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# Variation in expression of *Biomphalaria glabrata* *SOD1*: A potential controlling factor in susceptibility/resistance to *Schistosoma mansoni*

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## Abstract

The snail *Biomphalaria glabrata* kills the blood fluke *Schistosoma mansoni* by a mechanism involving production of hydrogen peroxide, the enzymatic product of cytosolic Cu/Zn superoxide dismutase (*SOD1*). This enzyme exhibits higher activity in blood cells (hemocytes) from a predominantly resistant strain of *B. glabrata* than in hemocytes from a susceptible strain. Additionally, *B. glabrata* *SOD1* polymorphisms have been associated with susceptibility/resistance to the parasite. To address the hypothesis that *SOD1* transcription levels differ in accordance with variation at the *SOD1* locus, quantitative PCR was performed using hemocyte-derived cDNA prepared from *SOD1*-genotyped snails. Here we report that individuals possessing the allele previously associated with resistance to *S. mansoni* express significantly higher levels of hemocyte *SOD1* transcripts than individuals lacking this allele. A causal relationship between *SOD1* expression and susceptibility/resistance to *S. mansoni* is supported by the correlation of transcript quantity with data (from a previous study) on the probability of infection.

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## 1. Introduction

As with any infectious disease, the outcome of an encounter between *Schistosoma mansoni* (Trematoda) and *Biomphalaria glabrata* (Mollusca) is the result of complex interactions between pathogen, host and the environment. There is sufficient evidence to infer that variation at more than one

locus within the snail genome plays a significant role in determining the likelihood of infection [1–4]. One approach to discerning the key factors that define the snail's innate immune response to this parasite is the use of association studies based on candidate genes. A recent study using this approach with the PR-1 strain of *S. mansoni* and a laboratory population of largely resistant 13-16-R1 *B. glabrata* revealed that polymorphisms at the locus coding for cytosolic Cu/Zn superoxide dismutase (*SOD1*; GenBank [DQ239578](http://www.ncbi.nlm.nih.gov/nuccore/DQ239578)) associate with susceptibility/resistance [5]. This finding reinforced results from a preceding investigation implicating a role for *SOD1*

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in schistosome resistance due to the fact that “resistant” 13-16-R1 hemocytes have significantly higher levels of both *SOD1* transcripts and superoxide dismutase activity than “susceptible” M-line Oregon hemocytes [6]. Additionally, that study emphasized the significance of constitutively expressed *SOD1*, since in vitro interaction/encapsulation of sporocysts by hemocytes did not influence *SOD1* mRNA levels. Together, these discoveries suggest the possibility of a direct functional relationship between variation at the *SOD1* locus and the probability of infection; or, to be more precise, variation in constitutive *SOD1* expression is a consequence of allelic differences in *SOD1* and this variation in expression is a controlling factor in determining the outcome of an infection. Thus, the major objective of the present study was to determine whether the presence or absence of any one *SOD1* allele is associated with either higher or lower levels of *SOD1* expression.

## 2. Materials and methods

### 2.1. Hemocyte collection, RNA extraction and *SOD1* quantitative PCR

Hemolymph (~75  $\mu$ L) was collected from individual 13-16-R1 snails via cardiac puncture, placed on Parafilm to allow shell debris to settle (~1 min), transferred to an untreated 96-well plate and spun for 10 min at 400 rpm. Each well contained hemolymph from a single snail. Hemocytes were allowed to adhere for 15 min at 26 °C and then washed three times with 150  $\mu$ L Chernin’s balanced salt solution (CBSS; 26 °C) [7]. Each well was examined microscopically and those wells with low numbers of hemocytes were excluded. Snails were processed in groups of 4–6 individuals to limit the amount of time between hemolymph collection and RNA extraction. Following CBSS removal, hemocytes were lysed in 100  $\mu$ L Trizol<sup>®</sup> (Invitrogen, Carlsbad, CA) and transferred to a 0.65 mL tube. RNA was purified in a three-step extraction as detailed below. At each step, the organic and aqueous phases were thoroughly mixed and then separated by centrifugation (5 min at 10,000g). First, 20  $\mu$ L of chloroform/isoamyl alcohol (24:1) was added to the lysate. A volume of 50  $\mu$ L was collected from the aqueous layer and added to a tube containing 50  $\mu$ L of acid phenol/chloroform (USB, Cleveland, Ohio) to remove contaminating DNA. To remove residual phenol, 30  $\mu$ L was collected from the aqueous layer

and added to 30  $\mu$ L of chloroform:isoamyl alcohol (1:1). A volume of 25  $\mu$ L was collected from the aqueous layer and added to 25  $\mu$ L isopropanol (containing 5  $\mu$ g of the nucleic acid carrier glycogen). RNA was precipitated (30 min at 4 °C), pelleted (2,000g for 20 min), allowed to air dry for 5 min, and dissolved in 9  $\mu$ L of RNase-free water. A volume of 7  $\mu$ L RNA was removed from each RNA sample and converted to cDNA using oligodT15 primers and Superscript<sup>®</sup> III Reverse Transcriptase (Invitrogen) following the manufacturer’s protocol. The remaining 2  $\mu$ L from each sample was used to quantify (in duplicate) total RNA using RiboGreen<sup>®</sup> (Molecular Probes, Eugene, OR) according to the manufacturer’s protocol. The mean of total RNA added to the reverse transcription reactions was 23.1 ng (16.7 standard deviation).

The resulting cDNA was used as template with *B. glabrata* *SOD1*-specific primers in quantitative PCR performed as previously described with the following exception: the total number of transcripts was normalized to the total amount of RNA [6]. This approach to normalization was taken since no housekeeping gene has been validated using molluscan hemocytes. Potential variability in reverse transcription efficiency was minimized by treating all samples identically, performing a three-step extraction to enhance RNA purity, and synthesizing cDNA immediately after RNA purification thereby reducing the possibility of RNA degradation. A previous study utilizing essentially the same reverse transcription protocol as employed here demonstrated that a total RNA input of between 3 ng and 1  $\mu$ g results in a linear relationship between starting amount of RNA and subsequent transcript abundance [8]. The total RNA input used in the present study was always less than 80 ng, thus eliminating the possibility of saturating the reverse transcription reaction.

### 2.2. Headfoot collection and *SOD1* genotyping

Immediately after hemolymph collection, the headfoot from each individual was removed for subsequent DNA extraction and allele-specific PCR genotyping as previously described [5].

## 3. Results

To determine if *SOD1* transcript abundance differed due to variation at the *SOD1* locus, quantitative PCR was performed using hemocyte-

Table 1  
*SOD1* mRNA transcripts/ng total RNA  $\times 10^{-4}$

Genotype	AA	AB	AC	BC	BB	CC
Mean	1.32	1.8	0.98	1.79	2.03	1.22
S.D.	1.11	0.89	0.55	0.80	0.00	0.81
<i>n</i>	12	18	15	13	1	3

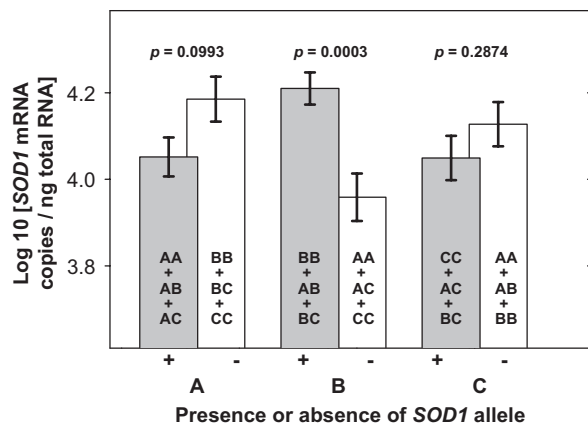


Fig. 1. Effects of the presence or absence of *SOD1* alleles on the abundance of hemocyte *SOD1* mRNA from individual 13-16-R1 snails. Quantitative PCR was performed on plastic-adhered hemocytes (10 min  $\times$  400 rpm followed by 15 min incubation). Serial dilutions of quantified PCR products were used to generate a standard curve. Total hemocyte RNA was determined in duplicate for each individual. Each bar represents the mean  $\pm$  SEM of the genotypes listed. Student's *t*-test was used to determine *p*-values (probabilities were not corrected for the number of alleles tested).

derived cDNA prepared from *SOD1*-genotyped individuals. Table 1 shows the mean value, standard deviation, and number of snails analyzed for each genotype. To perform an allele-wise analysis, the value from each individual was log-transformed and *t*-tests were used to determine if the presence of any allele corresponded to expression differences (Fig. 1). The presence or absence of either the A or C allele did not significantly affect *SOD1* transcript levels; however, individuals possessing the B allele had significantly higher transcript levels than individuals lacking the B allele ( $p = 0.0003$ ). Since the *SOD1* B allele had been previously associated with resistance to *S. mansoni* [5], these results raised an obvious question: is variability in susceptibility/resistance to *S. mansoni* a consequence of variability in constitutive *SOD1* expression by hemocytes? This is difficult to address directly in a single study. Constitutive transcript

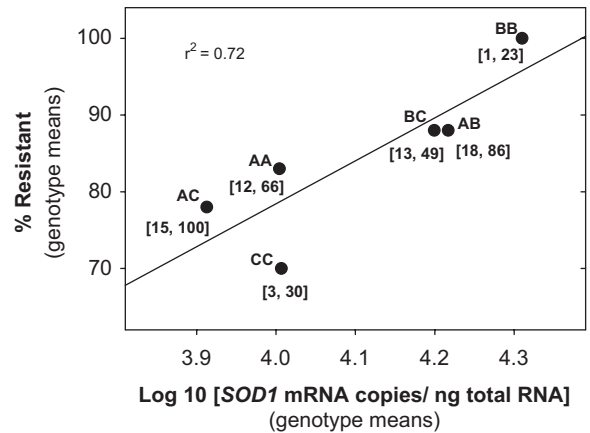


Fig. 2. Relationship between mean *SOD1* expression within 13-16-R1 genotypes and mean probability of *S. mansoni* infection of each genotype. The percent resistance values were derived from a previous phenotype/genotype study of the same 13-16-R1 population used in the present study [5]. Numbers in brackets represent *n* values for expression and phenotype/genotype studies, respectively. The slope of the regression line was significantly different than zero ( $p = 0.0316$ ).

level and susceptibility/resistance phenotype cannot be measured in the same individual, since hemocyte mRNA levels in parasite-exposed snails would be measured by terminal bleeding at some time point after exposure. Therefore, potentially confounding outcomes may arise due to transcriptional responses that occur as a physiological consequence of infection. Alternatively, the possibility of a correlation between constitutive *SOD1* expression and susceptibility/resistance can be assessed by utilizing the *SOD1* genotype as a common denominator between snails in an expression study and snails in a separate susceptibility/resistance study. To accomplish this, we compared the mean transcript level for each of the six *SOD1* genotypes with the mean "percent resistance" for each genotype. These genotype resistance values were derived from a previous phenotype/genotype analysis of 354 individuals from the same 13-16-R1 population utilized in the present study [5]. Fig. 2 illustrates the relationship between hemocyte *SOD1* expression and the likelihood of infection by *S. mansoni*. Although the small number of B and C homozygous individuals in the expression study precludes using this linear regression as a predictive model, a correlation seems apparent since the genotypes form two distinct clusters: individuals with and without the B allele. An analysis using only the genotypes with substantial sample sizes for *SOD1*

expression (i.e. AA, AB, BC and AB) confirms a significant correlation ( $r^2 = 0.96$ ; linear regression not shown). These results demonstrate that from the perspective of *SOD1* genotype, there is a correlation between *SOD1* expression in hemocytes and the probability of *S. mansoni* infection in 13-16-R1 snails. This supports the notion that the association of *SOD1* B allele with susceptibility/resistance may result from differences in *SOD1* expression.

#### 4. Discussion

There is increasing evidence that genetic variation at regulatory sites significantly influences the course of infectious disease [9]. These regulatory polymorphisms appear to be especially important in complex (i.e. polygenic) diseases where multiple genes in the host each contribute subtle phenotypic effects. The results presented here raise the possibility of a similar role for polymorphisms of the *B. glabrata* *SOD1* locus, since individuals possessing an allele previously associated with resistance to *S. mansoni* express significantly higher levels of hemocyte *SOD1* transcripts than individuals lacking this allele. Regulatory variants can be classified into two types, *cis* acting and *trans* acting, depending on the genomic locations of the mutation and the gene(s) whose expression is affected [10]. A *cis*-acting variant alters gene expression by affecting a locus on the same allele and is often associated with a promoter (5'-flanking region) [10] or with translational efficiency/mRNA stability (5'- or 3'-untranslated regions) [11,12]. A *trans*-acting variant is not allele-specific and involves one gene affecting the expression of another gene. The previously identified *B. glabrata* *SOD1* variants [5] utilized in this study were defined by sequence differences in the fourth intron (see GenBank accession no. DQ239578 for intron/exon structure). This location suggests these polymorphisms are likely serving as markers for yet unidentified sites affecting expression and not directly affecting expression on their own. However, the possibility of intronic regulation at this site or unidentified sites cannot be ruled out. A potential first step in determining the location of the polymorphism affecting *SOD1* regulation would be to determine if there is an allelic imbalance associated with hemocyte *SOD1* expression [13,14]. If such an imbalance exists, one would expect higher expression levels of the B allele compared to the A or C alleles within heterozygous individuals.

This would confirm the existence of a *cis*-acting polymorphism and suggest its likely location to be relatively close to or within the *SOD1* locus.

Regardless of its location, the polymorphism(s) affecting *SOD1* expression apparently has considerable impact on 13-16-R1 snails, since higher levels of expression and a higher probability of resistance to *S. mansoni* associate with the same allele. This suggests a causal relationship linking *SOD1* expression and susceptibility/resistance. This notion is supported by the demonstration that within the context of *SOD1* genotype, there is a correlation between *SOD1* expression and probability of infection. These results point toward *SOD1* playing a key role in the hemocyte-mediated response to the parasite, and this proposition is supported by additional evidence. First, scavenging of hydrogen peroxide (the enzymatic product of *SOD1*) reduces in vitro hemocyte-mediated killing of *S. mansoni* sporocysts [15]. Second, 13-16-R1 hemocytes have more *SOD1* transcripts and when stimulated produce more hydrogen peroxide than hemocytes from a susceptible strain (M-line Oregon) [6,16]. When taken together, the outcomes of these lines of investigation converge on hemocyte-derived hydrogen peroxide as a critical element of the *B. glabrata* "resistance mechanism," most likely as a component in cellular signaling pathways and/or oxidative killing mechanisms [17,18]. The findings reported here suggest that *SOD1* expression is one variable controlling hydrogen peroxide production and this leads to a direct connection between *SOD1* polymorphisms and susceptibility/resistance to *S. mansoni*. If this supposition is correct, it follows that any factor influencing either the production or consumption of hydrogen peroxide may also influence the likelihood of infection, and this reveals a number of additional candidate genes for future investigation.

In summary, we have determined that within the 13-16-R1 strain of *B. glabrata* there is an association between the *SOD1* B allele and increased *SOD1* expression. This suggests that increased *SOD1* expression is the driving force behind the association of this *SOD1* variant and resistance to *S. mansoni*. This notion is supported by our demonstration that, from the standpoint of *SOD1* genotype, there is a correlation between *SOD1* expression and the likelihood of *S. mansoni* infection.

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