruleoalba* species, and only one of them was from a dart biopsy of a living animal, collected in the Pelagos Sanctuary. *AHR* and *BCAP31* transcripts were significantly higher in the samples from the Adriatic Sea than from the Tyrrhenian Sea. *BCAP31* mRNA levels were also significantly higher in Adriatic samples than in the Ionian ones (Fig. 2). *CDC42* had the highest level of expression in the animals sampled in the Adriatic Sea and the lowest in those sampled in the Tyrrhenian Sea; conversely, *MTSS1* had higher levels of expression in the animals from the Ionian Sea, but in both instances the differences were not statistically significant with the available sample set (Fig. 2).

Gender-related differences were not significant, with only 2 specimens in the experimental male group (data not shown).

4. Discussion

This study reports about different levels of expression of some selected genes that may indicate different environmental quality in the