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Profiling of gender-regulated gene transcripts in the filarial nematode *Brugia malayi* by cDNA oligonucleotide array analysis

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Abstract

Microarray technology permits high-throughput comparisons of gene expression in different parasite stages or sexes and has been used widely. We report the first use of this technology for analysis of gene expression in filarial male and female worms. The slide array (comprised of 65-mer oligos representing 3569 EST clusters) was spotted with sequences selected from the extensive *Brugia malayi* EST database (http://zeldia.cap.ed.ac.uk/fgn/brugia.php). Arrays were hybridized with Cy dye labeled male and female cDNA. The experimental design included both biological and technical (dye-flip) replicates. The data were normalized for background and probe intensity, and the relative abundance of hybridized cDNA for each spot was determined. Genes showing two-fold or greater differences with $P < 0.05$ were considered gender-regulated candidates. One thousand one hundred and seventy of 2443 clusters (48%) with signals above threshold in at least one sex were considered as gender-regulated gene candidates. This included 520 and 650 clusters up-regulated in male and female worms, respectively. Fifty of 53 (94%) gender-regulated candidate genes identified by microarray analysis were confirmed by real-time RT-PCR. Approximately 61% of gender-regulated genes had significant similarity to known genes in other organisms such as *Caenorhabditis elegans*. Many *C. elegans* homologues of these genes have been reported to have reproductive phenotypes (sterility or abnormal embryo development) by RNA interference. This study has provided the first broad view of gender-regulated gene expression in *B. malayi*; this should lead to improved understanding of reproduction in filarial nematodes. More generally, this approach holds great promise as a means of studying stage-specific or tissue-specific gene expression in parasitic nematodes.

Keywords: Microarray; *Brugia*; Filarial; Gene expression; Reproduction; Nematode

1. Introduction

Filarial nematodes cause important tropical diseases in humans such as onchocerciasis ("river blindness" caused by *Onchocerca volvulus*) and lymphatic filariasis ("elephantiasis" caused by *Brugia malayi*, *B. timor* and *Wuchereria bancrofti*). The parasites are dioecious, and they exhibit marked sexual dimorphism. Brugia adult worms live in lymphatic vessels. The ovoviviparous females release microfilariae (MF) that are ingested by insects; these are essentially equivalent to the L1 stage of other nematodes. Microfilariae molt twice in competent insect vectors to become infective stage larvae (L3) that are infective to humans. L3 molt twice in the human host and to become adult worms that are reproductively active for years. Obviously, parasite reproduction is necessary for transmission, and transmission is necessary for reproduction. Improved understanding of the reproductive biology of filarial worms may provide new insights into the epidemiology of filariasis and lead to new tools for controlling these diseases. Basic research may identify new

Abbreviations: RT-PCR, reverse transcription polymerase chain reaction; RNA, ribonucleic acid; EST, expression sequence tag; MSP, major sperm protein; PMT, photomultiplier tube

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targets for intervention that interfere with egg, microfilaria, or sperm production [1,2]. Filariasis researchers stand to benefit from extensive functional genomic studies that have been performed on the free-living nematode *Caenorhabditis elegans* [3,4]. Since filarial parasites share many genes with *C. elegans* [5], information available on genes involved in reproduction in *C. elegans* is an important starting point for parallel studies in filarial worms [6]. However, there are important differences between filarial worms and *C. elegans*. For instance, while adult filarial worms exist as males (XY) and females (XX), *C. elegans* has males and hermaphrodites but no true females and no Y chromosome.

The large expressed sequence tag (EST) database and extensive genomic sequence information available for *B. malayi* [7–9] provide a solid foundation for studying the molecular biology of this organism using gene expression profiling and functional genomics approaches [10–13]. We have previously identified a small number of sex-regulated genes in *B. malayi* worms by differential display RT-PCR and real-time RT-PCR [11,12]. One of these genes (microfilaria sheath protein) was recently shown to be essential for normal production of microfilaria by RNA interference (RNAi) [13]. However, the pace of progress with this "molecule by molecule" approach is limited.

Microarrays can be used to rapidly assess and quantitate relative levels of expression of thousands of genes in parallel [14,15]. Early experience with small-scale arrays has demonstrated the potential value of this approach for studying gene expression in parasites [16–19]. We now report production and use of the first large-scale oligonucleotide microarray for a nematode parasite. We chose to first evaluate the array with a study of gender-linked expression because of prior work on this area in *B. malayi* and in *C. elegans*. Our results show that oligonucleotide microarray analysis is a reproducible, rapid, and highly efficient method for profiling gender-associated gene expression in *B. malayi*.

2. Materials and methods

2.1. Parasite materials

Adult *B. malayi* worms were isolated from the peritoneal cavity of infected jirds (*Meriones unguiculatus*) obtained from the NIAID Filariasis Repository (University of Georgia, Athens, GA). Male and female worms were carefully separated by size; broken worms were discarded. The worms were washed and immediately frozen at −80 °C.

2.2. RNA isolation and probe preparation

Worms (usually 30 female and 100 male adult worms per batch) were crushed under liquid nitrogen with a ceramic mortar and pestle and extracted in TRIzol reagent (Invitrogen, Carlsbad, CA) as previously described [12]. The accuracy of worm gender separation was assessed by RT-PCR with *B. malayi* major sperm protein primers (*BmMSP*) and *B. malayi* embryo-associated fatty acid-binding protein primers (*Bm-FAB*-1) [11]. RNA quality was assessed with a model 2100 Bioanalyzer (Agilent, Palo Alto, CA). cDNA was synthesized from 5 to 7 μg each of male and female total RNA samples using 3DNA capture sequence primers (3DNA Array 350 Detection system, Genisphere, Hatfield, PA) and SuperScript II Reverse Transcriptase (Gibco BRL, Gaithersburg, MD) for each probe according to standard protocols. cDNA was concentrated by Microcon YM-100 filter (Millipore) and either used immediately or stored at −80 °C. cDNA was synthesized from two different male and female RNA samples (independently prepared as biological replicates).

A two-step protocol was used for hybridization (3DNA Array 350 Detection system, Genisphere, Hatfield, PA). First, oligo arrays were hybridized to the cDNA probes in 2× SDS based-hybridization buffer and washed in 2× SSC, 0.2% SDS according to the manufacturer's protocol. Fluorescent Cy3- and Cy5-capture reagents were combined in hybridization buffer and added to each array. The arrays were incubated and washed as above. Each experiment consisted of a pair-wise competitive hybridization of cDNA samples from female and male worms and a reciprocal dye-flip replicate. Since biological duplicates were tested, a total of four DNA microarrays were used for comparison of the two types of cDNA.

2.3. Microarray fabrication

B. malayi clusters for arrays were selected from 8392 clusters generated by the Filarial Genome Project and posted at the website http://zeldia.cap.ed.ac.uk/fgn/brugia.php [7]. These clusters were derived from 15 cDNA libraries that represent the major *B. malayi* life cycle stages. They represent approximately 40% of the total number of predicted genes for *B. malayi* [8]. Clusters with multiple ESTs, or with detectable similarity to proteins in public databases using BLAST [20], and sequence permitting design of a unique 65-mer oligonucleotide were chosen for inclusion on the array. Oligonucleotides were synthesized from the consensus sequence of selected clusters ($n = 3569$) by standard methods by Illumina (San Diego, CA). The oligonucleotides (50 nM in 3× SSC with 0.75 M betaine) were printed in duplicate on MWG Epoxy slides (MWG Bioteche Inc, High Point, NC) by a locally constructed linear servo arrayer (after the DeRisi model, http://derisilabs.ucsf.edu/).

2.4. Data processing and analysis

Slides were scanned immediately after hybridization on a ScanArray Express HT Scanner (Perkin-Elmer, Boston, MA) to detect Cy3 and Cy5 fluorescence at 543 and 633 nm, respectively. Laser power was kept constant for Cy3/Cy5 scans, and photomultiplier tube values were 69 and 60 V, respectively. An additional scan was done for each slide with the PMT set for 54 and 46 V. The high PMT scan was done in order to maximize signal from low intensity spots. Likewise,