Localization of Tubulin from the Carcinogenic Human Liver Fluke, *Opisthorchis viverrini*

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Background: *Opisthorchiasis caused by Opisthorchis viverrini, is of considerable public health importance in Southeast Asia, particularly in Lao People's Democratic Republic and Thailand. The infection is associated with a number of hepatobiliary diseases including cholangitis, obstructive jaundice, hepatomegaly, cholecystitis, cholelithiasis and cholangiocarcinoma.*
Objective: *This study aims to localize the expression sites of O. viverrini tubulin using immunohistochemistry by monoclonal anti α-tubulin (MAαT) and anti β-tubulin (MAβT).*
Material and Method: *The adult worms of O. viverrini, and the adult worm of O. viverrini in biliary system of hamsters were fixed, cryo-sectioned and then immunohistochemically stained. The sections were incubated with MAαT or MAβT. A positive test required the observation of brown-staining in the fluke's organs.*
Results: *The immunohistochemistry of MAαT and MAβT in adult worms of O. viverrini, and the adult worm of O. viverrini in biliary system of hamsters strongly expresses in the sperm and seminal vesicles of the worm. MAαT and MAβT expressed slightly in sub-tegumental tissue, stromal parenchyma, muscle fibers, and miracidium in the mature egg of the worm. No staining in the spermatogonia, gut epithelium, immature egg, tegument or vitelline glands of the worm nor in the hamster bile duct epithelium was seen.*
Conclusion: *The present study indicates that O. viverrini tubulin is present in the reproductive organs and other important organs of the worm. Because it plays a key role in the biological processes of cellular motility and fertility it should be further studied in detail including the characterization, production of recombinant proteases, and their application in immunodiagnosis.*

Keywords: *Localization-Tubulin-Carcinogenic human liver fluke-Opisthorchis viverrini*

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Opisthorchiasis caused by *Opisthorchis viverrini*, is of considerable public health importance in Southeast Asia, particularly in Lao People's Democratic Republic and Thailand[1]. In Thailand, the first nationwide survey of the four regions of Thailand during the years of 1980-1981 revealed an overall prevalence of *O. viverrini* infection of 14%; the Northeast (34.6%), the Central (6.3%), the North (5.6%) and the South (0.01%) regions[2]. It is estimated that 6 million people are infected with the *O. viverrini*[3]. Human have been infected by ingesting undercooked fish containing infective metacercariae; this is very common in the northeastern and northern regions, particularly in rural areas[4-7]. The infection is associated with a number of hepatobiliary diseases including cholangitis, obstructive jaundice, hepatomegaly, cholecystitis and cholelithiasis[8,9]. Experimental and epidemiological evidence strongly implicates liver fluke infection in the etiology of cholangiocarcinoma (CCA) the bile duct cancer[10-12].

Tubulin is one of several members of a small family of globular proteins. The most common members of the tubulin family are α-tubulin and β-tubulin, the proteins that make up microtubules. Microtubules are assembled from dimmers of α- and β-tubulin, a heterodimer of two globular subunits that are highly conserved across species. Microtubules play a key role in many important biological processes, such as cell division, cellular motility, intracellular transport of materials, and cell configuration in eukaryotic cells[13]. Tubulins are a major target of medications treating cancer, gout, as well as fungal and helminth infections[14-16]. Tubulins are found in many parasites

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as components in structures such as muscle, tegument, egg, etc. Tubulin of human trematodes has been found[17-20] in the tegumental syncytium, the tegumental cell bodies and their cytoplasmic connections with the surface syncytium of *Fasciola hepatica*[17]. Tubulin was also demonstrated in the tegumental cell bodies, their cytoplasmic processes, and the basal layer of the tegumental syncytium of *F. gigantica*. In *Schistosoma mansoni* tubulin appears as vertical lines stretching across the whole thickness of the syncytium[18,19]. Researchers have identified five alpha-tubulin and six beta-tubulin isotypes that are expressed in adult *F. hepatica*. Phylogenetic analysis indicates that two of the alpha-tubulins and two of the beta-tubulins are distinctly divergent from other trematode and nematode tubulin sequences. *Clonorchis sinensis*, the Chinese liver fluke, it was reported that two beta-tubulin cDNAs, CsTB1 and CsTB3 have been successfully cloned. The CsTB1 and CsTB3 cDNA were 2,082 and 1,486 basepair long and encoded 445 and 444 amino acids, respectively. The two clones were identical in 78% in their coding sequences and 97% in deduced sequences[20].

Based on Laha et al[21], Approximately 5,000 randomly selected cDNAs from the adult stage of *O. viverrini* were characterized and accounted for 1,932 contigs, representing ~14% of the entire transcriptome, which is presently the largest sequence dataset for any species of liver fluke. Twenty percent of contigs were assigned GO classifications. Abundantly represented protein families include those involved in physiological functions that are essential to parasitism, such as anaerobic respiration, reproduction, detoxification, surface maintenance and feeding. GO assignments were well conserved in relation to other parasitic flukes, however, some categories were over-represented in *O. viverrini*, such as structural and motor proteins. An assessment of evolutionary relationships showed that *O. viverrini* is more similar to other parasitic flukes (*C. sinensis* and *S. japonicum*) compared with free-living (*Schmidtea mediterranea*) flatworms, and 105 sequences have close homologues in both parasitic species but not in *S. mediterranea*. Sripa and Kaewkes[22] have reported that a positive reaction to the immunoperoxidase staining for *Opisthorchis* antigens is recognized by reddish-brown deposits. Two antiserum preparations (i.e. anti-ES and anti-somatic antibodies) produce strongly positive staining in the tegument, muscle, digestive tract and reproductive organs, including eggs, of the liver fluke. The anti-ES antibody exhibited less activity for the parasite eggs than that of the anti-somatic. From this data and the existence of cross-reacting species in the excretory secretory (E/S) of *C. sinensis* makes the presence of such tubulin of *O. viverrini* highly probable. Therefore, we intended to localize the expression sites of *O. viverrini* tubulin using immunohistochemical staining by MAαT and MAβT.

Material and Method

The study protocol was approved by the Suranaree University Biotechnological Review Committee (2011). *O. viverrini* metacercariae were obtained from naturally infected cyprinoid fish captured from an endemic area of Khon Kaen province, Thailand. The fish were digested with pepsin-HCl at 37°C for 2 hours and then the metacercariae were collected and identified under a dissecting microscope. Moving viable metacercariae was used to infect hamsters, and then bed for each 2 weeks and a month. The adult worm of *O. viverrini* was recovered from infected hamsters, and the adult worm of *O. viverrini* in biliary system of hamsters, were mounted in paraffin (Sakura Tissue-Tek® TEC™) and sectioned as described[22]. The sections were deparaffinized in xylene three times for 5 minutes each. After deparaffinization, slides were dehydrated in decreasing concentrations of ethanol; absolute ethanol three times, 5 minutes each, 95% ethanol two times, 3 minutes each, 70% ethanol one time, 3 minutes, and a final rinse with tap water for 1-2 minute. Slides were then washed in PBS twice, 5 minutes each time, then rinsed with tap water for 5 minutes. Slides were then exposed to 30% H_2O_2 in methanol for 30 minutes to block non-specific endogenous peroxidase activity. Sections were then blocked for non-specific binding with normal horse serum diluted 1:20 in PBS/NaN_3 for 30 minutes at room temperature in a humid chamber. The sections were probed with MAαT or MAβT antibodies (Amersham International plc, Amersham, Buckinghamshire, England, UK) at a dilution of 1:100 in PBS/NaN_3 in a humid chamber at 4°C overnight. Sections were then incubated in a secondary antibody (HRP-goat anti-rabbit IgG) diluted 1:200 in PBS for 1 hour at room temperature. To develop color, slides were submerged in freshly prepared 0.05% diaminobenzidine tetrahydrochloride (DAB) in 0.003% H_2O_2 solution for 5 minutes. The reaction was stopped with tap water and slides counterstained with Mayer hematoxylin for 1 minute. After counterstaining, the slides were washed with tap water and dehydrated as follows: 70% alcohol once for 3 minutes; 95% alcohols twice, 3 minutes each;

final dehydration in absolute alcohol three times, 3 minutes each, and then slides were cleared and mounted. Slides were then examined and photographed with the aid of 100X and 400X objectives fitted to an Olympus model BX40 compound microscope connected to a digital camera (Nikon DXA1200C). A positive test required the observation of brown-staining in the fluke's organs.

Results

Immunohistochemistry staining and sequential tubulin localization in the adult worm of *O. viverrini*, and the adult worm of *O. viverrini* in biliary system of hamsters as detected by immunoperoxidase staining using MAαT and MAβT. Immunoperoxidase staining were original magnification with 200 and 1,000x.

1) Immunohistochemistry of αMAT in the adult worm of *O. viverrini* and the adult worm of *O. viverrini* in biliary system of hamsters: Negative control shows no staining in the fluke and bile duct epithelium (Fig. 1A, 1B). MAαT stained in sub-tegumental tissue, stromal parenchyma, muscle fibers, and miracidium in mature eggs (Fig. 1C, 1D, 2A-2D). MAαT strongly stains in sperm in both the testis and the seminal vesicle (Fig. 2). No staining was seen in spermatogonia, gut epithelium, immature egg, tegument or vitelline glands, nor in the hamster bile duct epithelium (Fig. 1A).

2) Immunohistochemistry of MAβT in the adult worm of *O. viverrini* and the adult worm of *O. viverrini* in biliary system of hamsters: Negative control shows no staining in the fluke and bile duct epithelium (Fig. 3A). MAβT also slightly stained in the sub-tegumental tissue and the stromal parenchyma, and strongly stained in sperm in the testis (Fig. 3B, 2C). No staining was seen in spermatogonia, gut epithelium, immature egg, tegument or vitelline glands, nor in the hamster bile duct epithelium (Fig. 3A).

Discussion

Tubulins are targets for medications treating cancer, gout as well as fungal and helminthic infections. One example would be colchicines in gout binds to tubulin and inhibits microtubule formation, arresting neutrophil motility and

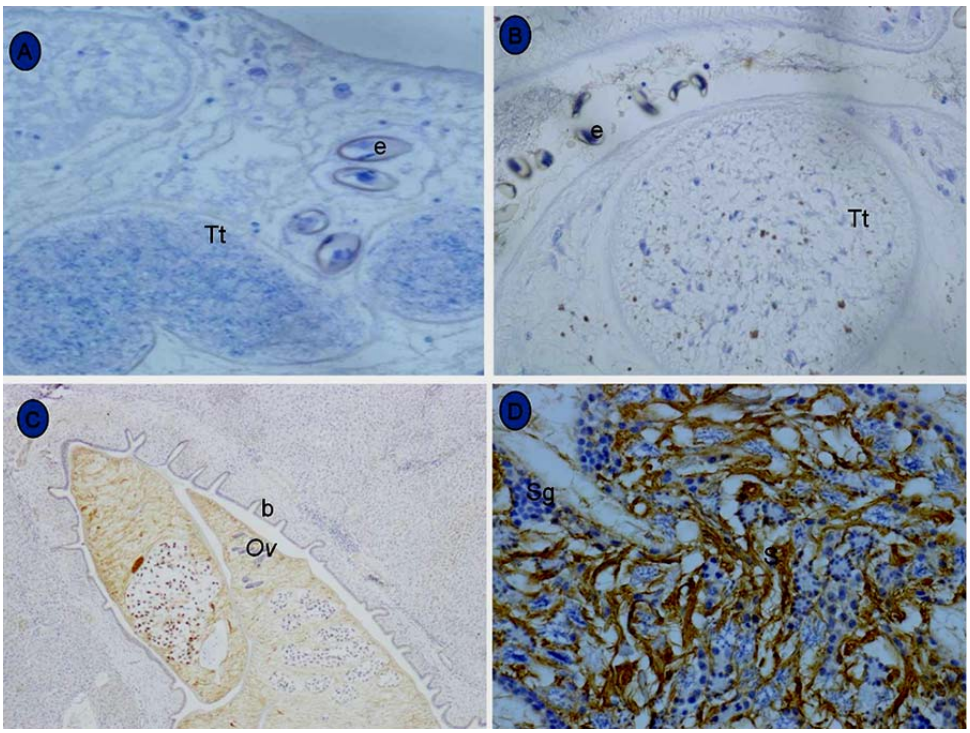

Fig. 1 Photomicrograph of immunohistochemistry staining and the expression sites of tubulin in the adult worm of *O. viverrini*. Negative control shows no staining in the fluke and bile duct epithelium (A, B). MAαT expresses in sub-tegumental tissue and stromal parechyma, muscle, and sperms (s) in both testis (Tt) (C, D). (A, B, D: Magnification 400X), (C: Magnification 100X).

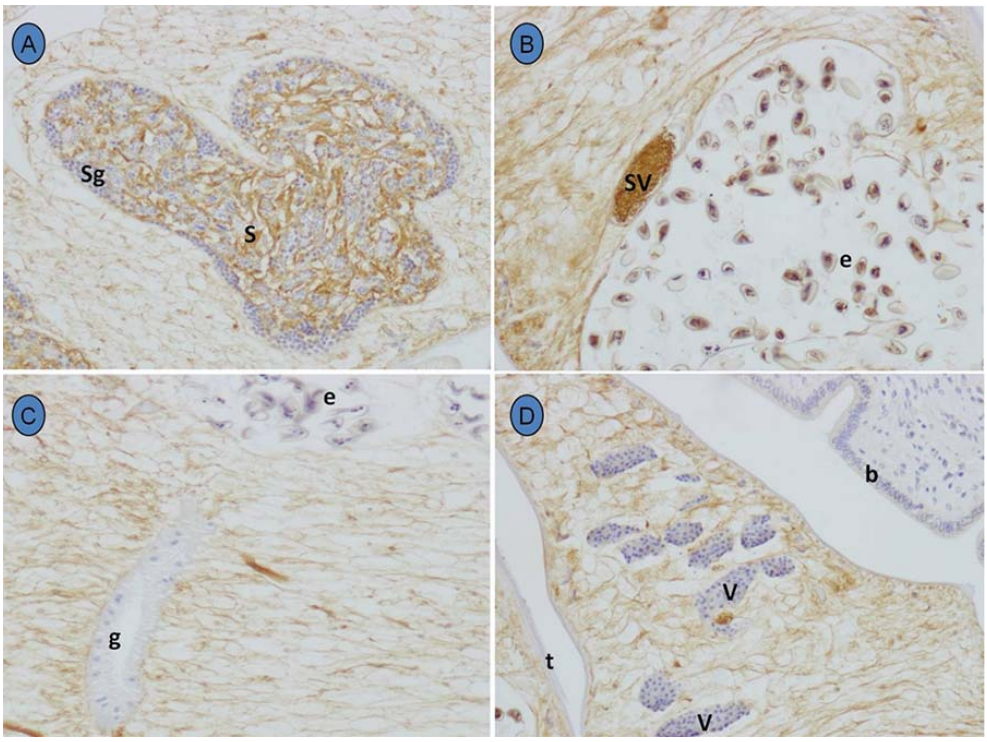

Fig. 2 Photomicrograph of immunohistochemistry staining and the expression sites of tubulin in the adult worm of *O. viverrini* as detected by immunoperoxidase staining. MAαT expresses in sub-tegumental tissue, stromal parenchyma, muscle fiber, and sperms in both testis (Tt) (A) and seminal vesicle (SV) (B), and miracidium in mature egg (e) (B). No staining in spermatogonia (Sg) (A), gut epithelium (g) (C), immature egg (e) (C), tegument (t) and vitelline glands (v) (D), and hamster bile duct epithelium (b) (D). (A-D: Magnification 400X).

decreasing inflammation. Another is the anti-fungal drug Griseofulvin which targets microtubule formation and also has applications in cancer treatment[14-16]. Tubulin is one of several members of a small family of globular protein. The most common members of the tubulin family are α-tubulin and β-tubulin, the proteins that make up microtubules. Microtubules are assembled from dimers of α- and β-tubulin. Tubulin was long thought to be specific to eukaryotes. To form microtubules, the dimers of α- and β-tubulin bind to GTP and assemble onto the (+) ends of microtubules while in the GTP-bound state. After the dimer is incorporated into the microtubule, the molecule of GTP bound to the β-tubulin subunit eventually hydrolyzes into GDP through inter-dimer contacts along the microtubule protofilament[13].

Many parasitic organisms contain tubulins as components of structures such as muscle fibers, tegument, tegumental syncytium, tegumental cell bodies, seminal receptacles, seminal vesicles, and testes[17-20,23]. The present study shows that tubulin strongly stains in numerous mature sperm within testes of the *O. viverrini* worm in the biliary system of hamsters, and the adult worm. These results were similar to where tubulin was localized in *Echinostoma caproni* and *Pseudodactylogyrus* sperm where it is used for reproductive processes[23,24]. In eukaryotic cells, there is plenty of evidence for the presence of tubulins in spermatocytes and spermatids during mammalian spermatogenesis[25-27] and also in mature spermatozoa[28-30]. However, because of the large diversity in the tubulin superfamily there is a gap in the field covering spermatogenesis and mature sperm undergoing changes prior to fertilization. Most eukaryotic cells can express multiple isotypes of α- and β-tubulin and this diversity is further expanded by a number of post-translational modifications[31]. Although the functional significance of tubulin diversity is still difficult to associate with consistent biological functions, there is increasing evidence that different α- and β-tubulin isotypes and post-translational modifications can impact microtubule

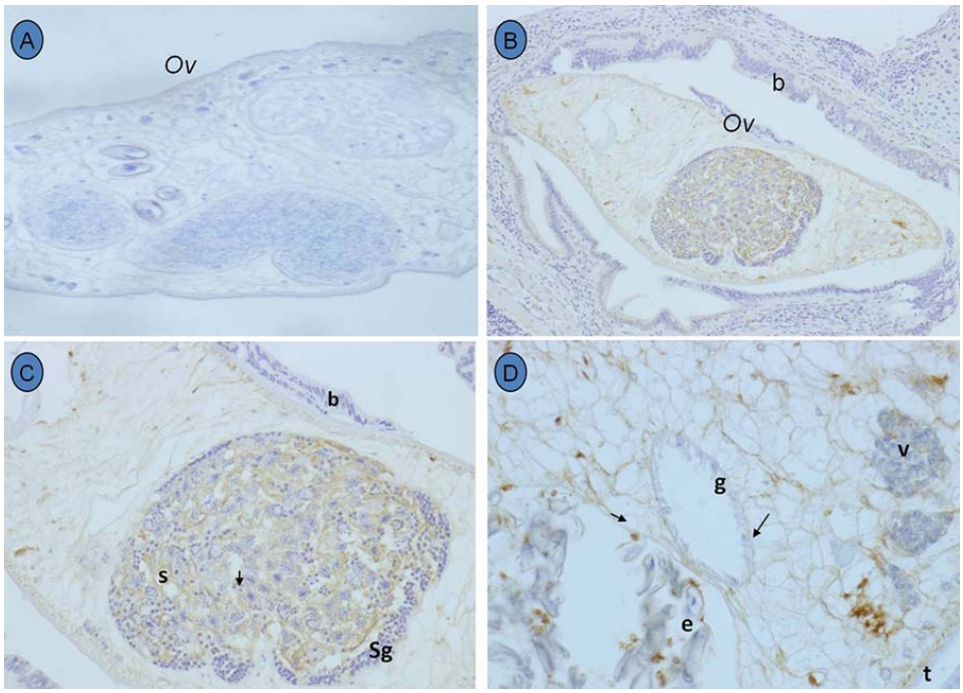

Fig. 3 Photomicrograph of immunohistochemistry staining and the expression sites of tubulin in the adult worm of *O. viverrini* as detected by immunoperoxidase staining. Negative control shows no staining in the fluke and bile duct epithelium (A). MAβT expresses in sub-tegumental tissue, stromal parechyma, muscle fibers, and sperms (s) in testis (B, C). No staining in spermatogonia (Sg) (C), gut epithelium (g) (D), immature egg (e) (D), tegument (t) and vitelline glands (v) (D), and hamster bile duct epithelium (b) (C). (A, B: Magnification 40X, C: Magnification 100X, D: Magnification 400X).

structure and function[26].

The present results also show that MAαT and MAβT are expressed in the sub-tegumental tissue and the stromal parenchyma of *O. viverrini*, which is also similar to the previously study of *Fasciola* spp. In *Fasciola* spp., the tegument plays crucial roles in maintaining homeostasis, including absorption of nutrients, exchange of waste molecules, regulation of ionic equilibrium, and protection from host immune responses[32]. In addition, tegument is the major site from which antigens are released to stimulate host immune responses[33,34]. *F. hepatica* tubulins have been reported and demonstrated in the tegumental syncytium and in the tegumental cell bodies and their cytoplasmic connections with the surface syncytium[17]. In *F. gigantica*, the presence of microtubules and actin filaments in the tegumental cells and their processes as well as in the syncytium could mediate the movement of secretory granules from the cell bodies toward the basal as well as the apical layer of the tegument[18]. In *O. viverrini* tubulins are now also shown to be localized in these vital organs similar to other important human trematodes.

Conclusion

The present study was the first reports that *O. viverrini* tubulin is expressed in vital organs, therefore, we intend to pursue further study to systematically detail the characterization and production of recombinant proteases as well as their applications in immunodiagnosis.

What is already known on this topic?

Many parasitic organisms contain tubulins as components of structures such as muscle fibers, tegument, tegumental syncytium, tegumental cell bodies, seminal receptacles, seminal vesicles, and testes.

What this study adds?

O. viverrini tubulin is expressed in vital organs, both MAaT and MAbT expressed slightly in sub-tegumental tissue, stromal parenchyma, muscle fibers, and miracidium in the mature egg of the worm. The

present study indicates that *O. viverrini* tubulin is present in the reproductive organs and other important organs of the worm.

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Potential conflicts of interest

None.

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การศึกษาตำแหน่งทูบุลินของพยาธิใบไม้ตับที่ก่อมะเร็งในมนุษย์ชนิดออร์พิสทอร์คิส วิเวอร์รินิ

ณัฏฐวุฒิ แก้วพิทูลย์, สรญา แก้วพิทูลย์

ภูมิหลัง: พยาธิใบไม้ตับออร์พิสทอร์คิส วิเวอร์รินิ เป็นปัญหาทางสาธารณสุขที่สำคัญในเขตทวีปเอเชียตะวันออกเฉียงใต้ โดยเฉพาะในประเทศสาธารณประชาธิปไตยประชาชนลาวและประเทศไทย การติดเชื้อพยาธิใบไม้ตับมีความสัมพันธ์กับการเกิดโรคเกี่ยวกับตับและทางเดินน้ำดี อาทิ ท่อน้ำดีอักเสบ ดีซ่าน ตับโต ถุงน้ำดีอักเสบ นิ่วในถุงน้ำดี และมะเร็งท่อน้ำดี

วัตถุประสงค์: เพื่อศึกษาตำแหน่งการแสดงออกทูบุลินของพยาธิใบไม้ตับออร์พิสทอร์คิส วิเวอร์รินิ ด้วยวิธีทางอิมมิวโนฮีสโตเคมโดยใช้โมโนโคลนอลแอนติบอดีต่ออัลฟาทูบุลินและเบต้าทูบุลิน

วัสดุและวิธีการ: พยาธิใบไม้ตับระยะตัวเต็มวัยและพยาธิใบไม้ตับระยะตัวเต็มวัยในระบบท่อทางเดินน้ำดีภายในตับของหนูนำมาดอง ตัดชิ้นสไลด์แล้วย้อมด้วยวิธีทางอิมมิวโนฮีสโตเคม โดยใช้โมโนโคลนอลแอนติบอดีต่ออัลฟาทูบุลินและเบต้าทูบุลิน อวัยวะของพยาธิส่วนที่ติดสีน้ำตาล คือการแสดงตำแหน่งของอัลฟาทูบุลินและเบต้าทูบุลิน

ผลการศึกษา: ผลการย้อมทางอิมมิวโนฮีสโตเคม พบตำแหน่งการแสดงออกของอัลฟาทูบุลินและเบต้าทูบุลินในพยาธิใบไม้ตับระยะตัวเต็มวัย และพยาธิใบไม้ตับระยะตัวเต็มวัยในระบบท่อทางเดินน้ำดีภายในตับของหนูสีน้ำตาลเข้มในอสุจิ ถุงน้ำเชื้อ พบการติดสีจางบริเวณเนื้อเยื่อหุ้มส่วนย่อยโครงร่างพยุงเนื้อต่อม เส้นใยกล้ามเนื้อ และไมราซีเดียมในไข่พยาธิ ไม่พบการติดสีที่เซลล์ต้นกำเนิดอสุจิ เนื้อเยื่อบุผิวท่อทางเดินอาหาร ไข่ที่ยังไม่สมบูรณ์เยื่อหุ้มหรือต่อมสร้างเปลือกไข่ของพยาธิ

สรุป: การศึกษานี้ชี้ให้เห็นว่าทูบุลินของพยาธิใบไม้ตับพบตามอวัยสืบพันธุ์และอวัยวะที่สำคัญของพยาธิ บริเวณส่วนที่สำคัญสำหรับการเคลื่อนไหว เคลื่อนที่ จึงควรศึกษาเพิ่มเติมเกี่ยวกับคุณลักษณะของจีน การสร้างโปรตีนลูกผสม และการประยุกต์ใช้ทางด้านการตรวจวินิจฉัยทางภูมิคุ้มกัน