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Original Article

Comparative Analysis of *Nanos* and *Ago* Genes Expression in the Germinative Cells Isolated from Germinal Layer and the Neck Region of *Echinococcus granulosus*

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Received 18 Mar 2024
Accepted 09 May 2024

Keywords:
Hydatid disease;
Development;
Differentiation;
Germinative cells;
Strobilation

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Abstract

Background: We aimed to evaluate the differential expression of *nanos* and *ago* genes in the protoscoleces, germinal layer, the neck, and the sucker regions of adult *Echinococcus granulosus*.

Methods: The study was conducted in 2018 at the Research Center for Hydatid Disease in Iran, Kerman University of Medical Sciences, Kerman, Iran. In the present study *E. granulosus* protoscoleces were cultured in a di-phasic medium to obtain strobilated worms. The strobilated worms were harvested and using a sterile razor blade, the neck region was separated. In the molecular study the neck sections were compared with the tissues derived from the suckers from the same worm. The primers were specifically designed for RT-qPCR on *nanos* and *ago*. The germinative cells were isolated from the cyst germinal layer and cultured in DMEM for further molecular studies. The Immunohistochemical profile was designed to explore the nature of *nanos* protein in the strobilated worms. Differences between and within groups were statistically assessed relative to the protoscoleces.

Results: An increasing *nanos* gene expressions were found in sucker, neck, cells and germinal layer in comparison to the protoscoleces. The expression of ago gene was decreased in sucker, cell and germinal layer, and increased in the neck region in comparison to the protoscoleces. The results showed that both genes were expressed in all developmental stages of *E. granulosus*.

Conclusion: *nanos* and *ago* genes were differentially expressed at different developmental stages of *E. granulosus* and may contribute to differentiation of the parasite.

Introduction

Asexual reproduction is rare in animal's kingdom but it does happen in almost all species of flatworms. Flatworms represent remarkable capacity for producing strobilar segment from the germinative region of their neck (1). Totipotent stem cells definitely have played an important role in developmental biology and evolution of life cycle of flatworm. It is thought these cells which are sources of new cells for renewing and reparation (2–4).

The characterizations of totipotent stem cells have been largely extensive in planarian more than other models (5). Planarians are known as renewing specialist and they owe this unique capability to a population of totipotent stem cell called neoblast. These cells have been found in almost all parts of planarians body. Neoblasts are the only planarians cell population that show mitotic activity and decisively contribute to tissue regeneration after physical damage and differentiate into all kind of somatic cell (6).

Like planarians, stem cells exist in parasitic flatworm and they are key player in high degree of morphological diversity and remarkable ability to regeneration. Neoblast cells are called germinative cell in tapeworms. In compare to widespread data that are available in neoblast, few studies have been done on other flatworm so the nature of germinative cell of adult worms is poorly understood (1).

Human cystic echinococcosis (CE) caused by the metacestodes of the dog tapeworm, *Echinococcus granulosus*, is one of the most important neglected zoonotic diseases. CE control is a serious public health challenge in endemic communities. Our knowledge on the molecular basis of parasite growth and development is crucial for disease control and management. Humans acquire infection through the ingestion of *E. granulosus* eggs, when the oncospheres penetrate the human intestine and the larval forms develop as hydatid cysts in the liver and lungs, producing thousands of protoscoleces by asexual reproduction (7). Further studies are required to understand the molecular basis of parasite development in vitro and in vivo. Some studies have been done on dynamism of cells division in cestodes. Since in adult worm new segment can be formed in the neck region and this process needs a high cell proliferation and differentiation, neck region is known as proliferating region (8).

Nanos gene, discovered first in *Drosophila* (9), plays an essential role in germ cells differentiation and migration (10). It has been demonstrated that *Nanos* and *Pumilio* interaction is required for anterior-posterior body patterning during early embryogenesis of adult *Drosophila* (11). Also in the flour beetle *Tribolium castaneum*, using hunchback mRNA translation interference, it was shown that *nanos* and *pumilio* have a significant role in posterior segmentation (12). In *C. elegans,* Subramaniam and Seydoux showed that nanos is essential for development and survival of early germ cells as well as the migration of primordial germ cells into the somatic gonad (13). Several studies present evidence for essential role of *nanos* protein in germ line development and maintenance (14).

Some genes including *DEAD box RNA helicase vasa* and Argonaute family gene *piwi* have been especially involved in determination of neoblast cells in planarians and vasa suppression usually contribute to infertility or death (15). One of the most striking family gene development in flatworms genome is a subfamily of *Piwi* genes encoding Argonaute protein such as ago. Silencing piwi protein in germline cells significantly decline the rate of cell division (16,17).

The use of molecular markers is generally admitted as one of the most valid methods to demonstrate the germinative nature of the cell. However for *E. granulosus* no study has used

molecular markers to identify germinative cells. Subgroups of germinative cells are usually having specific markers.

Since there is not enough information about nature of the neck cells for adult worm in this study, we used *nanos* and *ago* as molecular markers to identify germinative cell throughout neck region as well as in the protoscoleces, germinal layer, and the sucker regions of *E. granulosus* by real-time qPCR.

Materials and Methods

Ethics approval

This study was approved by the Research Ethics Review Committee of Kerman University of Medical Sciences, Iran under the code 940352. The study was conducted in 2018 at the Research Center for Hydatid Disease in Iran, Kerman University of Medical Sciences, Kerman, Iran.

Parasite collection and preparation

The protoscoleces of *E. granulosus* were obtained from hepatic hydatid cysts from sheep, slaughtered in Kerman municipal abattoir. After sterilizing the surface of the liver, hydatid fluid containing protoscoleces was aspirated using a 20 ml syringe and aseptically transferred into 50 ml tube. The suspension was left until protoscoleces settled down, the supernatant was discarded and the sediment was washed repeatedly in PBS solution (pH 7.2). The germinal layer was also removed from the cyst and washed three times with sterile normal saline. The percentage of live protoscoleces was determined by eosin exclusion test (18). The cysts with at least 90% viability rate were used for further experiments.

In vitro culture of E. granulosus

In the present study *E. granulosus* protoscoleces were cultured in a di-phasic medium to obtain strobilated worms. Di-phasic culture of protoscoleces was performed as described by Smyth and Davis with some modifications (18,19). Briefly, to obtain protoscoleces from brood capsules, the cyst contents were treatment with Hank's buffer (pH=2) containing 0.5% pepsin. Test tubes were shaken in water bath for 40 minutes at 38◦C. To enhance evagination, the protoscoleces were treated with a solution containing CMRL 1066 with 20 percent dog bile at 100 mg/ml concentration and incubated in shaker incubator for 1 hour (37◦C, 90 shakes/min). Finally the protoscoleces were rinsed 2-3 times with Hank's buffer and transferred into a di-phasic culture medium under sterile conditions. CMRL 1066 was used as the liquid culture medium as well as coagulated fetal bovine serum as the solid base. The medium was changed every week and was replaced with fresh medium. The parasite cultivation was continued for about 40-50 days until the worms strobilated with 2-3 proglottids. The strobilated worms were harvested from the flasks and using a sterile razor blade, the neck region was separated under dissecting microscope. In the molecular study, the neck sections were compared with the tissues derived from the suckers from the same worm.

Parasite material and cell culture

The parasites were aseptically collected from the hepatic and pulmonary hydatid cysts of infected livestock. The germinative cells were isolated from the cyst germinal layer and cultured in DMEM for further molecular studies. Briefly, the cyst layers were cut into pieces and washed several times with sterile PBS and antibiotics (100 U/ ml streptomycin and 60 U/ml penicillin G). The pieces were treated with 0.25% trypsin for 20 min at 4°C after continuous shaking (22 cycles/min). After trypsinization and mincing, germinal layer segments were cultured. Briefly, following 10 min centrifugation (2000g, 4°C), the pellet was resuspended in prewarmed culture medium supplemented with 20% FBS which was added to stop trypsinization. Cells were plated on 12-well plates with a density of approximately 105 cells/cm2 and cultivated in 2 ml/well of DMEM high glucose medium (Gibco) in 5%

CO2 at 37 °C. The culture medium was supplemented with 10% FBS and 10% hydatid fluid. The final medium pH was 7.5 The culture medium was changed weekly. The number of viable cells was determined by trypan blue exclusion analysis.

Immunohistochemical (IHC) evaluation to identify the patterns of nanos

To explore the nature of nanos protein in the strobilated worms, the IHC profile was examined as follows. Tissue sections of 5 µm in diameter were prepared and salinized to enhance the adherence of cells. Then, sections were gradually chilled to room temperature. Subsequently the slides were washed quickly in tris buffered saline at pH 7.4 and stained with monoclonal antibody against cell surface antigens of strobilated worms, including NOS-1 antibody (ab106535, abcam Company).

Molecular study

Total RNA was extracted from different developmental stages of the parasite (protoscoleces, germinal layer and neck region) using a commercial kit (RNeasy Mini Kit, Qiagen, Germany). RNA was quantified by measuring 260/280 nm absorbance ratios by spectrophotometry (Nano Drop ND-1000, Wilmington, DE). cDNA was then synthesized from 100 ng RNA in a total volume of 20 µl using miscript®II Reverse Transcriptase Kit (Qiagen, Germany) according to the manufacturer's instructions.

Real-time qPCR (RT-qPCR) was performed in a Rotor-Gene Q System (QIAGEN, Hilden, Germany). The primers were specifically designed for RT-qPCR on *nanos* and *ago* (Table 1). gapdh was used as the reference gene. Relative quantification of gene expression levels was carried out in duplicate by using SYBR green PCR Master Mix in 10 µl reaction volume. All samples were run for 40 cycles of 95 °C, 2 min for initiation, 94 °C, 5 sec for denaturation, and 60 °C, 10 sec for annealing.

Table 1: Primer sequences used for RT-qPCR analysis of Ago and Nanos genes of *Echinococcus granulosus*

Gene name	*Primer*	*Sequence (5' → 3')*	*PCR product*
Ago	Forward	TGGGTGGCATCAACTCCATC	138pb
	Reverse	TGTGGCAGAGTACCTGCTTG	
Nanos	Forward	GTCGGAAATGTGCCCTCTCA	152pb
	Reverse	GTATTAGGGCTCACCGACCG	
gapdh	Forward	TTCTCCAAGGAAGAGCTGGA	180pb
	Reverse	GCTGGTCGTGATCTGACTGA	

Statistical analysis

Differences between and within groups were assessed relative to the protoscoleces for statistical significance using Student t-test and one-way ANOVA using GraphPad Prism 7.0 Software (www.graphpad.com). For relative quantification, $2^{-\Delta\Delta CT}$ method was employed, using *gapdh* as the reference gene for each sample. *P* values less than 0.05 were considered significant.

Results

Parasite culture

The intact protoscoleces were evaginated within the first day of cultivation and were successfully developed to the strobilated forms. Developmental stages included intact protoscoleces, evaginated protoscoleces, first proglottid formation, second proglottid formation and full-grown 3-proglottid forms that were observed after 55 days of cultivation. Fig.

1 presents different stages of *E. granulosus* in in vitro culture based on different time of development. Light microscopy analysis from primary culture illustrates that cells divided constantly and 48 hours after incubation the cell number was doubled and the morphology suggested a stem cell-like features. As shown in the Fig. 1, several cell colonies within 8 days of initial plating were found. The cells were homogeneous resembling fibroblast morphology. Peripheral cell migration was also significant. The colonies were apparently heterogeneous with barrel-shaped spindles and round configurations.

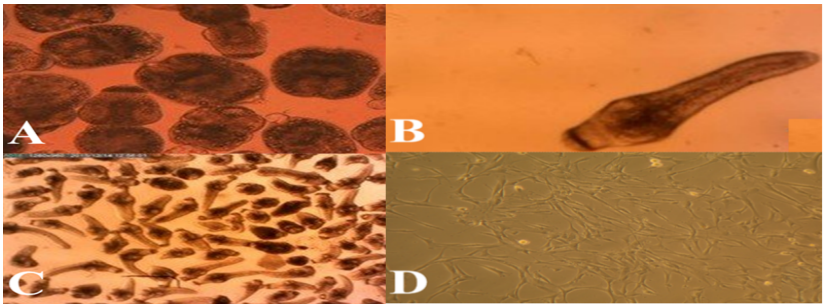

Fig. 1: Light microscopy images of different developmental stages of *Echinococcus granulosus* obtained in monophasic and diphasic culture media. A: Invaginated and newly evaginated protoscoleces on first day. B: Excretory canals formation. C: Proglottid formation. D: The cells isolated from the parasite germinal layer in primary culture

Figure 2 presents the expression levels of the *nanos* and *ago* genes in the protoscoleces, germinal layer, the neck, and the sucker regions of adult *E. granulosus*. An increasing gene expression was found in sucker (0.0625-fold), neck (0.203-fold), cells (0.25-fold) and germinal layer (0.008-fold) in comparison to the protoscoleces (0.004-fold, P <0.001). The expression of *ago* gene was decreased in sucker (0.03-fold), cell (0.014-fold) and germinal layer (0.002-fold), and increased in the neck region (0.088-fold) in comparison to the protoscoleces (0.07-fold, P <0.001, Fig. 2). The results showed that both genes were expressed in all developmental stages of *E. granulosus* (Fig.2). As shown in Fig. 3, the activation of *nanos* was detected using Immunohistochemical analysis. IHC analysis revealed that nanos protein is expressed in the neck region of *E. granulosus*.

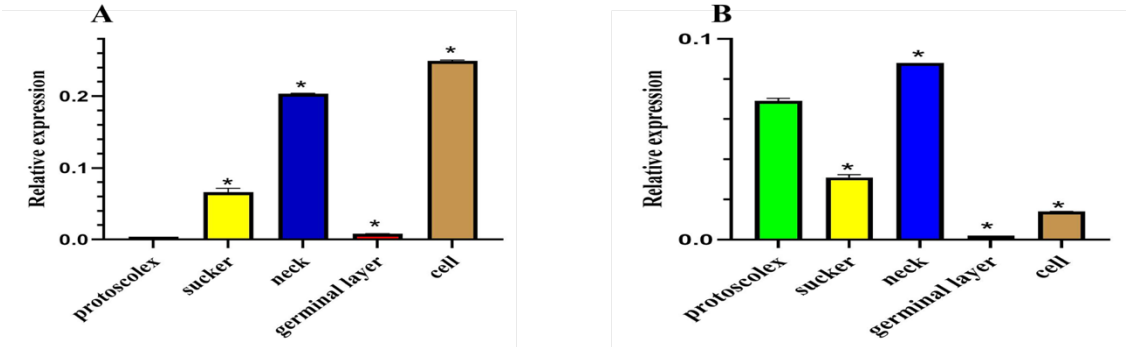

Fig. 2: Relative quantification of the expression levels of two genes, A. *nanos* B. *ago*, in the sucker, germinal layer, neck region and cells isolated from the germinal layer of *Echinococcus granulosus* using RT-qPCR. Relative expression of the genes in the protoscoleces were used as the reference. For relative quantification, 2−ΔΔCT method was employed, with *gapdh* as the reference gene

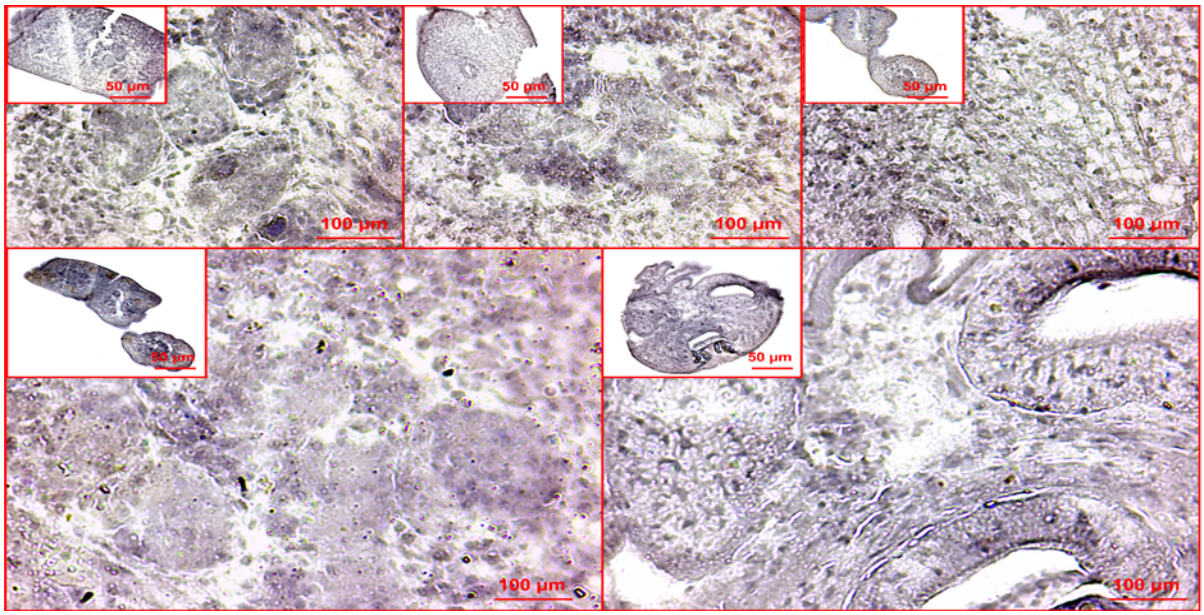

Fig. 3: Nanos-specific immunohistochemical analysis of cross-sections of the neck region of the strobilated forms of *Echinococcus granulosus*, revealing expression of nanos as a molecular marker of the germinative cell throughout the neck region

Discussion

In the present study using qPCR, we compared the expression of the molecular markers, *nanos* and *ago* in five stages of *E. granulosus*. Today different species of platyhelminths are considered as suitable models for studying the stem cell biology. Using protoscoleces as the reference stage, and comparing neck region with the suckers of strobilated worms we tried to identify the differences in the expression of *nanos* and *ago* genes. Looking into the *nanos* expression the findings suggested the stem cell nature of the cells derived from the germinal layer of *E. granulosus*. According to Koziol et al it have been shown germinative cells are the only proliferating cells causing the progression of all cells or parasites during metacestode growth and regeneration (2). Albani et al. have performed couple of studies on stem cells in *E. granulosus* (20). They developed stem cell cultures from *E. granulosus* germinal layer with more than 100 passages, with no indication of decreasing proliferation capacity. However no molecular studies was performed on stem cell markers (20). To date, most studies of germinative cell in *E. granulosus* have been accomplished on cell population, however understanding the generative process require a change of our focus from population based analyses to individual cells (21). Although these cells are morphologically identical, they are heterogenous at the molecular level. Unfortunately, there is no global molecular marker for germinative cells, nonetheless there are useful internal markers for identification of proliferated germinative cells in flatworms. In the present study two genes including *nanos* and *ago* were selected according to the existing information in the literature (22,23). Both gene markers were expressed in all stages of the parasite.

Nanos as a universal stem cell marker was found significantly overexpressed in the cells derived from the neck region compared to the sucker cells from strobilated worms. The expression of *nanos* mRNA has not been investigated in various developmental stages of *E. granulosus*. In *Schistosoma mansoni*, *nanos* role in the maintenance, proliferation and develop-

ment of the germinative cells has been established as some workers showed that *nanos* is expressed in germinal cells of sporocysts and the somatic stem cell as well as the vitelline cells in the adult schistosomes (24,25,26). It has been shown that *nanos* is essential for the reproductive organ development of both male and female *S. japonicum* particularly the testes, vitelline glans and ovaries (27,28). Using RNAi on the genes specifically expressed in the female vitelline glands, some studies showed that vitelline gland development was suppressed with significant changes in gene expression (29–31). Regarding the expression of *nanos*, we found that this gene was overexpressed in all groups of cells isolated from the sucker and neck regions as well as in the germinal layer comparing to the protoscoleces (4).

Nanos contributes to the fate maintenance of the primordial germ cells in *Bombyx mori*, *Caenorhabditis elegans*, *Danio rerio*, *Drosophila*, *Xenopus*, and mouse (32). The function of *nanos*, expressed in the early germarium, is crucial for producing egg chambers during *Drosophila* oogenesis (33). Silencing *nanos* in *Xenopus* lead to the and the loss of germ cells of genitalia and a decrease in primordial germ cells (34). Obviously further studies are required to improve our knowledge on the molecular and cellular biology of the stem cells derived from cestode parasites including *Echinococcus* species.

Regarding the expression of *ago* mRNA, significant difference was observed between protoscoleces and neck region cells. *Ago* gene was overexpressed in the cells of neck region however *ago* expression was significantly lowered in the cultured cells as well as in the sucker and germinal layer cells (Fig. 2). Both genes showed extensive expression in the neck region of the adult worms. The expression of ago gene in *E. granulosus* protoscoleces was found higher than the cells from the sucker region of the adult worm, while this gene shows an increased expression in the neck part of the adult worms.

Piwi genes encoding Argonaute proteins such as *ago* are one of the most striking family genes in the flatworms development and significantly contribute in the rate of cell division in germline cells. Argonaute family proteins are at the heart of RNA-induced silencing complex (RISC). Unfortunately our knowledge on the nature and functions of *ago* in *Echinococcus* species is poor. Various number of *Argonaute* genes has been reported among different organisms, ranging from one in *Schizosaccharomyces pombe* to 27 in *Caenorhabditis elegans* (35). A couple of studies determined the full-length sequences of the three Argonaute proteins as well as the molecular characteristics of *SjAgos* with the description of Argonaute orthologs in *S. japonicum* (36,37). Differential expression of *SjAgos* during the parasite development was also reported by Chen et al. They suggested that *SjAgos* play a role in different small RNA regulatory pathways, and can be involved in regulating schistosome development (36). In addition, germline-specific expression was demonstrated in the *ago* transcripts of both adult female and male *S. mansoni* (38). This indicates a role for ago in the genome stability of germline cells by retrotransposons silencing in adult schistosomes.

Conclusion

Trying to find new cure for echinococcosis needs a deeper understanding of host-parasite interactions and mechanism of parasite development. Regarding the critical role of *nanos* and *ago* genes in germ cell proliferation, self-renewal and differentiation, the present study investigated the differential expression of these genes in four developmental stages of *E. granulosus*. Higher expression of *nanos* gene was found in sucker, neck, and germinal layer cells, while the expression of *ago* gene was only increased in the neck region in comparison to the protoscoleces. Our data could potentially contribute to improve our understanding of the parasite biology, host-parasite relation and parasite development.

Acknowledgements

The authors wish to express their gratitude to the Vice chancellor for Research and Technology, Kerman University of Medical Sciences, Kerman, Iran, for the financial support of the study, Grant No. 940351 and 940352. Special thanks also go to Dr. Ebrahim Saedi for his technical assistance.

Conflict of Interest

The authors declare that there is no conflict of interests.

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