Results in Immunology 2 (2012) 83–87

Contents lists available at SciVerse ScienceDirect

Results in Immunology

journal homepage: www.elsevier.com/locate/rinim

Short Communication

Expression of RAS-like family members, c-jun and c-myc mRNA levels in neoplastic hemocytes of soft-shell clams *Mya arenaria* using microsphere-based 8-plex branched DNA assay

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ARTICLE INFO

Article history:
Received 16 February 2012
Received in revised form
20 March 2012
Accepted 29 March 2012
Available online 7 April 2012

Keywords:
Hemocytes
Multiplex
RAS
c-myc
c-jun
Tetraploidy

ABSTRACT

The molecular mechanisms by which disseminated neoplasia (DN) is developed in soft shell clams *Mya arenaria* remain largely unknown. This study aims at quantifying Rho-like GTPase, RAS-Rho, RAS-related C3 botulinum (RAS C3), c-jun as well as c-myc transcript levels in clams sampled at North River (Charlottetown, Prince Edward Island, Canada). The transcripts were quantified using multiplex gene analysis (Quantigene® 2 Plex, Affymetrix) in 3 groups of clams: (1) Group C (healthy clams considered as control) with a low percentage of tetraploid hemocytes ($<10\%$); (2) Group D (disease in development): individuals presenting a percentage of tetraploid cells ranging between 10% and 50%; (3) Group E (established disease): clams with a high percentage of tetraploid hemocytes ($>50\%$). Data showed a down-regulation of Rho-like GTPase, Rho-like subfamily, RAS C3, c-jun and an up-regulation of c-myc gene expression. It is believed that a deregulation of the expression of these genes could partly unravel the molecular mechanisms involved in the development of DN in soft shell clams *Mya arenaria*. Further investigations should be pursued to determine the role of these gene products in clams' hemocytes.

1. Introduction

Infectious diseases constitute one of the major causes of economic loss in the aquaculture industry. In 1997, the World Bank estimated disease-related losses at approximately 3 billion US$ per year. In 1999, a high mortality of soft-shell clams, *Mya arenaria*, ranging between 20% and 90% in some areas, was recorded in Prince Edward Island and was found to be related to the development of "disseminated neoplasia" (DN) [11]. Neoplastic hemocytes lose their pseudopodia and become rounded affecting thus their capacity of motility and phagocytosis [1]. Based on the DNA contents, neoplastic cells exhibited double DNA content and were assessed as tetraploid in comparison to normal hemocytes [14]. Flow cytometry was used as a tool to determine the tetraploidy level of hemocytes in clams [14]. Using tetraploidy status as an indicator of the disease, clams sampled in North River (Prince Edward Island, Canada) present high prevalence in comparison to clams sampled in other sites in Canada [4].

The molecular actors involved in the development of the disease of DN in *M. arenaria* still remain unknown. Studies demonstrated that the sequestration of p53 by mortalin would constitute one of the mechanisms of DN induction in clams [17,16,15,2], whereas Holbrook et al. [9] stipulated that molecular mechanisms regulated by p53 were disrupted by a high expression of the mouse double minute 2 (MDM2) proto-oncogene. In order to identify the molecular actors involved in the development of the disease, a subtractive suppressive hybridization approach has been performed in healthy and diseased clams, as well as in organisms during the development stages of the disease [20]. In our SSH cDNA bank, RAS-like family members such as RAS-C3, RAS-Rho, Rho-like GTPase, c-jun and c-myc were identified as regulated transcripts during the development of the disease [20]. RAS-like family members play a pivotal role in cell cycle (cytokenesis, quiescence) by activating c-jun while c-myc stimulates cell cycle progression and proliferation. Therefore, this study aims at quantifying the level of these transcripts at different stages of tetraploidy. In this study, levels of these transcripts were quantified using microsphere-based 8-plex branched DNA assay.

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2211-2839
http://dx.doi.org/10.1016/j.rinim.2012.03.003

2. Materials and methods

2.1. Sampling

Approximately five-cm-long specimens of *M. arenaria* were collected at low tide with a hand rake at 15–20 cm depth in North River (46°15′01″N, 63°10′42″W) (Charlottetown, Prince Edward Island, Canada), known as an endemic area for disseminated neoplasia. After being washed with seawater, clams were transported to our aquatic facilities at the Atlantic Veterinary College (Charlottetown, Prince Edward Island, Canada). Upon arrival, animals were kept in tanks with static seawater at 18 °C and a salinity of 28 before analysis.

2.2. Flow cytometry

Flow cytometry (FCM) analysis was used to assess the ploidy status of *M. arenaria* hemocytes according to the methods described by Delaporte et al. [4]. Briefly, hemolymph (500 μL) was withdrawn from individual clams using a 3 mL syringe fitted with a 25-gage needle. Hemocytes were fixed in 2.5 mL of cold absolute ethanol and stored at −20 °C for at least 30 min. Fixed cells were centrifuged (400 g for 10 min at room temperature), and supernatants were discarded. Hemocyte pellets were re-suspended in 0.01 M phosphate-buffered saline (PBS) and cells were left to re-hydrate for 30 min at room temperature. After two washes in PBS and centrifugation (400 g for 10 min at room temperature), cells were re-suspended in 380 μL of PBS solution and transferred to flow cytometer tubes using an 80 μm nylon mesh filter. Propidium iodide (PI, 50 $\mu g\,mL^{-1}$) and DNAse-Free RNase A (50 $\mu g\,mL^{-1}$) were added to each tube before incubating the mixtures in the dark for 30 min until optimal PI staining. PI fluorescence, which is related to the DNA content of each cell, was detected on an orange photo-multiplicator of a FACS-Calibur flow cytometer (BD Biosciences) at a wavelength ranging between 550 and 600 nm. For each sample, 10,000 particles were counted at a low flow rate (15 $\mu L\,min^{-1}$). For each cell event, a single pulse of PI fluorescence was represented according to its area and width. The pulse width was compared to the pulse area in order to discriminate cells in the phase G2/M from doublets of G0/G1 cells having the same DNA quantity. To gate single hemocytes, PI fluorescence intensities were plotted as an FL2-area vs. FL2-width dot-plot. The region R1 was drawn in order to discriminate single cells from doublets. The single cells gated in R1 were plotted on an FL2-area histogram and were used to estimate the percentage of normal and tetraploid hemocytes in the analyzed cell population [4].

2.3. Total RNA extraction

Hemolymph (2 mL) was withdrawn from individual clams using a 3 mL syringe fitted with a 25-gage needle. Total RNA from hemocytes was extracted using a Qiagen RNeasy Mini Kit according to the manufacturer's protocol (Qiagen, ON, Canada). RNA was quantified using a NanoDrop spectrophotometer (Thermo-Fisher Scientific, DE, US) and RNA quality was assessed using the Experion RNA StdSens Analysis Kit (Bio-Rad Ltd. ON, Canada). Only samples with high quality (RNA Index Quality higher than 8) and RNA concentration higher than 100 ng μL^{-1} were selected for further analysis. Only 6 samples from group C (0–10%), 7 samples from group D (10–50%) and 3 samples from group E (> 50%) met these conditions and were therefore selected for microsphere-based multiplex branched DNA downstream analysis.

2.4. Microsphere-Based Multiplex Branched DNA Assay

The basic principle of our assay is based on two novel technologies. First, the target-specific probes are coated to the beads and the hybridization is performed in the liquid system. Second, signal amplification is performed by using branched DNA technology, which enables a high sensitivity of detection similar to quantitative real time RT-PCR [6,22]. In addition, this technology enables a high-throughput analysis by quantifying multiple mRNA targets from the same and unique sample [10,8]. Microsphere-based multiplex branched DNA assay is widely used in transcript profiling and validation against quantitative real time RT-PCR has been performed [3].

The assay uses 3 probe sets, namely Capture Extenders (CEs), Label Extenders (LEs), and Blockers (BLs), which are all capable of specifically hybridizing the RNA targets (Affymetrix Inc, CA, US) (Table 1). Different fluorescent beads are coated with CEs, thus enabling hybridization and discrimination among the different RNA targets. LEs probe sets are designed to hybridize the branched DNA allow amplification of the signal. Each branched DNA contains multiple hybridization sites for biotinylated Label Probes that bind Streptavidin-conjugated R-Phycoerythrin (SAPE). The combined fluorescence signals resulting from both the capture beads and SAPE are read on a Luminex 100 flow cytometer (Luminex Corp., Austin, CA, US).

In this study, 8 transcripts have been selected from the subtractive suppressive cDNA library previously generated in Siah et al. [20]. The transcripts selection was based on their involvement in the development of tumors. Three of the 8 are housekeeping previously validated as the best housekeeping for accurate gene expression analysis related to DN in *Mya arenaria* [19]. The microsphere-based multiplex-branched DNA assays were performed according to the recommended procedure of QuantiGene Reagent System (Affymetrix Inc., CA, US). Briefly, 20 μL extracted total RNA at 100 ng for each sample was mixed with 80 μL of mixture containing probe sets (5 μL) with capture beads (1 μL), lysis buffer (33.3 μL), blocking reagent (2 μL) and nuclease-free water (38.7) for each well.

Wells were incubated at 55 °C for 16 h and washed 3 times with 300 mL of washing buffer. Two series of hybridizations at 55 °C for 1 h with 100 mL of a 1:1000 dilution of branched DNA amplifier and 100 mL of 3′-alkaline phosphatase-conjugated Label Probe oligo respectively were performed and followed by 3 washes with 300 mL of washing buffer after each incubation. To develop the amplified signal, the alkaline phosphatase substrate dioxetane was added to the wells and incubated at 50 °C for 1 h. The signal was detected using the Luminex 100 machine (Luminex Corp., Austin, CA, US).

2.5. Data analysis and statistics

Three replicate assays ($n=3$) were performed for each experimental sample. The performance for the assay for each transcript was determined using a 2-fold serial dilution ($n=5$). Nuclease-free water was used for the background quantification instead of the total RNA. The average median fluorescence was subtracted from the background and normalized to the housekeeping genes. Statistical significance of biological comparison was tested using one-way ANOVA. Significance was defined at $p < 0.01$.

3. Results and discussion

3.1. Tetraploidy status

Sixty clams were sampled from May to October 2010 in North River (Charlottetown, PEI, Canada) and the tetraploidy status of each individual was assessed using flow cytometry. The clams were ranked into 3 categories based on their tetraploid status: (1) Group C (healthy clams considered as control) with a low