THE MITOGENIC EFFECT OF *OPISTHORCHIS VIVERRINI* EXCRETORY/ SECRETORY PRODUCT AND ITS ACTIVATED SIGNAL TRANSDUCTION PATHWAYS

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Abstract. Our group has studied the responses of fibroblast cell line, NIH-3T3, to *O. viverrini* ES product using a non-contact co-culture. The results indicated a marked increase in cell proliferation with either absence or presence of serum in the media compared with the co-culture without parasites. The ES product increased cell proliferation by stimulating the expression of phosphorylated retinoblastoma (pRB) and cyclin D1, the key proteins in driving cells through the G1/S transition point of the cell cycle. In addition, the whole gene expression data from cDNA array indicated 239 genes with 2-fold and more up-regulated by *O. viverrini* ES product compared to those in cells without exposure to the parasitic product. This finding may clarify, in general, how this parasitic product affects human epithelium during cholangiocarcinogenesis. The understanding of the exact signal transduction pathways activated by *O. viverrini* ES product particularly in hyperproliferative fibroblasts will provide a novel target for chemoprevention and treatment of fibrosis in this cancer.

INTRODUCTION

Opisthorchis viverrini infection or opisthorchiasis remains a major public health problem in Thailand, especially in Southeast Asia, including northeastern Thailand, Lao PDR, Vietnam, and southern China (Sithithaworn and Haswelll-Elkins, 2003). The highest incidence is in the northeastern part of Thailand, with the rate of 188 per 100,000 (Sriamporn *et al*, 2004). The morbidity of *O. viverrini* infection does not directly relate to the parasite itself but relates to the carcinogenic capability of the parasites. The International Agency for Research on Cancer (IARC) has accepted that *O. viverrini* is a risk factor for bile duct cancer or cholangiocarcinoma (IARC, 1994). The mechanism by which this parasite causes the normal bile duct epithelium to transform into cancer cells involves several mechanisms (Watanapa and Watanapa, 2002). Three main irritations, including mechanical, immunological and biochemical, caused by the parasites have been proposed and demonstrated by many investigations. The mechanical process activated by *O. viverrini* sucker causes the epithelial cells to be expelled and then induces hyperproliferative cells. This effect has been demonstrated both in animal and human models (Sripa, 2003). For the immunological process, a significant degree of humoral and cell mediated immune responses to the parasite can be detected both in patients and animal models (Wongratanacheewin *et al*, 2003). Although the pathology of parasites by their direct contact and indirect suppressed immune response have been clearly shown as described, the effect of the biochemical substance released from the parasites has not been demonstrated. This may be the limitation of the studies in animal models or even in *O. viverrini*-infected people; the problem of eliminating direct contact and isolating the immune responses. We have designed an *in vitro* model to demonstrate the effect of *O. viverrini* excretory/secretory (ES) product on cells. The mechanisms by which the parasites initiate activation of cell proliferation and the activated intracellular signal transduction pathways have been proposed in this review.

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MITOGENIC EFFECT OF *O. VIVERRINI* ES PRODUCT

The non-contact co-culture technique using double-chamber culture plates was used to culture

cells and adult parasites together under the same conditions but with no direct contact. The double-chamber culture plate is composed of lower and upper chambers in the same cavity. The cells that we used were the mouse fibroblast cell line, NIH-3T3. We used these plates in the lower chamber, as a model, because of their sensitivity to the external stimuli. The 1.5-2 month-old parasites were then added in the upper chamber of the culture plate. The porous membrane of the upper chamber allows only the *O. viverrini* ES product to pass through the membrane and act on the cells in the lower chamber (Fig 1). After a two-day co-culture, an increased cell proliferation was observed in the co-culture treatment with parasites when compared to the control without parasites (Thuwajit *et al*, 2004) (Fig 2). This effect could be observed in media with either the presence or absence of a serum supplement.

The significant increase of viable NIH-3T3 cell numbers when co-cultured with viable parasites in serum-free medium indicated that ES product from *O. viverrini* might act as the growth factor, similar to supplementation with calf serum, which can induce the NIH-3T3 cell to proliferate. However, the efficacy of the ES product was not equal to serum (Fig 2). An explanation might be that, first, the concentration of growth stimulant in ES product is lower than that in serum. Secondly, the pathways that ES product used to control cell proliferation are different from those used by serum. The numbers of dead cells, including apoptotic cells, were measured, and the results showed no differences of cell death in co-culture

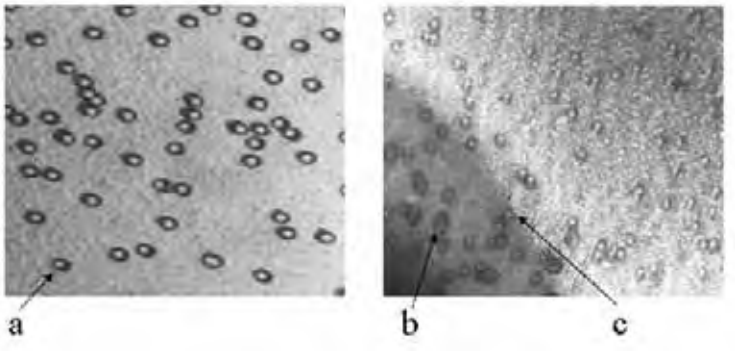

Fig 1- The porous membrane of the double-chamber culture plate comparing the pores sizes (a), *O. viverrini* eggs (b) and a part of an adult parasite (c).

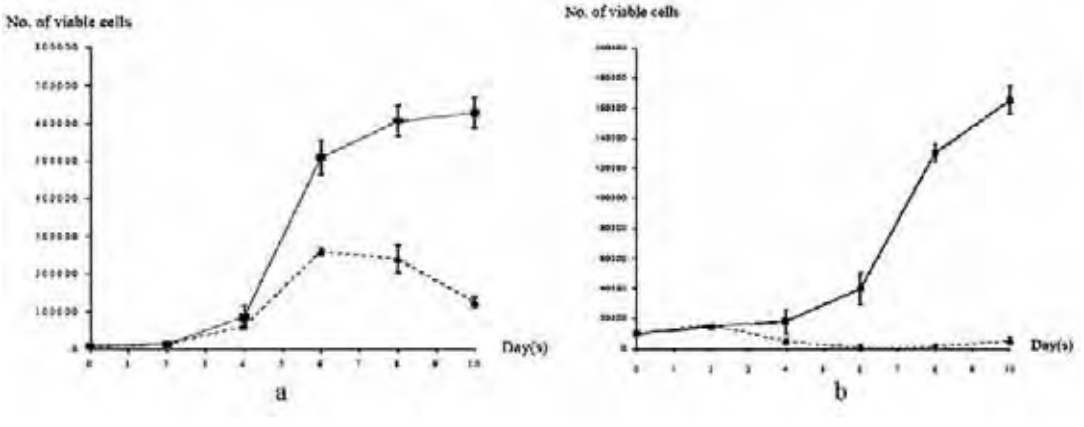

Fig 2- Growth curves of cells treated with and without adult *O. viverrini*, either cultured in media containing serum (a) or without serum (b).

compared to the non-co-culture ones (Thuwajit *et al*, unpublished data). An increased expression of cyclin D1 and phosphorylated Rb was observed in cells of both serum and ES product treatments. The similar expression profiles of pRB and cyclin D1 were previously shown in human fibroblasts treated with serum (Thuwajit *et al*, 2004). In conclusion, ES product from *O. viverrini* seems to act as growth stimulant(s) that can induce fibroblast cell proliferation. The increase in cell proliferation can be explained by the capability of ES product to stimulate the entrance of cells through G1/S transition point into cell cycle by over-expression of pRB and cyclin D1.

Many studies have demonstrated the effects of substances released from several parasites. For *Fasciola hepatica*, cathepsin L has been reported for its capacity to inhibit lymphocyte cell proliferation (Prowse *et al*, 2002). This facilitates the parasite to mask the immune response and help parasites to stay in the body. Moreover, mucin was also detected in the secretory product of *F. hepatica*. This can help the parasite to move thorough the tissues (Barnal *et al*, 2004). The secreted product of *Acanthocheilonema viteae*, a filarial nematode, has been demonstrated to have an anti-inflammatory effect via the reduction of cytokine production from macrophages (Goodridge *et al*, 2001). The larval stage of *Taenia taeniaeformis* could also induce gastric mucosa cell proliferation in SCID mice leading to gastric mucosal hyperplasia (Lagapa *et al,* 2002). For *O. viverrini*, there have been a few reports on the ES product and its effect (Sripa 2003; Thuwajit *et al*, 2004). The reason why parasites release such mitogens is still unclear. We believe that, first, the intended effect of the parasites may be to induce host cell proliferation as their source of energy, nutrients, and so forth. Second, the mitogens may be synthesized aimlessly but are a part of the molecules that act like mitogens.

MORPHOLOGICAL CHANGE OF *O. VIVERRINI*-TREATED FIBROBLAST

In addition to the cell proliferation induction of *O. viverrini* ES product, this product can also induce changes in NIH-3T3 cell shape (Fig 3). These results indicated that the loss of cell-to-cell contact in the refractive shape, after exposure to ES product, could allow cells to proliferate continuously; whereas, those with normal flat fibroblastic shape did not. The refractive shape NIH-3T3 can be observed in both types of media, either with or without serum, used in the non-contact co-culture systems. This might indicate that the shape change phenomenon was induced by the ES product, not by either the presence or absence of serum. The same shape change of NIH-3T3 cells was previously presented as the result of *h-ras* over-expression that could induce collapse of the actin filament that resisted the antiproliferation signals mediated by cell-cell contact and led to the increased cellular proliferation (Ritter *et al*, 1997). Moreover, *raf*-1 could induce NIH-3T3 cell proliferation (Kerkhoff and Rapp, 1997). We performed real time RT-PCR of *h-ras* and *raf*-1 genes, and the result demonstrated an increased *h-ras* expression level but not significantly in cells treated with *O. viverrini* ES product, while *raf*-1 expression was decreased (Fig 4). Since their actions involve the capability to induce other proteins to be phosphorylated (Woods *et al*,

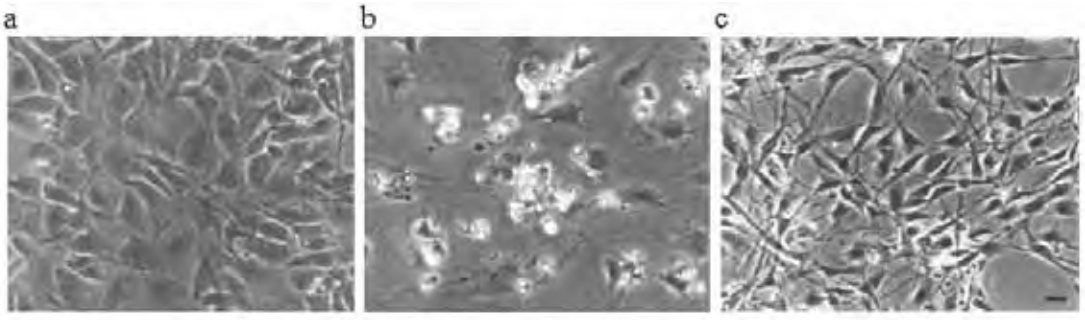

Fig 3- NIH-3T3 cells cultured in different conditions as detected by inverted microscopy. Cells in serum containing medium (a), serum-free medium (b) and co-cultured with parasites (c) (scale bar = 10 μm).

2001), these kinase enzymes may not be needed to increase the mRNA expression level. The kinase activity measurement will be of great interest to explore. The cascade of *h-ras* after growth factor stimulation is to send the signal to the downstream molecules, *raf*-1, and then activate either the MAPK-dependent pathway (Janulis *et al*, 2001) or MAPK-independent pathway (Laird *et al*, 1999). They may activate or increase expression levels in parasitic product induced fibroblasts.

EXPRESSION PROFILE IN *O. VIVERRINI* ES PRODUCT TREATED CELL

To understand the intracellular mechanism parasites used to activate cell proliferation, whole gene expression profiles were investigated using a mouse cDNA array (Thuwajit *et al*, 2006). A total of 15,000 genes and ESTs were tested. *O. viverrini* caused widespread alteration in gene expression. Most of the two-fold up-regulated expression genes have a cell proliferation-related function. They were categorized in groups as proteins that play roles in energy and metabolism, signal transduction, protein synthesis and translation, matrix and structural function, transcription control, cell cycle, and DNA replication. Only the signal transduction genes will be focused on in this review paper.

Growth factors or mitogens usually activate cells through signal transduction pathways. To know which intracellular signal transduction pathway *O. viverrini* utilized, the signal transduction genes were focused and categorized as corresponding to the stimulations. We selected the pathways stimulated by either PDGF or EGF, PDGF, EGF, and TGF-β. Regarding both the cDNA array and a validated real time RT-PCR, the most up-regulated expressions are *tgf*β 1*i*4 and *eps* 8, which are the representatives of TGF-β- and EGF-stimulated signal transduction pathways, respectively (Thuwajit *et al*, 2006). In addition, the receptors for PDGF and EGF are classified in different types as tyrosine kinase and serine/threonine kinase receptors. The cDNA array data represented the up-regulated expression level of these two types of receptors (increases around 1.59-4.42 fold). All the data support the possibility that TGF-β and/or EGF signal transduction pathways will be utilized by the parasites to activate cell proliferation.

ROLE OF THE TGF-β ASSOCIATED SIGNAL TRANSDUCTION PATHWAY IN *O. VIVERRINI* ES PRODUCT-INDUCED CELL PROLIFERATION

Though both TGF-β and EGF have been proposed as candidate signal transduction pathways activated by *O. viverrini* ES product, the highest up-regulation level was observed

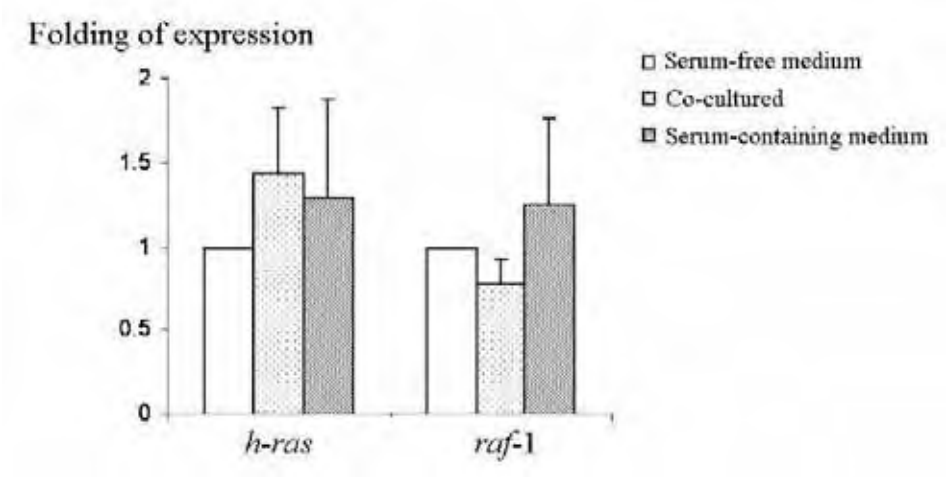

Fig 4- Expressions of *h-ras* and *raf*-1 genes in cell treated with *O. viverrini* compared to those in media with or without serum (using the level of gene expression in serum-free medium = 1).

in the pathway of TGF-β. Moreover, our study demonstrated a statistically significant increased Smad 4 mRNA in the same fashion as *tgf*β 1*i*4 (Fig 5). Because Smad-4 is a molecule in the TGF-β signal transduction pathway (Lutz and Knaus, 2002), it is possible that *O. viverrini* ES product-induced cell proliferation is through this pathway. It has been shown that TGF-β can induce fibroblast proliferation and is the major cause of hyperproliferative and hyperactive fibroblasts causing the fibrosis in many pathological conditions (Javelaud and Mauviel, 2004). TGF-β stimulates cell proliferation via the activation of either the Smad-dependent or the Smad-independent pathway according to cell types (Kaminska *et al*, 2005). For fibroblast cell proliferation, the Smad-dependent pathway has been proposed to play important role (Flanders, 2004). Given these above results and the published data, it may be of great interest to propose the importance of the TGF-β signal transduction pathway in *O. viverrini* induced cell proliferation. Through using Smad-dependent or -independent pathways of TGF-β, the most important upstream molecule in this signal transduction pathway is TGF-β receptor type II (TBRII) (Lutz and Knaus, 2002; Kaminska *et al*, 2005). In order to know whether the TGF-β signal transduction pathway plays a role in *O. viverrini* ES product induced cell proliferation, RNAi was used to knockdown the expression of TBRII on the cell membrane of fibroblasts before treatment with the parasitic product. The real time RT-PCR data demonstrated the successfulness of TBRII siRNA treatment with a statistically significantly decreased expression level of TBRII (Fig 6). Cell proliferation induced by *O. viverrini* ES product was decreased in TBRII siRNA treated cells compared to those with a normal expression level of TBRII (Fig 6). This result may confirm the possibility that fibroblasts are activated by *O. viverrini* ES product to increase cell proliferation using the TBRII-mediated signal pathway. Though the exact molecules existing in the parasitic product have yet to be identified, it has been proposed from this present finding that these pathway entities may be a component of the molecules acting like TGF-β. However, further experiments will be needed for a definitive conclusion.

ROLE OF ES PRODUCT ON HUMAN FIBROBLASTS

Using mouse fibroblasts as a model to study the effect of *O. viverrini* ES product has both advantages and disadvantages. The sensitive response of the NIH-3T3 mouse fibroblast cell line to external stimuli is the most important advantage. However, mouse fibroblast may respond to the same stimulus differently from human fibroblast cells. The human fibroblast cell line derived from normal colon was used to confirm the similarity of response between mouse and human cells. The human fibroblast cells were cultured with adult parasites in double-chamber culture plate. The

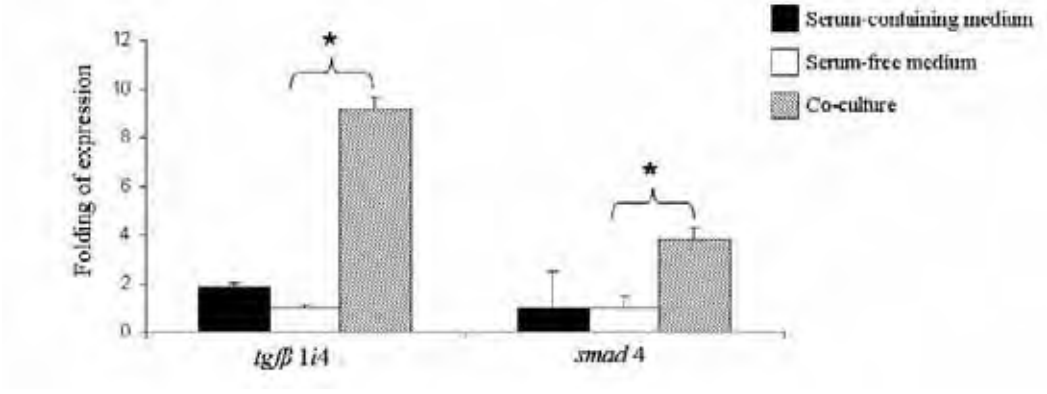

Fig 5- The expression levels of TGRb 1*i*4 and Smad 4 genes in cells exposed to *O. viverrini* ES product compared to those treated in serum-containing and serum-free media (* p-value <0.05) (using the level of gene expression in serum-free medium = 1).

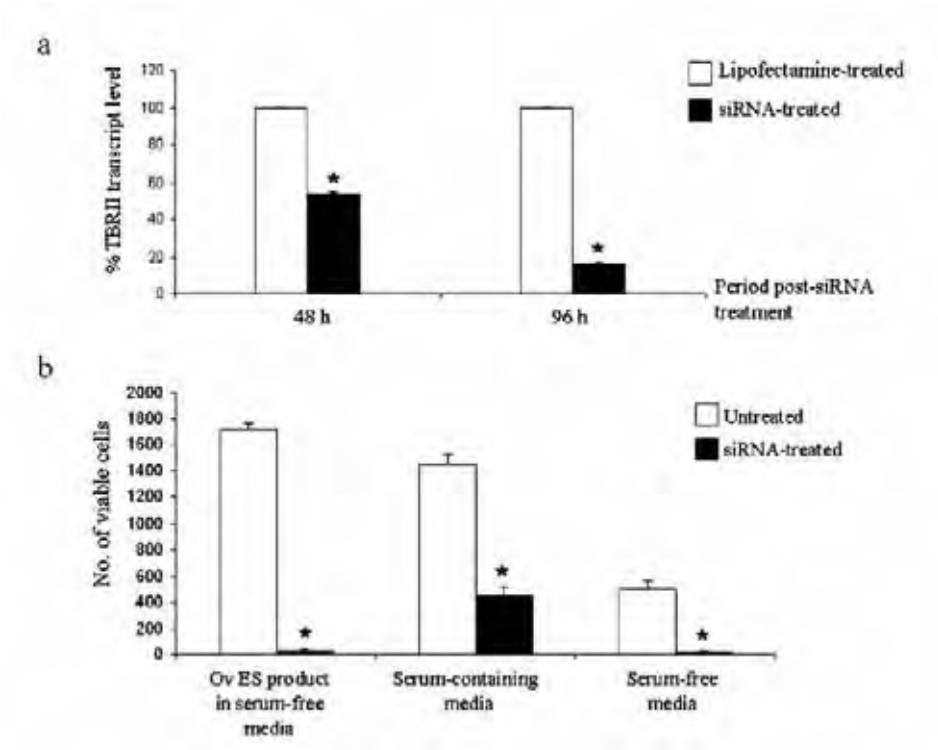

Fig 6- Real time RT-PCR of TBRII in fibroblast cells with and without treatment with TBRII siRNA (a) and the growth activated by *O. viverrini* ES product compared between cells with and without treatment with TBRII siRNA (b) (* p-value<0.05).

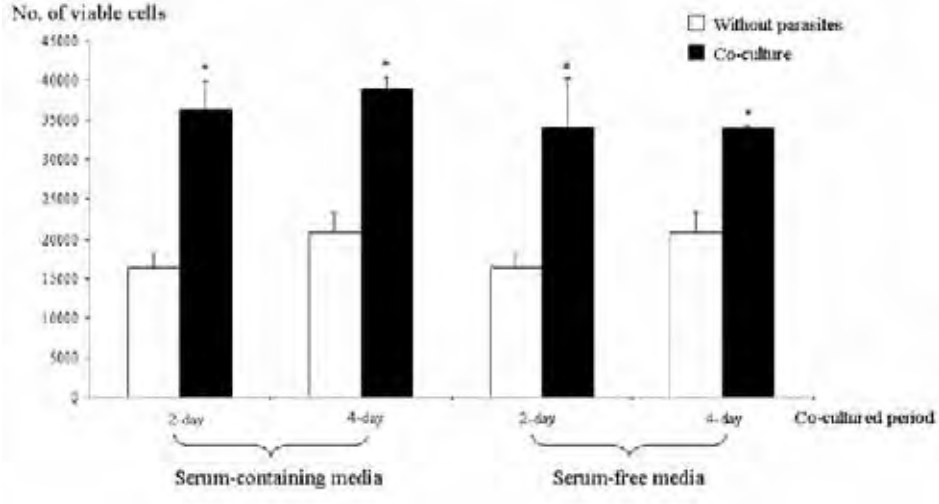

Fig 7- Cell proliferation effect of *O. viverrini* ES product on human fibroblast cell line (* p-value <0.05).

induction of human fibroblast cell proliferation was observed in both 2- and 4-day co-culture periods, with statistical significance (Fig 7). The cell proliferation induction was observed in conditions of both using media with and without serum. However, further experiments are needed to confirm these observations, it may imply that the *O. viverrini*-activated signal transduction

pathways detected in mouse fibroblasts can be similar or the same in human fibroblasts as well.

We have proposed the mechanism of *O. viverrini* ES product-induced fibroblast cell proliferation as an additive mechanism to a variety of demonstrated mechanisms, that is, mechanical and immunological processes induced by the parasites. *O. viverrini*-activated fibroblasts themselves are not enough to play only one important role in this process; however, the two-way interaction between the activated fibroblasts and the bile duct epithelium has been proposed and demonstrated in many kinds of carcinoma, including colon, prostrate, and breast (Olumi *et al*, 1999; Maffini *et al*, 2003; Nakagawa *et al*, 2004). The most abundant forms of fibroblasts in cancer tissues are called myofibroblasts (Kunz-Schughart and Knuechel, 2002). They are characterized by increased cell proliferation and collagen production. Our data indicated that *O. viverrini* ES product could induce cell proliferation and cDNA; microarray data also demonstrated incremental collagen production (Thuwajit *et al*, 2006).

CONCLUSION

We think that chronic infection by *O. viverrini* causes exposure of the bile duct epithelium and the surrounding fibroblasts to the parasitic product. For the epithelium, the increased cell proliferation causes vulnerable, incorrect DNA-containing cells to propagate and, if the damage occurs at oncogene sites or tumor suppressor genes, cancerous cells will result. For fibroblasts, *O. viverrini* ES product caused the hyperproliferative cells that may then secrete many substances, especially several growth factors. These mitogens activated fibroblasts themselves as the autocrine stimulus and acted on bile duct epithelium as the paracrine initiator. Taken all together, the proposed roles of activated-fibroblasts, that is, *O. viverrini* ES product-activated fibroblast during cholangiocarcinogenesis should be considered.

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