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Effects of curcumin on the parasite *Schistosoma mansoni*: A transcriptomic approach

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ABSTRACT

Schistosomiasis remains a severe problem of public health in developing countries. Several reports show that praziquantel, the drug of choice for treating schistosomiasis, can select *Schistosoma mansoni* strains resistant to the drug. Thus, developing new drugs against this parasitosis is a highly desirable goal. Curcumin, a phenolic compound deriving from the plant *Curcuma longa*, has been shown to have anticancer, anti-inflammatory and antiparasitic effects. Recently, our group has demonstrated that curcumin causes the separation of *S. mansoni* adult worm pairs, eggs infertility, decreased oviposition and parasite viability, leading to death. In the present work, we have investigated the effects of curcumin on *S. mansoni* gene expression in adult worms through microarray analyses. Our results showed 2374 genes that were significantly and differentially expressed, of which 981 were up-regulated and 1393 were down-regulated. Among the differentially expressed genes there were components of important signaling pathways involved in embryogenesis and oogenesis such as Notch and TGF-β. Gene networks most significantly enriched with altered genes were identified, including a network related to Cellular Function and Maintenance, Molecular Transport, Organ Development, which is connected to the TGF-β signaling pathway and might be related to the effect of curcumin on pairing of adult worm pairs, egg production and viability of worms. qPCR validation experiments with 7 genes have confirmed the expression changes detected with arrays. Here we suggest that transcriptional repression observed in Notch and TGF-β pathways could explain the effects on oviposition and egg development described in the literature.

1. Introduction

Schistosomiasis has been estimated to afflict as many as 207 million people in 76 countries, with 779 million more being at risk of infection [1] causing more than 208,000 deaths per year [2]. Praziquantel is the drug of choice for the treatment of schistosomiasis. However, the existence of resistant strains [3–8], the reduction in cure rates and the failure of treatment after praziquantel administration [9] reinforce the need to develop new safe and effective methods against schistosomiasis.

Research with medicinal plants constitutes a very viable strategy for drug discovery [10]. Curcumin is the active component from the rhizome of herb *Curcuma longa*, possessing many pharmacological and biological activities. This compound has anti-inflammatory, antioxidant, antiviral, anti-infectious, and anti-carcinogenic effects [11–14]. In addition, the use of curcumin as parasiticidal agents has been extensively studied. It has activity against *Leishmania* [15–18], *Giardia lambia* [19] and *Trypanosoma* [20,21]. The first studies about curcumin effects in *Schistosoma mansoni* showed the schistosomicidal effect of the oil extract of *C. longa* against *S. mansoni* infected mice [22,23]. Our group described *in vitro* schistosomicidal activity of curcumin against *S. mansoni* adult worms [24]. Then, the schistosomicidal activity of curcumin *in vivo* was published [25]. Recently, El-Agamy et al. [26] showed that curcumin has potent antifibrotic activity in suppressing and reversing *S. mansoni*-induced liver fibrosis.

Considering these observations and knowing that this compound can modulate the expression of many genes, as described in other organisms, we proposed to investigate the effects of curcumin on *S. mansoni* adult worms through microarray analyses. These analyses could determine the possible signaling events related to

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parasite development, enabling the identification of possible targets for new therapeutical approaches against schistosomiasis.

2. Materials and methods

2.1. Ethics statement

In accordance with accepted national and international principles for laboratory animal welfare all experiments were authorized by the Ethical Committee for Animal Care of University of São Paulo.

2.2. Parasite culture and treatment

The LE strain of *S. mansoni* was maintained by passage through *Biomphalaria glabrata* snails and Balb/c mice. After 56 days, *S. mansoni* adult worms were recovered under aseptic conditions from mice previously infected with 200 cercariae by perfusion of the livers and mesenteric veins [27]. The worms were maintained in RPMI 1640 medium (Invitrogen) supplemented with penicillin (100 UI/mL), streptomycin (100 μg/mL) and 10% bovine fetal serum (Gibco). Adult worms were separated in two groups with 20 worm pairs each. The first, control group was maintained in RPMI 1640 culture medium with 1% DMSO, since curcumin was dissolved in DMSO. In the second group, 15 μM curcumin (Sigma–Aldrich, St. Louis, MO, USA) was added to the culture medium. Both groups were maintained at 37 °C, 5% CO_2, during 24 hours. Under these conditions less than 20% of worm pairs had separated. Four biological replicas were assayed; for each replica, 5 groups of 20 worm pairs were pooled for each experimental condition (treated or untreated).

2.3. Total RNA extraction and microarray experiments

Total RNA from adult worms, treated or untreated with curcumin, was extracted with Trizol reagent (Invitrogen, Life Technologies Inc., Carlsbad, CA, USA), using the recommended protocol. After Trizol extraction, RNAs were treated with DNAseI (QIAGEN, Hilden, Germany) and subsequently purified using Qiagen RNAeasy mini kit (QIAGEN). The integrity of RNA samples was evaluated using microfluidic electrophoresis in the Bioanalyzer equipment (Agilent Technologies, Santa Clara, CA, USA) and quantified in the NanoDropTM 1000 spectrophotometer (Thermo scientific, Wilmington, DE, EUA). Gene expression analysis was performed using the 4 × 44 K oligoarray platform, an oligonucleotide microarray slide containing around 44,000 probes representing *S. mansoni* gene fragments, designed by Verjovski-Almeida et al. [28] and manufactured by Agilent Technologies; the platform probe annotation is available on gene expression omnibus (GEO) under the accession number GPL8606.

Parasites were kept in sets of 20 worm pairs each, and they were exposed to either curcumin or vehicle. Subsequently, five sets of worm pairs were pooled to comprise one biological replicate of either treatment or control experiments. We performed a total of four biological replicates, and for each biological replicate two technical replicates were obtained. For these two technical replicates we used 500 ng of total RNA each, to generate amplified cRNA that contained either Cy5- or Cy3-labeled dCTP according to the Agilent Quick Amp Labeling Kit (Agilent Technologies); this kit essentially produces a linear amplification and labeling of poly-A RNA with a T7-RNA polymerase. Hybridization was performed by the combination in one microarray of a technical replica from a treated sample labeled with one dye *vs.* a technical replica from a control sample labeled with the opposite dye (dye-swap approach); 825 ng of amplified Cy5- or Cy3-labeled cRNA was used for each hybridization. Overall, eight microarrays were hybridized (two slides of 4 × 44 k elements each). The slides were washed and processed according to Two-Color Microarray-Based Gene Expression Analysis (Quick Amp Labeling) Protocol (Agilent Technologies) and scanned on a GenePix 4000B scanner (Molecular Devices, Sunnyvale, CA, USA). Data were extracted using Feature Extraction software (Agilent Technologies). Raw data is available in GEO under the accession number GSE37647.

2.4. Processing and analysis of microarray data

Genes were considered as expressed when they had a mean signal intensity of the array spot that was significantly higher (2-sided *t*-test) than the mean signal of the local background (IsPosAndSig column from Feature Extraction data output). Genes were kept in further analyses when they were detected as expressed in at least 75% of all replicas from at least one biological condition (treated or control). The intensities were normalized by LOWESS algorithm [29]. Intensity correlations among different curcumin *vs* control microarrays ranged from 0.86 to 0.96 (average correlation = 0.92); among technical replicates of the same condition (treatment or control) the correlations were from 0.91 to 0.99 (average correlation = 0.97). Correlations among biological replicas were in the range from 0.91 to 0.99 (average correlation = 0.95).

The log 2 ratio of intensity data between treated and control for each gene was calculated. With these ratios, we used Significance Analysis of Microarray (SAM) [30] as the statistical test, to identify differentially expressed genes. We used SAM one-class approach and genes were considered as significantly differentially expressed at a cutoff *q*-value $\leq$ 0.03 and with a Fold Change > 1.5 (*i.e.*, log2 (Treated/control) > |0.58|). Hierarchical clustering of selected genes was generated using Spotfire Decision Site software (TIBCO Software Inc., Palo Alto, CA, USA). For a gene that was represented in the array by multiple probes, we picked a single representative probe (see the list of selected Feature Numbers in Supplementary Table S1) by selecting the probe with the lowest variation coefficient (obtained from intensity data of previous replicated experiments with the 44 k oligoarray). The ontology annotation of all differentially expressed genes (3433) was achieved using the Blast2GO online annotation pipeline [31] as described by Nawaratna et al. [32].

Functional analysis was performed using Ingenuity Pathway Analysis (IPA, http://www.ingenuity.com/). For this purpose we annotated *S. mansoni* genes encoding putative homologs to human proteins; the putative homolog should have similarity with a BlastX *e*-value lower than 10^{-1} ° and coverage of at least 60% of the human homolog. The RefSeq number of each human homolog was associated to each *S. mansoni* gene and the expression data was uploaded to Ingenuity Pathway Analysis System version 7.6. We included all gene/protein relationships described as experimentally observed and/or predicted with high confidence.

2.5. Real-time quantitative PCR (q-PCR) validation of microarray data

One μg of DNAse-treated Total RNA was used as template to synthesize cDNA using the ThermoScript™RT-PCR System (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. cDNA synthesis was performed in the presence of oligodT primer and Reverse Transcriptase (Thermoscript RT-PCR® System, Invitrogen), according to the manufacturer's protocol; 0.5 μl of RT reaction was used for each PCR reaction. Reactions were carried out with SYBR green PCR Master Mix (Applied Biosystems) for 40 cycles in a total volume of 25 μl and according to the manufacturer's instructions using the AB 7500 real-time PCR system (Applied Biosystems). The experiment was performed on 3 independent biological replicates; for each one, 3 technical replicas were performed. The results were analyzed by comparative CT method using the AB 7500 software.