Isolation and Characterization of *vasa* Gene of Triploid and Diploid Human Lung Flukes (*Paragonimus westermani*)

Keun Hee Lee[1†], Hak Sun Yu[1,2†], Jae Won Hur[1], Sung Suk Yu[3], Sun Hee Choi[1], Sang Kyun Park[1], Sun Joo Lee[1], Dong Il Chung[4], Hyun Hee Kong[4], Mee Sun Ock[5] and Hae Jin Jeong[1,2*]

[1]*Department of Parasitology, School of Medicine, Pusan National University, Busan, Korea*
[2]*Pusan National University Hospital Medical Research Institute, Busan, Korea*
[3]*Department of Life Science Gwangju Institute of Science and Technology, Gwangju, Korea*
[4]*Department of Parasitology, School of Medicine, Kyungpook National University, Daegu, Korea*
[5]*Department of Parasitology, Kosin University College of Medicine, Busan, Korea*

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In this study, we isolated, characterized, and compared the *vasa* homologous genes of diploid and triploid *Paragonimus westermani* and localized VASA homologous proteins in both lung fluke types. Open reading frames of *Pw-vasa-2n* and *Pw-vasa-3n* were of 1812 bp, and encoded deduced proteins of 622 amino acids with calculated molecular weights of 69.0 kDa and 68.9 kDa and pI's of 9.11 and 9.03, respectively. A comparison of these two VASA deduced protein sequences showed that only 6 of the 622 amino acids differed. The deduced sequences of Pw-VASA-2n and Pw-VASA-3n contained eight consensus sequences characteristic of the DEAD-box protein family and their N-terminal regions contained four arginine-glycine-glycine (RGG) motifs. These two lung fluke VASA-like proteins were more similar to those of other VASA proteins than to those of other DEAD-family proteins isolated from several organisms (planarian, zebra fish, mouse, and human). *vasa* homologous gene transcription and VASA protein expressions in triploid type lung flukes was slightly stronger than in the diploid type. Immunostaining showed that testes and a portion of the ovaries of both diploid and triploid lung flukes reacted strongly to anti-Pw-VASA antibody.

Key words – *Paragonimus westermani*, diploid, triploid, *vasa*, immunohistochemistry

Introduction

Of the 43 species of *Paragonimus* recorded worldwide, 3 species including, *P. westermani*, *P. pulmonalis*, and *P. iloktsuenensis* are known to occur in Korea [15,25]. *P. westermani* has long been considered to be the most important causative agent of paragonimiasis in Asia, and in Korea in particular. Human infections are caused by eating raw or insufficiently cooked crabs or crayfish, which serve as intermediate hosts [2].

Miyazaki [15] examined spermatogenesis of 20 species in 894 adult lung flukes from several different countries around the world. He divided *P. westermani* into two types based on the contents of seminal receptacles, i.e., a bisexual type and a parthenogenetic type. In the bisexual type receptacles were filled with sperm, while in the parthenogenetic type, they were filled with vitelline cells. In Korea, the majority of flukes reported have been of the parthenogenetic type [9], although Park et al. firstly reported a bisexual type of *P. westermani* in crayfish in Korea in 2001 [18].

P. westermani exists in both diploid and triploid forms, and the triploid form is presumed to be the more pathogenic form in humans [1]. Significantly, the phenomena of the pathologies caused by these two forms differ; triploid flukes mainly form cysts in the lungs, but diploid flukes cause lesions in the pleural cavity and pleura [16]. Park et al. also examined the chromosome karyotype, and some genes of two flukes isolated in Haenam and Youngam provinces (Korea) [17,18]. The results obtained showed that lung flukes isolated in Haenam province were diploid, while those from Youngam province were triploid. These results supported the hypothesis that diploid lung flukes have functional sexual organs and normally reproduce sperm and eggs, whereas triploid lung fluke cannot reproduce sperm because reproductive organs do not function [6]. This reproductive organ dysfunction may be due to mutations or the reduced expressions of some re-

***Corresponding author**
Tel : +82-51-240-7747, Fax : +82-51-241-0860
E-mail : jeonghj@pusan.ac.kr
†K. H. Lee and H. S. Yu equally contributed to this study

over 30 amplification cycles (30 sec at 95°C, 30 sec at 50°C and 30 sec at 72°C). Sequences for the primers are listed in Table 1. *vasa* mRNA expression levels were normalized versus β-actin mRNA.

Expression and antibody analysis

Recombinant Pw-vasa-2n and Pw-vasa-3n proteins (rPw-VASA-2n and rPw-VASA-3n) were expressed in *E. coli* by cloning the presumed mature coding sequences (1866 bp) into pGEX 4T-2 expression vector (Amersham, Biosciences, UK). Protein expressions were induced in BL21 (DE3) cells using 0.1 mM IPTG for 3 h at 37°C. Bacteria were pelleted and sonicated in PBS and mixed with a 50% slurry of Glutathione Sepharose 4B equilibrated with 1×PBS, and incubated for 30 min at room temperature with slight agitation to allow binding of GST-fusion proteins. Eluted proteins were dialyzed against PBS, and recovered at a final concentration 1 mg/ml. Rats were immunized with 100 ug of rPw-VASA-2n or rPw-VASA-3n (first in complete Freund's adjuvant, the second in incomplete Freund's adjuvant), respectively. Serum samples were collected 14 days following the boost. For western blots, whole lung fluke proteins were extracted using total protein isolation kit (Intron) and mixed with SDS-loading buffer. Samples were subjected to SDS-PAGE and electrotransfer, probed with a 1/200 dilution of rat anti Pw-VASA-2n or anti Pw-VASA-3n and normal rat serum, and treated with peroxidase anti-rat Ig and ECL substrate.

Immunohistochemistry

Triploid lung fluke worms were fixed with 10% formalin and embedded into paraffin blocks. Individual paraffin sections were deparaffinized and dehydrated through a series of xylene-ethanol solutions. To inactivate endogenous peroxidase activity, specimens were treated with methanol containing 3% H_2O_2 for 30 min. For antigen retrieval, sections were immersed in citrate buffer (pH 6.0) and boiled for 15 min. After boiling, sections were washed in PBS (pH 8.2) containing 3% normal rat serum for 2 hr, and then incubated overnight at 4°C with primary antibodies; rat anti-Pw-VASA-3n (1:200 dilutions). Goat HRP conjugated anti-rat IgG (Santa Cruz Biotech, Santa Cruz, CA) was used as the secondary antibody at 1:500 dilution in PBS (pH 8.2). For HRP-conjugated antibody, sections were stained with 1 mg/ml 3, 3-diaminobenzidine (DAB) in 0.1M Tris-HCl (pH 7.5) containing 0.02% H_2O_2 and counterstained with Hematoxylin.

Results

Sequence analysis and recombinant protein expression

Partial *vasa*-like genes were amplified from the adult worm cDNAs of *P. westermani* diploid and triploid types. These genes consisted of 314 nucleotides, and their deduced amino acid sequences contained the PTRELA and DEAD domains. We generated full-length *vasa*-like genes from cDNA using RACE PCR and full-length PCR. The open reading frames of *Pw-vasa-2n* and *Pw-vasa-3n* were predicted both of 1812 bp, and these were assumed to encode putative proteins of 622 amino acids with calculated molecular weights of 69.018 kDa and 68.930 kDa and pI's of 9.11 and 9.03, respectively. A comparison of the two predicted VASA protein showed that only 6 of the 622 amino acids differed (Fig. 1). The deduced amino acid sequences of Pw-VASA-2n and Pw-VASA-3n contained eight consensus motifs characteristic of the DEAD-box protein family, and contained four arginine-glycine-glycine (RGG) motifs (Fig. 1). The sequence AQTGSGKT corresponds to the consensus sequence AQTGXGKT (domain I), which is known as the ATPase A motif. The sequence DEAD (domain V), known as the ATPase B motif, was found at residues 311 of Pw-VASA-2n and Pw-VASA-3n. The sequence SAT and the HRIGRTGR domain (domain VI and VIII), which have been shown to participate in RNA-binding and unwinding activity in eIF4A, were also found to be conserved in the predicted amino acid sequences of Pw-VASA-2n and Pw-VASA-3n. Other domains PTRELA (domain II), GG (domain III), TPGR (domain IV), and ARGRD (domain VII), were well conserved. A conserved domain search using the Conserved Domain Database [14] demonstrated the presence of HELICc and DEADc domains in both genes. The HELICc domain was found to start at the 149th residue and to terminate at the 368th residue, whereas the DEADc domain spanned the 378th to 508th residues (Fig. 2). These results show that the two isolated VASA-like proteins have similar or identical functions. The identity rates of these two proteins was found to be highest with VASA proteins in planarian (61% and 57% similarity with diploid and triploid VASA proteins, respectively), in zebra fish (52%, 47%), in mouse (48%, 48%), and in man (48%, 48%). These similarities were

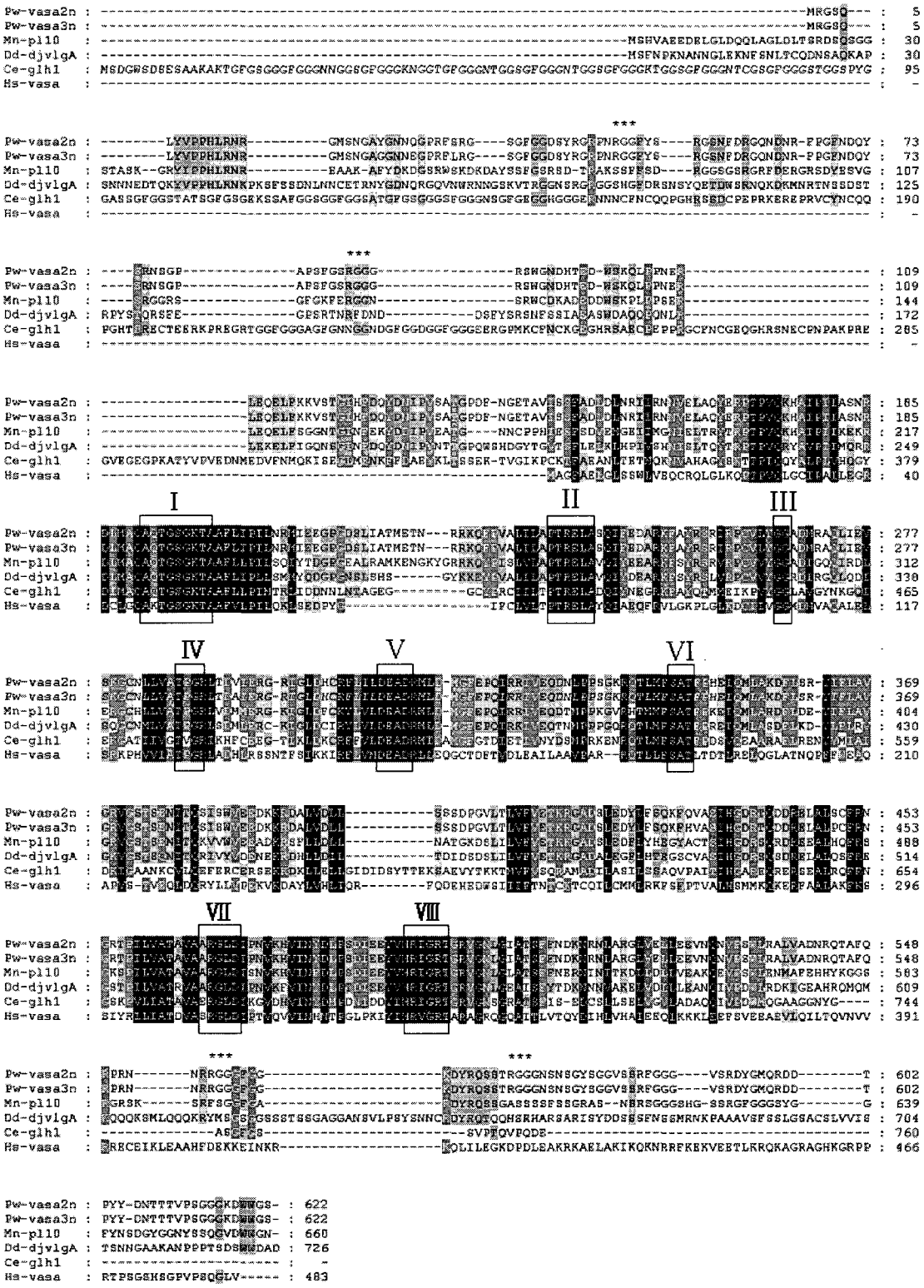

Fig. 1. Multi-alignment of VASA amino acid sequences from planarians to human. Residues conserved in 100% and 99% - 50% of the sequences are highlighted in black and gray color, respectively. The eight conserved homology boxes present in all DEAD box protein are indicated by I-VIII Domain search in diploid and triploid *P. westermani. Mn-pl10* Mouse (**j04847**); *Dd-DjvlgA Dugesia dorotocephala* (**AB017002**); *Ce-glh1 Caenorhabditis elegans* (**p34689**); *Hs-vasa* human (**AAF72705**). Asterisk marks RGG motif.

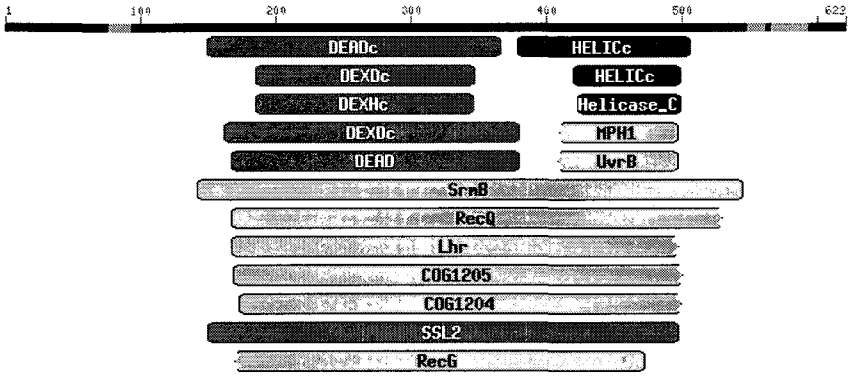

Fig. 2. Conserved domain search in VASA of diploid and triploid *P. westermani*. DEADc (DEAD-box helicases): A diverse families of proteins were involved in ATP-dependent RNA unwinding, needed in various cellular processes including splicing, ribosome biogenesis and RNA degradation. HELICc (Helicase superfamily c-terminal domain): associated with DEXDc-, DEAD-, and DEAH-box proteins; SSL2 (DNA or RNA helicases of superfamily II): Transcription / DNA replication, recombination, and repair.

substantially higher than those of other DEAD-family proteins, e.g., p68 in man (39%, 39%) or eIF4A in mouse (32%, 32%, respectively). An analysis of the phylogenic relationships between members of the DEAD-box protein family showed three distinct clusters: the VASA sub-family (including the predicted lung flukes VASA sequences), the eIF4A sub-family, and p68 sub-family (Fig. 3).

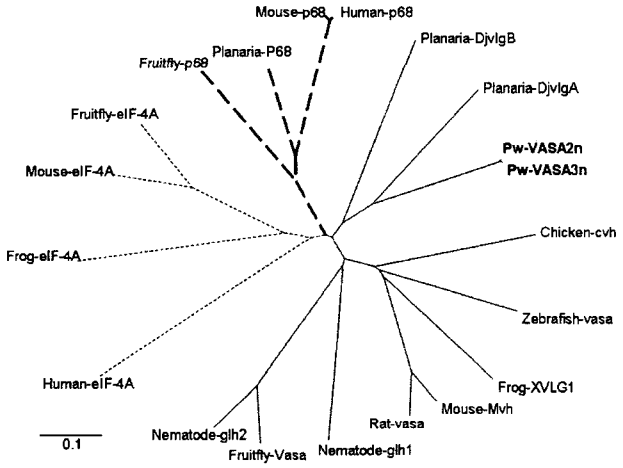

Fig. 3. Phylogenetic tree derived by aligning amino acid sequences of VASA proteins of both lung fluke types with those of other VASA proteins. Solid, thick dotted and fine dotted lines were represented as VASA, P68, and eIF-4A groups respectively. Human-eIF-4A (**d26528**); Frog-eIF-4A (**x92421**); Mouse-eIF-4A (**x03040**); Fruitfly-eIF-4A (**x69045**) ; Fruitfly-P68 (**x52846**); Planaria- P68 (**AB016997**); Mouse-P68 (**x65627**); Human-P68 (**x52104**) ; Zebrafish-vasa (**ab005147**); Mouse-Mvh (**d14859**); Rat-vasa (**s75275**); Nematode-glh1 (**p34689**); Nematode-glh2 (**AAB03510**) ; DjvlgA (**AB017002**); DjvlgB (**AB017003**); Chicken-cvh (**AB004836**); Fruitfly-vasa (**NM165103**); Frog-XVLG1 (**s69534**).

Comparison of vasa gene and VASA protein expression in the two lung fluke types

To compare *vasa* gene and VASA protein expression levels, RT-PCR and western blot analyses were performed. The *vasa* gene transcription level of triploid type lung fluke was similar to that of the diploid fluke (Fig. 4). VASA proteins were exclusively detected as a single band (about 69 kDa for both flukes) by western blotting against both lung fluke total extracts (data not shown) VASA protein was expressed by the triploid fluke at slightly higher levels than by the diploid fluke. This result was consistent with RT-PCR results.

Localization of VASA-like protein in *Paragonimus westermani*

It has been shown in many animals that VASA proteins are specifically expressed in germ line cells and that they are differently expressed during the life cycle. Therefore, to determine the expression patterns of VASA proteins in the

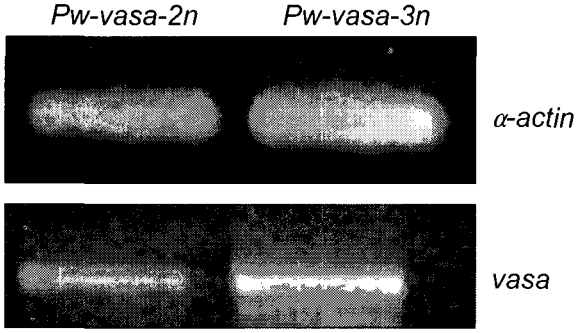

Fig. 4. Transcription level of *vasa* gene of diploid and triploid *P. westermani* measured by RT-PCR.

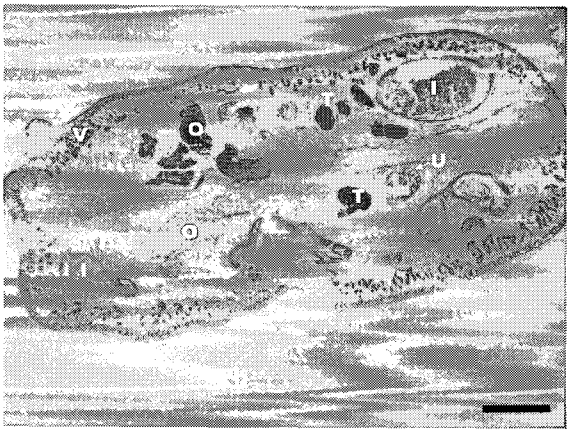

Fig. 5. Immunohistochemical expression of VASA in diploid *P. westermani*. Testes (T) show strong positive reactivity for VASA, but other organs including ovary (O), uterus (U), vitellaria (V), and intestine (I) show negative immunoreactivity. (bar = 1 mm)

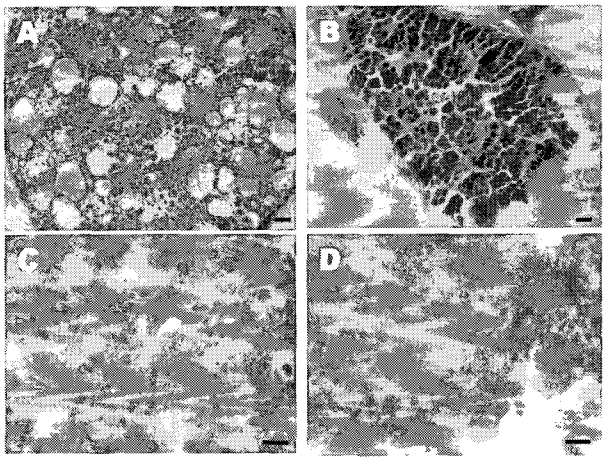

Fig. 6. The histology and immunohistochemical reactivity for VASA in testes of diploid and triploid *P. westermani*. Microscopically, a testis of diploid lung fluke has staged reproductive cell include mature spermatozoa (A), but no evidence of spermatozoa formation is noted in testes of triploid lung fluke (B). A and B were stained with H&E. Immunohistochemical studies for VASA show intense cytoplasmic staining in testes of diploid (C) and triploid (D) lung flukes. (bar = 10 μm)

two flukes, we performed whole mount immunohistochemistry experiments. After immunostaining, only the testes of diploid lung fluke type were found to strongly react with anti-Pw-VASA antibody (Fig. 5). Other organs (ovary, intestine, vitelline, uterus, eggs, and suckers) of both lung flukes exhibited no immunoreactivity for VASA. Triploid lung fluke type was detected the same immunohistochemical reactivity as seen in diploid lung fluke type (data not shown). Cytoplasms of various reproductive cell types in testes of both lung fluke types were reacted with anti-Pw-VASA antibody (Fig. 6). Host lung tissues did not reacted with anti-Pw-VASA antibody.

Discussion

In this study, we isolated, characterized, and compared the *vasa* homologous gene of diploid and triploid *Paragonimus westermani* and localized VASA homologous proteins in both lung flukes. The predicted amino acid sequences encoded by these genes shared eight well-conserved domains of DEAD box family proteins, suggesting that they are ATP-dependent RNA helicase, including ATP-binding, RNA-binding, and in-winding modalities [19,20]. In addition, the RGG repeat sequences, which are specifically conserved in the *vasa* subfamily, were found in both *Pw-vasa-2n* and *Pw-vasa-3n*. However, another conserved sequence EARKF in planarians and other organisms was present as DARKF in *Pw-vasa-2n* and *Pw-vasa-3n*. Although they show motif differences, both genes were categorized as *vasa* family by phylogenic analysis. Sequence homolog analysis revealed that both *Pw-vasa-2n* and *Pw-vasa-3n* are closer to planarian *DjvlgA* and *DjvlgB* than to *vasa* related genes in other animals, which suggests that *Paragonimus* are closer genetically and evolutionary to planarian than other to organisms.

Early research demonstrated that it is usual for *Paragonimus* adults to occur in pairs in lung cysts [24]. Eggs were laid within these cysts and eventually discharged to the outside via the respiratory passages. An exception to this rule of paired worms in cysts is provided by *P. westermani*, as unpaired worms were often found in lung cysts in humans and in experimental carnivore infections, and this lead to the assumption that the worms are self-fertilizing [5]. This anomaly was explained by the discovery of diploid and triploid forms of *P. westermani* in East Asia [15]. Diploid sexually reproducing forms require partners for sperm exchange and cyst formation. In contrast, triploid worms, which reproduce parthenogenetically, are individually capable of cyst formation and egg production (although more than one worm may occupy a cyst in cases of heavy infection). Spermatogenesis in triploid *P. westermani* is aberrant and normal spermatozoa are not produced [6]. Moreover, their results show that *P. pulmonalis* (*P. westermani* triploid type) spermatogenesis is almost completely inhibited by aberrations in the process. The spermatozoa found in the testis of triploid type lung fluke

may degenerate, because no spermatozoa were observed in seminal receptacles. We hypothesized that aberrant spermatogenesis in triploid *P. westermani* is due to a defect in one or some reproduction related genes, especially in the *vasa* gene. In the present study, although the triploid type had an intact form of the *vasa* homologous gene, its expression level was slightly higher than observed in the diploid type. This over expression phenomenon may be elicited by abnormalities of other *vasa*-related gene (*oskar* or *tudor* gene) functions. In *Drosophila*, VASA protein is assumed to interact with other maternal factors, such as, Oskar protein, Tudor protein, and mitochondria large rRNA [10,12] and to function at the translational level in, e.g., the storage and transporation of some specific mRNAs. It is our intention to further study relationships between *vasa* expression and other reproduction-related genes.

Several reports have suggested that *vasa* genes evolved in the reproductive system [3,7,21], and in fact, the majority is expressed only in the reproduction system and at special periods during the developmental period. However, we could not determine when *vasa* gene expression peaked during the lung fluke life cycle, because of we were unable to obtained adequate egg and metacercaria mRNA for northern hybridization analysis. However, immunohistochemistry located VASA-like proteins to the testes only in each type lung fluke. Our results are consistent with several previous reports in mouse, man, and planaria [3,7,21].

In conclusion, in the present study we isolated *vasa* genes for the first time from the diploid and triploid types of *P. westermani*. Our observations suggest that they function in the reproductive system, much as has been reported in other organisms. In particular, the triploid type expressed slightly higher levels of VASA protein than the diploid type. Although, we found no abnormality in the VASA proteins of triploid lung flukes, it remains possible that abnormalities in other genes or proteins related to VASA may cause incomplete spermatogenesis in these animals.

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초록 : 폐흡충의 이배체와 삼배체 *vasa* 유전자 분석 및 특징

이근희[1†] · 유학선[1,2†] · 허재원[1] · 유성숙[3] · 최선희[1] · 박상균[1] · 이선주[1] · 정동일[4] · 공현희[4] · 옥미선[5] · 정해진[1,2*]
([1]부산대학교 의과대학 기생충학 교실, [2]의과학연구소, [3]광주과학기술연구소, [4]경북대학교 의과대학 기생충학 교실, [5]고신대학교 의과대학 기생충학 교실)

폐흡충은 국내를 비롯한 아시아에서 폐흡충증을 일으키는 중요한 기생충이다. 이러한 폐흡충은 이배체 와 삼배체가 국내에 알려져 있다. 이배체 폐흡충은 양성생식을 하고 삼배체 폐흡충은 단위생식을 하는 것으로 알려져 있다. 그러나 이러한 원인에 대하여서는 알려진 바가 없다. 생식유전자 중 초파리에서 최초 분리되었고 포유동물에서도 그 기능이 밝혀진 *vasa* 유전자가 가장 유명하다. 이 유전자는 생식세포의 분화에 관여하며 종에 따라서는 정자생성에도 관여하는 것으로 알려져 있다. 이번 연구는 삼배체 폐흡충의 단위생식과 *vasa* 유전자와의 관계를 규명해 보고자 하였다. 폐흡충의 이배체와 삼배체 성충으로부터 *vasa* 유전자 전체의 염기서열을 얻을 수 있었다. 두 염기서열의 경우 8개의 ATP-binding domain이 관찰되었고 helicase가 결합할 것으로 예상되는 RGG motif도 관찰되었다. 총 622아미노산 서열로 구성될 것으로 보이며 이배체는 69.018 kDa, 삼배체는 68.930 kDa 크기의 단백질을 만들 것으로 예상되었다. Vasa 재조합 단백질은 GST와 fusion되어 93 kDa 크기에서 관찰되었다. mRNA의 발현은 이배체에 비해 삼배체가 다소 높았다. Anti-Pw-VASA 항체를 이용한 면역조직화학법을 수행한 결과 이배체와 삼배체는 다른 기관에서는 면역반응력을 보이지 않고 고환에서만 면역반응력을 볼 수 있었다. 결과를 종합해 보면 *vasa* 유전자는 이배체 삼배체 모두 가지고 있었으며 정상적으로 발현되었다.