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BASIC RESEARCH

Gene expression profiling defined pathways correlated with fibroblast cell proliferation induced by *Opisthorchis viverrini* excretory/secretory product

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Abstract

AIM: To investigate the mechanism of fibroblast cell proliferation stimulated by the *Opisthorchis viverrini* excretory/secretory (ES) product.

METHODS: NIH-3T3, mouse fibroblast cells were treated with *O. viverrini* ES product by non-contact co-cultured with the adult parasites. Total RNA from NIH-3T3 treated and untreated with *O. viverrini* was extracted, reverse transcribed and hybridized with the mouse 15K complementary DNA (cDNA) array. The result was analyzed by ArrayVision version 5 and GeneSpring version 5 softwares. After normalization, the ratios of gene expression of parasite treated to untreated NIH-3T3 cells of 2-and more-fold upregulated was defined as the differentially expressed genes. The expression levels of the signal transduction genes were validated by semi-quantitative SYBR-based real-time RT-PCR.

RESULTS: Among a total of 15 000 genes/ESTs, 239 genes with established cell proliferation-related function were 2 fold-and more-up-regulated by *O. viverrini* ES product compared to those in cells without exposure to the parasitic product. These genes were classified into groups including energy and metabolism, signal transduction, protein synthesis and translation, matrix and structural protein, transcription control, cell cycle and DNA replication. Moreover, the expressions of serine-threonine kinase receptor, receptor tyrosine kinase and collagen production-related genes were up-regulated by *O. viverrini* ES product. The expression level of signal transduction genes; *pk*C, *pdgfrα*, *jak* 1, *eps* 8, *tgfβ* 1i4, *strap* and *h ras* measured by real-time RT-PCR confirmed their expression levels to those obtained from cDNA array. However, only the up-regulated expression of *pkC*, *eps* 8 *and tgfβ* 1i4 which are the downstream signaling molecules of either epidermal growth factor (EGF) or transforming growth factor-β (TGF-β) showed statistical significance ($P < 0.05$).

CONCLUSION: *O. viverrini* ES product stimulates the significant changes of gene expression in several functional categories and these mainly include transcripts related to cell proliferation. The TGF-β and EGF signal transduction pathways are indicated as the possible pathways of *O. viverrini*-driven cell proliferation.

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Key words: Gene expression profile; *Opisthorchis viverrini*; Excretory/secretory product; cDNA array; Fibroblast; Cell proliferation; Signal transduction; Cholangiocarcinogenesis

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INTRODUCTION

An *Opisthorchis viverrini* infection or opisthorchiasis is the most important health problem in Southern Asia, including Northeastern Thailand, Loas, Vietnam and southern China[1]. It is a definite cause of bile duct cancer or cholangiocarcinoma (CC) in humans and has been classified as a carcinogen. Parkin *et al*[2] performed

a case-control study of patients and estimated that two-thirds of CC cases in Thailand were caused by *O. viverrini* infestation. Nowadays, CC remains a major public health problem in many parts of Southeast Asia. The incidence rate of CC around the world is different with increasing tendency[3,4]. The highest incidence is in the Northeastern part of Thailand with the rate of 188 per 100 000[5]. The pathogenesis of *O. viverrini*-associated CC may be a complex process, involving several mechanisms. The effect of parasites on the bile duct epithelium is classified, as both mechanical and chemical irritations. The mechanical irritation is the direct contact of parasites to the bile duct epithelium; the chemical irritation is believed to be caused by the excretory/secretory (ES) product released from the flukes. These chronic irritations caused by the parasite later result in the proliferation of bile duct epithelium and leads to epithelial hyperplasia[6]. This is an important step in the genesis of cancer because hyperplastic cells are vulnerable to a carcinogen and can easily turn to adenomatous and finally cancerous cells[7]. Moreover, the alterations of immune response and the stromal cells which are mainly composed of fibroblasts were observed in opisthorchiasis and CC in both animal models and human. The fibrosis and mononuclear cell infiltration with lymphocyte aggregation and, additionally, ductal dilatation were observed in the gall bladders and extrahepatic bile ducts of the hamsters infected with *O. viverrini*[8]. In addition, the chronic inflammation and fibrosis of the bile ducts may contribute to the strikingly enhanced susceptibility to CC among people with heavy liver fluke infection[9]. The malignant transformation of bile duct epithelium *via* dysplasia was detected in the abnormal intrahepatic biliary tree of the patients with congenital hepatic fibrosis[10]. There is the important relationship between the gradual decreases of inflammation with a concomitant increase in fibrosis after *O. viverrini* re-infection in the experiment hamsters[11]. All of these data provide evidence of the importance of fibrosis in the formation of cholangiocarcinoma.

Fibrosis is the excessive accumulation of extracellular matrix proteins produced from the active and accumulated fibroblasts. It occurs in most types of chronic diseases including chronic liver disease. Liver fibrosis is considered a model of the wound-healing response of the liver to repeated injury[12]. Activated hepatic stellate cells, portal fibroblasts, and myofibroblasts have been identified as the major collagen-producing cells in the repeatedly injured liver[13]. In the fibrogenesis, the increased numbers and then the accumulation of fibrogenic cells including the fibroblasts and myofibroblasts are the main observations. As aforementioned, there is the correlation of fibrosis with the development of CC. The interaction of *O. viverrini* and the host immune cells has been proven to be the main causative issue for chronic inflammation in opisthorchiasis. The cytokines released from the immune cells will lead to the formation of fibrosis later. Moreover, direct contact of *O. viverrini* which have been strongly proven to induce bile duct epithelium erosion and cell proliferation replacement that occurs later on, may indirectly induce the surrounding fibroblasts around the affected area to proliferate[14]. The importance of hyperproliferative fibroblasts regarding the induction of epithelial cancer has been increasingly reported[15,16]. Though this phenomenon has not been investigated in CC, the direct effect of *O. viverrini* ES product to induce fibroblast cell proliferation has recently been reported *in vitro*[14,17]. So far, there are no data for the explanation of the fibroblast cell proliferation induced by the *O. viverrini* ES product.

From all the evidence described above, it would appear to be of great interest to study the mechanism of how *O. viverrini* ES product induces fibroblast cell proliferation. In order to know the response of cell to *O. viverrini* ES product, the gene expression analysis of fibroblast cell non-contact co-cultured with adult *O. viverrini* was performed. The expression level of genes was compared to those without *O. viverrini* ES product treatment. The cell proliferation-associated gene expression was discussed in relation to their roles in *O. viverrini* ES product-induced fibroblast cell proliferation. The expression levels of some signal transduction genes were also analyzed and validated by real-time RT-PCR to indicate the possible signal transduction pathway(s) utilized by *O. viverrini* in the induction of fibroblast cell proliferation.

MATERIALS AND METHODS

Parasite preparation

Opisthorchis metacercariae were obtained from naturally infected cyprinoid fish captured from fresh-water reservoirs in the endemic area of Khon Kaen province, Thailand. Pepsin-HCl was used to digest the flesh to obtain metacercariae, which were then introduced to the 6-8-wk-old hamsters *ad libitum*. After 1 mo, the adult *O. viverrini* were collected from the bile ducts of the hamsters and were washed several times with PBS containing 100 mg/L penicillin and 100 kU/L streptomycin. To prevent microbial contamination, the adult *O. viverrini* were then incubated for 30 min in PBS containing antibiotics before being used in the non-contact co-culture technique. The animal holding protocol was approved by the Animal Ethics Committee of Khon Kaen University (AEKKU011/04), based on the Ethics of Animal Experimentation of National Research Council of Thailand.

Cell culture and non-contact co-culture

To prepare the fibroblasts stimulated with *O. viverrini* ES product, the non-contact co-culture between the fibroblasts and the adult parasites was performed. The mouse fibroblast cell line, NIH-3T3, was bought from the American Type Culture Collection (ATCC; Manassas, VA, USA). It was maintained in DMEM (Gibco, Gaithersburg, MD, USA) containing 100 mL/L calf serum (CS) (Gibco, Gaithersburg, MD, USA), designated as the complete medium, followed by incubation in a 50 mL/L CO_2 incubator at 37℃ and passaging twice a week. Trypan blue staining was used to measure the viability of cells. The cells with more than 95% viability were collected for performing non-contact co-culture.

The NIH-3T3 cells and the intact viable *O. viverrini* were non-contact co-cultured in a 24-well transwell (Costar, Corning Incorporated, Cambridge, NY, USA). The

parasites were incubated in the upper chamber containing the 8-μm porous plate, which allowed the ES product to diffuse to the lower chamber where the NIH-3T3 cells adhered. The cell proliferation induction of ES product to the NIH-3T3 cells was confirmed by the viable cell count using trypan blue staining. Briefly, 10 000 NIH-3T3 cells were plated onto the lower chamber of a 24-well double-chamber culture plate for 2 d with complete medium to let most of cells adhere to the well. Then the media were changed to DMEM without CS, serum-free medium. Five viable adult *O. viverrini* were added to the upper chamber of each well. The NIH-3T3 cells cultured in complete medium and serum-free medium were used as positive and negative controls, respectively. The cell numbers obtained from the *O. viverrini* treatment cultured for 2 d was compared to those of the negative and positive controls with the same culturing time.

Hybridization and array data analysis

The in-house prepared mouse cDNA array was prepared by Dr. Kazuhiko Uchida, Department of Biochemistry and Molecular Oncology, Comprehensive Human Science, Graduate School, University of Tsukuba, Japan. It consists of 15 K of either mouse or human genes/ESTs. Firstly, the total RNA extraction from NIH-3T3 cells treated and untreated with adult *O. viverrini* ES product was performed. Briefly, total of 2-5 × 10^6 cells were collected in RNAlater solution (Ambion®, Hilden, Germany) and processed for total RNA with a commercial kit (ISOGEN; Nippon Gene, Tokyo, Japan) according to the manufacturer's protocol. The RNA quality was confirmed by subjecting the samples to electrophoresis on formaldehyde-agarose gel and staining the 18S and 28S RNA bands for visualization. Then, a 2-μg portion of the intact total RNA was dissolved in 8 μL of RNase-free water, and 2 μL of oligo (dT) primer (1 g/L; Invitrogen, Carlsbad, CA, USA) was then added. The sample was heated for 10 min at 70℃ and immediately chilled on ice. The following reagents were then added to the sample: 5 μL of first-strand cDNA synthesis buffer, 1 μL of DTT (0.1 mol/L final concentration), 1.5 μL of deoxynucleotide triphosphates (20 mmol/L dATP, dGTP, and dTTP), 1.5 μL of SuperScript (200 MU/L; Invitrogen), and [α-^{33}P]dCTP (111 PBq/mol; Amersham). The reaction was incubated at 37℃ for 90 min. Labeled cDNA was then denatured for 3 min at 95℃. The array was prehybridized at 42℃ for 2 h in MicroHyb solution (Invitrogen, Carlsbad, CA, USA) with 0.5 mg/L poly(dA) and 1 mg/L *COT1* DNA (Invitrogen, Carlsbad, CA, USA). The probes were hybridized at 42℃ overnight in the same solution. The membrane array was washed twice at 50℃ with 2 × SSC-10 g/L SDS for 20 min and at room temperature with 0.5 × SSC-10 g/L SDS for 15 min. The membrane array was exposed to an imaging plate which was scanned with a BAS 5000 Imaging Analyzer (Fuji Film, Tokyo, Japan). Spot intensity was quantified with ArrayVision version 5.0 software (Imaging Research, Ontario, Canada). GeneSpring version 5.0 software (Silicon Genetics, Redwood, CA) was used to normalize values for each gene for data analysis.

Real-time RT-PCR

The mRNA was isolated using a Total RNA Extraction Miniprep System (Viogene, Fegersheim, France) according to the manufacturer's instructions. The DNA contaminated in the extracted RNA was destroyed by DNaseI (Promega Corporation, Madison, WI, USA) at 37℃ for 30 min. The DNaseI inactivation reaction was performed at 75℃ for 10 min. The first-strand cDNA was synthesized from 1 μg of mRNA using the Reverse Transcription System (Promega Corporation, Madison, WI, USA). Semi-quantitative PCR was performed with the SYBR-based method in 12.5 μL reaction in Rotor Gene RG-3000 (Corbett Research). After the denaturing of cDNA at 95℃ for 10 min, the reaction profile was subjected to 50 amplification cycles, each cycle consisted of denaturation at 95℃ for 30 s, annealing at different temperatures for each gene (52, 55, 58, 58, 54, 52, 52, 52, 65, and 60℃ for *abl* 1, *pk*C, *pdgfr*α, *jak* 1, *eps* 8, *tgf*β 1*i*4, *strap*, *csnk* 1α1, *h ras, and* $\beta_2 m$, respectively) for 30 s, and extension at 72℃ for 45 s. After the PCR, a melting curve was constructed by increasing the temperature from 50 to 99℃. β_2-microglobulin (β_2m), whose expression was found to be directly proportional to the amount of mRNA that presented in the sample stimulated by any growth factors[18], was used as the internal control. The standard curve between the CT of each gene expression and the amount of the starting total RNA was performed. The CT of the samples was then compared to the corresponding standard curve and quantitated the amount of the starting mRNA in that sample. The RT-PCR was performed three times for each gene.

Table 1 shows the PCR primers designed by the Gene Fisher Program according to the mRNA sequences of each gene in the GenBank database. The primer sequences can then be categorized into groups of growth factors they belong to, in this case, the signal transduction molecules. The result was analyzed for the statistical significance by STATA version 8 (StataCorp LP, Texas, USA).

RESULTS

Gene responding to O. viverrini ES product

Using a mouse cDNA array, we examined the gene expression profile of cell proliferation stimulated by *O. viverrini* ES product. A total of 15 000 genes and ESTs were tested. *O. viverrini* caused widespread alteration in gene expression. The ratios of gene expression between treated and untreated with the parasite ES product equal and greater than 2 were focused on because of their striking changes. Among all genes/ESTs in 15K cDNA array, 885 genes fitted to this criterion. Among these genes, only 536 genes had a variety of molecular functions and only 239 genes within these 536 genes had cell proliferation-related functions. These 239 genes were categorized in groups as proteins playing roles in energy and metabolism, signal transduction, protein synthesis and translation, matrix and structural protein, transcription control, cell cycle and DNA replication. The percentage distribution of genes in each group is 25.2, 21, 18.9, 17,

Table 1 List of signal transduction genes[1] validated the expression by real-time RT-PCR, their corresponding PCR primers[2], annealing temperature and product sizes

Group of stimulating growth factor	Gene	Primer sequence	Annealing temperature (℃)/ product size (bp)
PDGF /EGF	*abl* 1	Forward: 5′-TGAAGTTGGTGGGCTGCA-3′ Reverse: 5′-TTTTCACTGGGCCCGCA-3′	52/148
	pkC	Forward: 5′-GGCATGCCTTGTCCTGGAGA-3′ Reverse: 5′-TGTAGCCCTGCCTCGAGAGA-3′	55/149
	jak 1	Forward: 5′-CAGCCTCGCTCGTCCTT-3′ Reverse: 5′-CCAAGCCCTTCAGAGCT-3′	58/147
	raf 1	Forward: 5′-TTTCCCGGATGCCTGCTA-3′ Reverse: 5′-TCCTGCTGTCCCATGCA-3′	62/147
	h ras	Forward: 5′-TGGTGGGCAACAAGTGTGA-3′ Reverse: 5′-CCGCAATTTATGCTGCCGAA	65/150
PDGF	*pdgfrα*	Forward: 5′-TGCGGGGAAGGACTGGA-3′ Reverse: 5′-GTGAGGAGACAGCTGAGGA-3′	58/122
EGF	*eps* 8	Forward: 5′-CCGCTCCGTGGGTATGGA-3′ Reverse: 5′-ACGTCCGACACACTGCTGA-3′	54/146
TGF-β	*tgfβ* 1*i*4	Forward: 5′-GCAAGTGTGGTAGCTATCGA-3′ Reverse: 5′-CAGATTGTTCTCCTGCTCC-3′	52/136
	strap	Forward: 5′-GCCGGGAGATACAGGAGA-3′ Reverse: 5′-TGCTTATGAGCCGGGTCA-3′	52/153

[1]They are classified based on their functions as the downstream signaling molecules of the specific growth factor. [2]Primers for the internal control (β_2m) are: 5′-CATGGCTCGCTCGGTGA-3′ (forward) and 5′-AATGTGAGGCGGGTGGAA-3′ (reverse). The annealing temperature and the product size of RT-PCR for β_2m are 60℃ and 148 bp, respectively. PDGF: platelet-derived growth factor; EGF: epidermal growth factor; TGF-β: transforming growth factor-β.

12.6, 3.3, and 2, respectively. The examples of genes in each group are summarized in Table 2.

Response of signal transduction-related genes to O. viverrini ES product

A total of 59 genes/ESTs categorized by their molecular function as the signal transduction genes showed 2-fold and more up-regulation after *O. viverrini* ES product stimulation. They are the downstream signal transduction molecules for a variety of growth factors. The signal transduction genes that corresponded to the stimulation of either PDGF or EGF, PDGF, EGF and TGF-β were categorized and summarized in Table 3. The most up-regulated expressions were *tgfβ* 1*i*4 and *abl* 1 which are the representatives of TGF-β- and either PDGF- or EGF-stimulated signal transduction pathways, respectively.

In addition to the different signal transduction molecules responded to different growth factors, the receptors for PDGF/EGF and TFF-β are classified in different types as tyrosine kinase and serine-threonine kinase receptors, respectively. The cDNA array data represented the different up-regulated expression levels between these 2 types of receptors (Table 4).

Nine signal transduction genes, which responded to the specific growth factor as mentioned in Table 3, were selected and tested for their expression levels by semi-quantitative RT-PCR. The single peak of the melting curve for each gene confirmed the appropriate PCR condition (data not shown). The result indicated similar responses in both cDNA array and RT-PCR analyses of *pkC*, *jak* 1, *h ras*, *pdgfrα*, *eps* 8, *tgfβ* 1*i*4, and *strap*. However, the significant (P < 0.05) up-regulation was only found for those of *pkC*, *eps* 8, and *tgfβ* 1*i*4. The opposite result of mRNA expression level was detected in *abl* 1 and *raf* 1 (Figure 1).

DISCUSSION

Cell proliferation is regulated by the intricacy of cell growth regulators, signaling cascades, and mediators of cell cycle progression including cyclins, cyclin-dependent kinases (CDKs). Anti-proliferative genes coding for the apoptotic protein, cyclin/CDK inhibitors are important in controlling cell proliferation. Addition of growth factors induces cell proliferation including the fibroblasts[19]. The changes in gene expression that accompany this proliferative response have been the subject of many studies, and the responses of dozens of genes have been characterized[19]. A recent study indicated the effect of *O. viverrini* ES product in induction of fibroblast cell proliferation *in vitro*[17]. We have examined the effect of this parasitic product in the gene expression profile using a cDNA array. The NIH-3T3 cells were used because it not only had the marked response to *O. viverrini* ES product[17], but also being classified as the mesenchymal cells as the real target of *O. viverrini* ES product; the stellate cells and myofibroblasts. Among all genes/ESTs in 15K cDNA array, 885 genes showed 2-fold and more up-regulation after stimulation by *O. viverrini* ES product. Among these genes, only 536 genes had a variety of established molecular functions. Moreover, 239 of these 536 genes had cell proliferation-related functions and were primarily focused into groups based on their specific functions because of their striking increased changes (⩾ 2-fold).

Most of the genes which increased their expression by *O. viverrini* ES product stimulation were the proteins controlling the enzymatic metabolism and biosynthesis. The expressions of glyceraldehyde-3-phosphate dehydrogenase, alpha-enolase and aldehyde dehydrogenase involve the glycolysis pathway. Cytochrome C oxidase and ATP synthase involve energy production from the electron transport chain. These phenomena could be easily understood because high levels of energy production and synthesis of biological macromolecules are required for the stimulation of quiescent fibroblasts by *O. viverrini* ES product into the cell cycle progression. These data suggest the possibility that ES product acts like a growth factor to stimulate cell proliferation basically by the stimulation of energy production-related gene expression[20].

In cell proliferation, the DNA replication is needed. Many proteins, including RPII215 polymerase and topoisomerase II, required for chromosome segregation at mitosis; and DNA primase, required for DNA replication, all increased their expressions in *O. viverrini* ES product-treated cells. These increased expressions will help cells undergo into cell cycle and consequently cell division. Ribonucleotide reductase is associated with the anabolic pathway of deoxynucleotide. The increased expression of this enzyme causes an increase in the deoxynucleotide triphosphate (dNTP) pool in the nucleus, which facilitates DNA synthesis and repair[21]. In addition, when cells are activated by the growth factors and then commit to enter

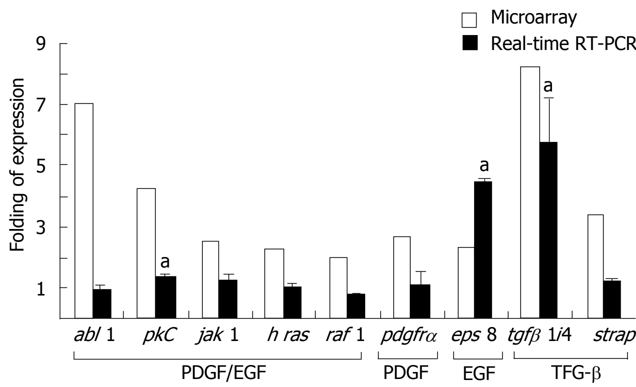

Figure 1 RT-PCR analysis of signal transduction genes up-regulation by *O. viverrini* ES product. The results of DNA array and RT-PCR are shown in comparison for each gene (mean ± SD, [a]$P < 0.05$).

Table 2 Proliferation-related genes with 2-fold and more[1] up-regulation in fibroblast cell proliferation activated by *O. viverrini* ES product compared to the negative control (not exhaustive)

A Energy and metabolism	Glyceraldehyde-3-phosphate dehydrogenase (*gapd*), cytochrome C oxidase, alpha-enolase, ATP synthase beta subunit[2], thioredoxin, aldehyde dehydrogenase
B Signal transduction	Transforming growth factor beta-1-induced transcript 4 (*tgfβ 1i4*), tyrosine kinase (*abl* 1), interleukin 1 receptor-associated kinase, MAP kinase phosphatase (*mkp* 6), colony stimulating factor 3 receptor, protein kinase C, Janus kinase 2 (*Jak* 2), casein kinase I alpha (*csnk 1α*1), *c-myc*
C Protein synthesis and translation	Polyubiquitin C (*Ubc*) gene, ribosomal protein S27a[2], ribosomal protein L27, elongation factor Tu, RNA polymerase 1-2, translation initiation factor 4 gamma[2]
D Matrix and structural protein	Lysophospholipase 1 (*lypla* 1)
E Transcription control	Transformation/transcription domain-associated protein (*trrap*)[2], telomeric repeat binding factor 1 (*terf* 1), transcription factor EB (tcfeb), DEAD-box RNA helicase (*ddx* 21)[2]
F Cell cycle	Kinesin family member 3a (*kif 3a*), mitotic kinesin-linked protein 1[2], cyclin-dependent kinase 4 (*cdk* 4), *cdk* 5, cyclin B1
G DNA replication	RPII215 polymerase II large subunit, topoisomerase II alpha, DNA primase p58 subunit (*prim* 2), ribonucleotide reductase

[1]Sequence in each group is ordered from high to low expression increases; [2]represents human gene.

Table 3 Up-regulation of signal transduction-related genes in NIH-3T3 treated with *O. viverrini*, categorized in groups according to the response to the specific growth factor

Related growth factor (Accession number)	Gene name/production	Folding
PDGF/EGF		
AW544655	Tyrosine kinase (*v abl*)	7.12
C80388	Protein kinase C (*pk*C)	4.23
AU042564	Casein kinase I-alpha isoform (*csnk* 1*α* 1)[1]	3.81
C87299	Casein kinase I-epsilon (*csnk* 1*ε*)	2.88
AU046274	Ras-GTPase activating protein SH3-domain binding protein (*c3bp*-pending)	2.73
C87788	*JAK*1 protein tyrosine kinase	2.56
AU017366	*H ras*	2.26
AW552623	*N ras*	2.01
PDGF		
AW537708	PDGF receptor, alpha-polypeptide (*pdgfr α*)	2.64
EGF		
C88280	Epidermal growth factor receptor pathway substrate (*eps* 8)	2.32
TGF-β		
AW546174	Transforming growth factor beta-1-induced transcript 4 (tgf*β* 1*i*4)	8.24
AU016757	*c myc*	3.70
C79202	Serine/threonine kinase receptor-associated protein (*strap*)	3.40

[1]Represents human gene.

Table 4 cDNA array analysis showing up-regulation of genes encoded for the kinase receptors

Receptor kinases	Gene names (accession number)	Folding
Serine-threonine kinase	Serine/threonine kinase 11 (C85710)	4.42
	Serine/threonine kinase 19 (AW557191)	3.28
	Serine/threonine kinase 4 (AU020804)	2.88
Tyrosine kinase	TYRO3 protein tyrosine kinase 3 (AW556118)	2.60
	JAK1 protein tyrosine kinase (C87788)	2.56
	Protein tyrosine kinase 9 (AW544421)	1.83
	Downstream of tyrosine kinase 1 (AW557123)	1.59

the cell cycle, the cascade of gene expression within the cells is needed. This means it is important that the initial gene expressed to be protein for the expression of the later ones. This is the action of protein classified as the transcription factor. In cells treated with *O. viverrini* ES products, a variety of transcription factors are activated.

Cyclins and CDKs are the important proteins in cell cycle progression[22]. The response of cell to *O. viverrini* ES product indicated the increased expression of many CDKs and cyclins. CDK4 and 5 are important at the G1 phase, whereas cyclin B1 is crucial in G2 phase. The increased expression of phosphorylated cyclin D1 was reported in fibroblast proliferation induced by *O. viverrini* ES product[17], thereby supporting the increased expression of

CDK4 in this study. This result supports the importance of cyclin D1-CDK4 expression in the re-entry of the serum-stimulated fibroblasts into the cell-division cycle[23]. Moreover, *O. viverrini* ES product induced the expression of kinesins which are a family of microtubles and are involved in many crucial cellular processes including cell division[24]. The kinesin family member 3a (*kif 3a*) and mitotic kinesin-like protein are crucial for spindle assembly and function, chromosome segregation, mitotic checkpoint control, and cytokinesis in higher eukaryotes[25]. Cells cannot divide without the appropriate functions of these genes.

Rapid and efficient production of macromolecule in protein synthesis and degradation of superfluous proteins play important role in cell cycle progression[26]. The increased RNA polymerase which determines the efficiency of mRNA production supports the capability of other genes to increase their expressions. The ribosomal proteins as the component of ribosomes, translation initiation factors and elongation factors increased their expressions which normally occur to promote proteins synthesized for cell division. In addition, the expression of polyubiquitin C (*ubc*) increased to get rid of the superfluous proteins already synthesized and that have reached their expired period. The increased expression of these genes is crucial for the turnover of the appropriate proteins for the right stimuli at the right time.

Matrix and structural proteins are important in supporting cell proliferation[27]. In this study, some matrix and structural proteins increased their expression level by *O. viverrini* ES product stimulation. Lysophospholipase D (lysoPLD) catalyses the production of lysophosphatidic acid (LPA) from the lysophosphatidylcholine (LPC). LPA has been demonstrated to be a potent inducer of cell proliferation of multiple cell lineages[28]. Taken together, all the data presented in this study confirm that *O. viverrini* ES product can activate cell proliferation resulting in the response of gene expression profile like a serum or growth factor[29].

Array analysis is not only crucial in understanding the effect of the ES product on cells but also to predict what should be the growth factor available in *O. viverrini* ES product. In this work, we took the advantage of this technology to decipher the possible genome expression circuit induced by *O. viverrini* ES product. The comparison of *O. viverrini* ES product-induced gene expression profile with that of established growth factor profiles may be useful to know the cell signaling pathway utilized by *O. viverrini* ES product in induction of cell proliferation. The *O. viverrini* ES product stimulated cell proliferation like the response of cells to the calf serum[19]. There are many types of growth factors contained in the calf serum including platelet-derived growth factor (PDGF), epidermal growth factor (EGF), transforming growth factor-β, and insulin-liked growth factor (IGF). Each of them stimulates a different signal transduction profile which is the most important feature used to distinguish the types of growth factor-stimulated cell responses[30,31]. In this study, the signal transduction genes selected from the array data can be categorized based on their functions as the downstream signal transduction molecules of both PDGF and EGF (PDGF/EGF), only PDGF, only EGF, and only TGF-β, according to the previous reports[30,31]. Though PDGF and EGF are different growth factors using different cell surface receptors, they used, in part, the same signal transduction pathway[32] including *abl* 1, *pk*C, *csnk* 1*a*1, *ras* and *jak* 1. The results of this study showed that *O. viverrini* ES product stimulated the expression of these genes. It may be possible that *O. viverrini* ES product activated cell proliferation *via* PDGF/EGF-driven signaling pathway. Moreover, *pdgfrα*, *eps* 8, and *tgfβ* 1*i*4, the specific genes for PDGF, EGF, and TGF-β, respectively, were found to be up-regulated as well. Based on the increased expression, *tgfβ* 1*i*4 was ranked in the uppermost expression level. It is supposed that *O. viverrini* ES product stimulated cell proliferation *via* TGF-β signaling pathway as well.

The difference between signal transduction pathways activated by TGF-β and PDGF/EGF is mainly focused on their different types of receptors. The receptors of TGF-β are membrane-bound receptors exhibiting intrinsic serine-threonine kinase activities, whereas the tyrosine kinase receptor is activated by EGF or PDGF[30,31]. From cDNA array data, the activation of serine-threonine kinase and tyrosine kinase receptors encoded gene expression showed the involvement of both types of receptors in the stimulation by *O. viverrini* ES product. This evidence still supports the possibility that TGF-β and EGF/PDGF can be the candidate signal transduction pathway induced by *O. viverrini* ES product.

Since the uncertain mRNA expression level was detected by cDNA array analysis, RT-PCR was used to measure the exact expression. From the list of up-regulated signal transduction-related genes, we selected 9 genes for additional analysis to validate the array expression data. The result showed marked increase of *tgfβ* 1*i*4 expression. It has been proven that *tgfβ* 1*i*4 is a direct target of TGF-β[33,34] and also is the transcriptional modulator stimulated by TGF-β. The *tgfβ* 1*i*4 gene expression was transcriptionally activated by TGF-β, phorbol 12-myristate 13-acetate, and serum but not appreciably by EGF[34]. The up-regulation of *tgfβ 1i4* expression induced by *O. viverrini* ES products may indicate the stimulation of the TGF-β-activated signal transduction pathway. The normal expression of *strap*, the inhibitor of the TGF-β signal transduction cascade[35], supports this conclusion.

For the EGF and PDGF-stimulated signal transduction pathways, the expressions of the common genes, including *abl* 1, *jak* 1, *h ras* and *raf* 1, were not significantly increased. Moreover, the specific gene related to PDGF-stimulated signal transduction, *pdgfrα*, was expressed at the same level as in cells without *O. viverrini* ES product treatment. This result excludes the potential of *O. viverrini* ES product in stimulating cell proliferation through the PDGF-mediated signal transduction pathway. For the EGF-stimulated signal transduction pathway, *eps* 8 is the transcription factor required in the EGF-stimulated cell proliferation and is confirmed to be the gene product that represents a novel substrate for tyrosine kinase receptors[36]. Adoptive expression of *eps* 8 cDNA in fibroblastic or hematopoietic target cells expressing the EGFR resulted in an increased mitogenic response to EGF, implicating the *eps* 8 product

in control of mitogenic signals activated by EGF[36]. That the *O. viverrini* ES product markedly stimulated the expression of this gene represents the possibility that this parasitic product stimulates cell proliferation *via* EGF-mediated signal transduction cascade. There are 4 main pathways induced by EGF, including the activations of Ras/Raf/ERK, phosphatidylinositol 3-kinase (PI3K), phospholipase C-γ (PLC-γ)/protein kinase C (PKC) and c *jun*-N-terminal kinase (JNK). These pathways lead to different cellular actions[37]. Only the Ras/Raf/ERK and JNK-transduced signal transduction pathways involve cell proliferation. In this study, the expression of *h ras* and *raf* 1 was slightly up-regulated by *O. viverrini* ES product, indicating *O. viverr*ini ES product stimulates cell proliferation *via* other pathways rather than the common pathway of Ras/Raf/ERK. The up-regulated expression of *pk*C represents the possibility of cell proliferation activated by *O. viverrini* ES product *via* the EGF-stimulated PKC signal transduction pathway. This is opposed to the data that *pk*C-mediated signal transduction pathway stimulated by EGF does not involve in control of cell proliferation[37]. Moreover, the array data showed the increased expression of JNK (2.78-fold, data not shown) represents the possibility that EGF-associated JNK pathway may be the pathway activated by *O. viverrini* ES product.

Taken all together, *O. viverrini* ES product activates cell proliferation *via* either TGF-β- or EGF-mediated signal transduction pathways. These two signal pathways have been proved to be strongly correlated with cancer development[37,38]. TGF-β also mediates tumor-promoting effects, through differential effects either on tumor or stromal cells[38]. Its signaling pathway is being evaluated as a prognostic or predictive marker for cancer patients. Over-expression of EGF has been found in many cancer types[37]. The roles of TGF-β and EGF in cholangiocarcinogenesis need further investigations. Regardless of the exact signal transduction pathway stimulated by *O. viverrini* ES product, the induction of fibroblast cell proliferation and the accumulation of extracellular matrix (i.e. collagen) have been proven to be associated with the fibrogenetic process[13]. The increased expression of collagen type I, III, and IV (between 2-3-fold increase) was observed in cDNA array analysis (data not shown). This supports the hypothesis that *O. viverrini* ES product can stimulate cell proliferation and collagen production leading to the fibrosis formation. Since the fibrosis has been detected in cholangiocarcinoma in both animal models and human[9-11], it is possible that *O. viverrini* ES product-induced fibrosis may be one component in cholangiocarcinogenesis. Moreover, it may be proposed that the ES product-induced fibrosis may act synergistically with the immunological response against the parasites to promote CC.

In summary, this study demonstrates the gene expression profile obtained from cDNA array analysis to determine the possibility of *O. viverrini* ES product as the growth factor in induction of fibroblast cell proliferation. Signal transduction-related genes may have a significant expression increment in cells exposed to this parasitic product. Among this set of genes, *tgfβ* 1*i*4 and *eps* 8 were found to have the strongest expression levels with corroboration data obtained by both cDNA array and RT-PCR. These two genes are the specific signal transduction molecules for TGF-β- and EGF-stimulated pathways. It may therefore be proposed that *O. viverrini* ES product activated cellular proliferation *via* the pathways of these growth factors. The issues of whether one type of growth factor is capable to cross-stimulate the two pathways or two types of growth factors are available in the *O. viverrini* ES product, needs future experiments to be clearly elucidated. This report is an important resource to indicate the mechanism of *O. viverrini* ES product-stimulated fibroblast cell proliferation *via* TGF-β- or EGF-stimulated signal transduction pathways. Since the activated fibroblast has been shown to initiate non-tumorigenic epithelium to carcinoma[16], *O. viverrini* ES product-stimulated fibroblast may play a part to change bile duct epithelium into CC. The ongoing research in understanding the signaling pathways stimulated by *O. viverrini* ES product and roles of fibroblasts in the development of CC will provide a novel target for chemoprevention and treatment of fibrosis in this cancer which may delay the formation of CC.

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