รายงานการวิจัย

เรื่อง

ชื่อโครงการวิจัย (ภาษาไทย) การแยกและติดตามโปรตีนด้วยกระแสไฟฟ้าสองมิติเพื่อระบุชนิดไบโอมาร์คเกอร์ในมะเร็งท่อน้ำดีชนิดที่เกี่ยวข้องกับการติดเชื้อพยาธิใบไม้ตับ: จากหนูแฮมสเตอร์สู่มนุษย์

(ภาษาอังกฤษ) 2D blotting for identification of biomarkers in Opisthorchiasis -associated cholangiocarcinoma: evidence from hamster to human

ชื่อแผนงานวิจัย (ภาษาไทย) ตัวตรวจชีวภาพในการคัดกรองกลุ่มเสี่ยงต่อโรคมะเร็งท่อน้ำดีที่สัมพันธ์กับการติดเชื้อพยาธิใบไม้ตับ

(ภาษาอังกฤษ) Biomarker Discovery for screening of risk group in Opisthorchiasis-associated cholangiocarcinoma

1. ผู้รับผิดชอบและหน่วยงาน ประกอบด้วยหน่วยงานหลักและหน่วยงานสนับสนุน

คณะผู้วิจัยและสัดส่วนที่ทำงานวิจัย		สัดส่วนที่รับผิดชอบ
1.1 หัวหน้าโครงการ	นางสาวธิดารัตน์ บุญมาศ	40 %
1.2 ผู้ร่วมวิจัย	นางสาวกุลธิดา เวทีวุฒาจารย์	30 %
1.3 ผู้ร่วมวิจัย	นางสาวพรทิพย์ เหลื่อมหมื่อนไวย์	30 %

หน่วยงานหลักที่รับผิดชอบ ภาควิชาปรสิตวิทยา ภาควิชาชีวเคมี คณะแพทยศาสตร์ มหาวิทยาลัยขอนแก่น 123 ถนนมิตรภาพ อ.เมือง จ.ขอนแก่น 40002 โทรศัพท์ 043-348-387 โทรสาร 043-202-475

2.ประเภทการวิจัย : การวิจัยประยุกต์

3.สาขาวิชาการและกลุ่มวิชาที่ทำการวิจัย: สาขาวิทยาศาสตร์การแพทย์

4. คำสำคัญ (keywords) ของแผนงานวิจัย

การแยกและติดตามโปรตีนด้วยกระแสไฟฟ้าสองมิติ (2D blotting)

ไบโอมาร์คเกอร์ (Biomarker) มะเร็งท่อน้ำดีชนิดที่เกี่ยวข้องกับการติดเชื้อพยาธิใบไม้ตับ (Ov-associated CCA)

5. Introduction

Cholangiocarcinoma (CCA) is a bile duct carcinoma arising from bile duct epithelium within the intrahepatic and extrahepatic bile duct. The highest prevalence of CCA is in northern part of Thailand where is the endemic area of *Opisthorchis viverrini* (OV) infestation (Sithithaworn, Haswell-Elkins, 2003; IARC, 2011) and crucial health problem of people in this region. CCA is an incurable and rapidly lethal disease if the tumors cannot be removed completely. In surgical patients, the cure depends on the tumor location and the tumor can be completely or partially resected. In non-resectable patients, the 5-year survival rate is 22% in general (Farley et al., 1995). The median overall survival rate for resected patients was 18.9 months versus 5.0 months for patients not resected (Schiffman et al., 2011). The patients may receive palliative chemotherapy or radiation therapy to enhance the cure. Moreover, CCA chemotherapy and radiation therapy are not effective for treatment of late stage of this disease (Pederson et al., 1997; Pazdur et al., 1999; Czito et al., 2006; Farhat et al., 2008). Most patients come to hospital and get the first diagnosis at an advance stage because it is asymptomatic in the early stage (Uttaravichien et al., 1999; Mairiang et al., 2006). Therefore, the prognosis of CCA is poor and short survival time within a year of diagnosis (Green et al., 1991; Andrews et al., 2008; Schiffman et al., 2011).

In order to find candidate biomarkers, hamster CCA cell lines were analyzed using one-dimensional SDS-PAGE, followed by identification with matrix-assisted laser desorption/ ionization time-of-flight mass spectrometry (MALDI-TOF MS).

6. Materials and methods

Parasite preparation

Fresh water fish were bought from the local market in Khon Kaen province northeast Thailand where the endemic area of opisthorchiasis. The fresh fish were digested by 0.25% pepsin- 1.5 % HCl solution, incubated at 37°C for 1 h, filtered through 1000, 300, 250 and 106 μm sieves and metacercariae were recovered via sedimentation in normal saline in a sedimentation jar. OV metacercariae, which have a double-walled cyst, oval shape, and oral and ventral suckers, could be clearly seen under a dissecting microscope. Each hamster was infected with 50 metacercariae by intragastric intubation.

Animals

Syrian hamsters, 1.5 to 2 months old, from the Animal Unit, Faculty of Medicine, Khon Kaen University, were divided into three groups (5 hamsters per group at age 1, 2, 3 and 6 months, as follows: group 1; uninfected (control); group 2; OV infected group (OV) and group 3; OV infected plus NDMA administered group (OVN*)*. Hamsters were sacrificed at 1, 2, 3 and 6 months post-infection. Individual serum was collected from each hamster in each group. CCA mass were collected from liver of group 3 and then used for hamster CCA cell line establishment. All work was conducted with the approval of the Khon Kaen University Animal Ethics Committee (AEKKU51/2555).

Hamster opisthorchiasis preparation

All OV infected groups as assigned above were infected with 50 *O.viverrini* metacercariae and then until sacrificed. Three ml of whole blood from heart puncture were collected, processed and then sera were kept in -20 ^{0}C for immune-western blotting assay.

Administration of NDMA for induction of cholangiocarcinoma

NDMA (final concentration at 12.5 ppm in drinking water) was administered every day from day 1 of the experiment for 2 months (first experiment) or for 1 month (second experiment) for reducing the mortality rate of both control and treated groups.

Hamster CCA cell lines preparation

Primary cholangiocarcinoma Syrian hamster cell lines obtained from cholangiocarcinoma of Syrian hamster and then were culture in 10% Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Invitrogen), 100 U/mL penicillin, 100mg/mL streptomycin and 0.25 mg/mL amphotericin B (antibiotic-antimycotic, invitrogen). Cells were maintained in an incubator with humidified atmosphere of 95% air and 5% CO_2 at 37°C. Cell culture techniques were performed as standard protocols.

Hamster CCA cell line protein extraction

Cells were allowed to grow until 80-90% confluent and were subsequently harvested by using trypsin digestion and then washed 3 times with phosphate buffered saline. Approximately 3x10^{6} of cells were then spin down in a 1.5 mL microcentrifuge tube by centrifugation (4ºC, 10 minutes, 220g) and then 500 µl of PBS was added to the hamsters CCA cell lines pellets into 1.5 mL centrifuge tube then broken cells on ice using ultra-

sonicator (Vibra Cell™, Inc, USA) and centrifuged. Aspirated the supernatant to a new 1.5 ml centrifuge tube and then measured the protein concentration using spectrophotometer at 280 nm and kept in -80 ^{0}C before use.

Gel electrophoresis and immunowestern blotting using hamster CCA and OV sera

15 µg whole cell lysate with an equal volume of 2x electrophoresis sample buffer and boil for 2–3 minutes. Loaded up to 10 µl of lysate into each well of 12% commercial gels of 0.75 mm thickness (Amersham™ ECL™ Gel 12% (GE healthcare Bio-Science, Sweden). One gel was developed the color with *Coomassie* blue (AppliChem, Germany) and another gel was transfer proteins to a nitrocellulose membrane (Bio-Rad Laboratories, USA) using an electro-blotting apparatus according to the manufacturer´s protocols. For *coomassie* blue stained gel was dried and kept for gel digestion. For protein transferred nitrocellulose menbrane was block non-specific binding by incubating membrane in 0.5% skim milk at 4° C overnight in a covered. Membrane was washed three times for 5 minutes each with TBST (10mM Tris, 154mM NaCl, pH=7.5 plus 0.1% Tween 20) TBS. Incubated membrane with each OV serum (dilution of 1:100 in 0.5% skim milk) at 1,2 and 3 month(s) and OVN serum at 1, 2, 3 and 6 months at 37 ^{0}C for 1.5 hr and then washed membrane three times for 5 minutes each with TBST Incubated membrane with 2nd antibody hamster IgG peroxidate (Rabbit Envision (Dako North America, Inc.) with dilution of 1:10000 in 0.5% skim milk at 37 ^{0}C for 1.5 hr and finally washed membrane three times for 5 minutes each with TBS and developed Gel electrophoresis ECL Gel Box (GE healthcare Bio-Science, Sweden) then taken the photos using Molecular Imager® Gel Doc™ XR (Bio-Rad Laboratories, Inc.).

Gel electrophoresis and immunowestern blotting for α HSP 90

Preparation of immunoprecipitation

In brief, solubilize hamster CCA antigen in 50 µl of immunoprecipitation buffer and added a molar excess of hamster sera or α HSP to the protein solution containing the antigen of interest. Adjusted the volume of the sample to 0.2 ml with immunoprecipitation buffer and incubated the sample overnight at 4°C. Added appropriate amount of immobilized protein A or G to the antigen-antibody complex. Incubated the sample with gentle mixing for 2 hours at room temperature. Washed the immobilized protein A or G-bound complexes with 0.5 ml of the immunoprecipitation buffer, followed by centrifugation for 2-3 minutes in a microcentrifuge. Discard the supernatant. Repeat this wash procedure at least 6 times. Elute d the bound antigen-antibody complex from the immobilized protein A or G by incubation for 5 minutes with 50 µl of the elution buffer. Centrifuge the sample, collect the supernatant, and incubate the gel with another 50 µl of the elution buffer (5 minute incubation). Then, centrifuge the sample and collect the supernatant. Combine the 2 x 50 µl supernatant samples. Immediately adjusted the protein sample to a physiological pH by addition of a suitable, more concentrated buffer such as 1.0 M Tris, pH 7.5 (10 µl of this buffer to 100 µl of the supernatant should be sufficient). Desalt the eluted fraction. The sample is ready for gel electrophoresis.

Gel electrophoresis and immunowestern blotting

Fifteen µg whole cell lysate - bind with protein G and mixed with an equal volume of 2x electrophoresis sample buffer and boil for 2–3 minutes. Loaded up to 10 µl of lysate into each well of 12% commercial gels of 0.75 mm thickness (Amersham™ ECL™ Gel 12% (GE healthcare Bio-Science, Sweden). One gel was developed the color with *coomassie* blue

(AppliChem, Germany) and another gel was transfer proteins to a nitrocellulose membrane (Bio-Rad Laboratories) using an electro-blotting apparatus according to the manufacturer´s protocols. For *coomassie* blue stained gel was dried and kept for gel digestion. For protein transferred nitrocellulose membrane was block non-specific binding by incubating membrane in 0.5% skim milk at 4° C overnight in a covered. Membrane was washed three times for 5 minutes each with TBST. Incubated membrane with (Anti- Hsp 90 alpha antibody (Abcam® discover more, England) at 37 ^{0}C for 1.5 hr and then washed membrane three times for 5 minutes each with TBST, incubated membrane with 2nd antibody rabbit IgG peroxidate (Rabbit Envision (Dako North America, Inc.) with dilution of 1:10000 in 0.5% skim milk at 37 ^{0}C for 1.5 hr and finally washed membrane three times for 5 minutes each with TBS and developed Gel electrophoresis ECL Gel Box (GE healthcare Bio-Science, Sweden) then taken the photos using Molecular Imager® Gel Doc^{TM} XR (Bio-Rad Laboratories, Inc.).

Protein analysis

In-Gel Digest Procedure

Gel-associated contaminants that perturb protein digestion in Coomassie blue-stained gels have been replaced by washing for 40 min with 50% acetonitrile, drying for 10 min at room temperature, and then rehydrating with a protease solution. The washing and drying steps result in a substantial reduction of the gel slice volume that, when next swollen in the protease solution, readily absorbs the enzyme, facilitating digestion. The Coomassie blue staining procedure has also been modified by reducing acetic acid and methanol concentrations in the staining solution and by eliminating acetic acid in the destaining

solution. The peptides resulting from the in-gel digestion are easily recovered by passive elution, in excellent yields for structural characterization. Digested proteins were performed using multidimensional liquid chromatography (LC) and tandem mass spectrometry (MS/MS) to separate and fragment peptides.

7. Result

1D gel electrophoresis and immuno-western blotting from hamster sera

Figure 1 shows the result of coomassie blue-stained gels using hamster CCA lysate and Fig. 2 show membrane-transferred hamster CCA lysate and incubated with hamster sera. The cross reaction was found about 64-98 kDa in all serum groups but different in the consistency of band. Normal hamster serum was very light stained and increased with the time manner of OV infection 1, 2 and 3 months post infection. For CCA model, the cross reaction band was gradually increased in dominate at OVN at 3 and 6 months.

Protein identification

The coomassie blue-stained gels was estimated band with nitrocellulose membrane band and cut for protein analysis. Protein identification as differentially expressed in hamster CCA cells. Ions score is -10*Log (P), where P is the probability that the observed match is a random event. Individual ions scores > 34 indicate identity or extensive homology ($p<0.05$).

These differentially expressed X protein bands were furthermore identified by in-gel trypsin digestion and the subsequent MALDI-TOF MS on the basis of peptide mass matching. The results have identified 2 different protein products. The identification was based on 4 to 13 matching peptides, which were statistically necessary for a confident

match (Table 1). Two proteins including HSP 90α and Elongation factor 2, only HSP 90α were further study. HSP 90α was observed in all groups, normal control, OV alone and OVN but the expression was difference in density by up regulated in OVN at 3 months of CCA development.

Table 1: Individual ions scores > 34 indicate identity or extensive homology ($p<0.05$).

Accession No.	Protein name	PI	Mr	MOWSE score	Matches	Sequence coverage (%)
HS90B_MOUSE	Heat shock protein HSP 90-beta	9.4	83571	1412	73	49
HS90A_RAT	Heat shock protein HSP 90-alpha	5.51	85161	1111	62	45
HS90A_CRIGR	Heat shock protein HSP 90-alpha	4.48	85195	1022	57	41
EF2_MOUSE	Elongation factor 2	1.67	96222	649	29	24
EF2_CRIGR	Elongation factor 2	1.67	96205	629	29	24

HSP90 α expression profile in hamster opisthithorchiasis and cholangiocarcinogenesis

The expression of HSP 90 α was observed in all groups, uninfected normal control, OV infected (OV) and OV infected and NDMA administered (OVN) groups in different level. Regarding to uninfected control, the HSP90 α in OV infected was 2- 5 fold and OV infected and NDMA administered groups was 2-14 fold. The highest expression was observed at 2 M of both OV and OVN groups but in OVN group was higher than OV group with statistic significant difference ($P<0.000$). At 6 M, the α HSP90 expression was lower than at 2 M in both groups but still higher than uninfected normal control . In OVN group was higher than OV group with statistic significant difference ($P<0.000$).

Statistical analyses

We used SPSS software version 16.0 (SPSS Inc., USA) to conduct all statistical comparisons. Non- parametric statistical analyses were applied to analyze the difference between two independent groups by means of Mann-Whitney U test. Two-tailed P values of 0.05 or less were considered significant. Receiver Operating Character-istic (ROC) analysis was used to detect the optimal cut-off points (i.e. those with the highest total accuracy) for sepa-rating CCA from other tested groups.

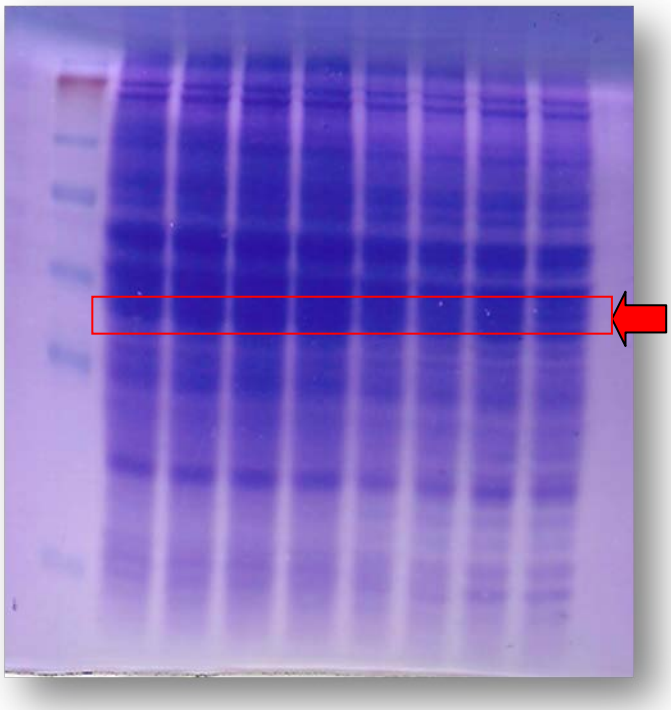


Figure 1 Gel electrophoresis

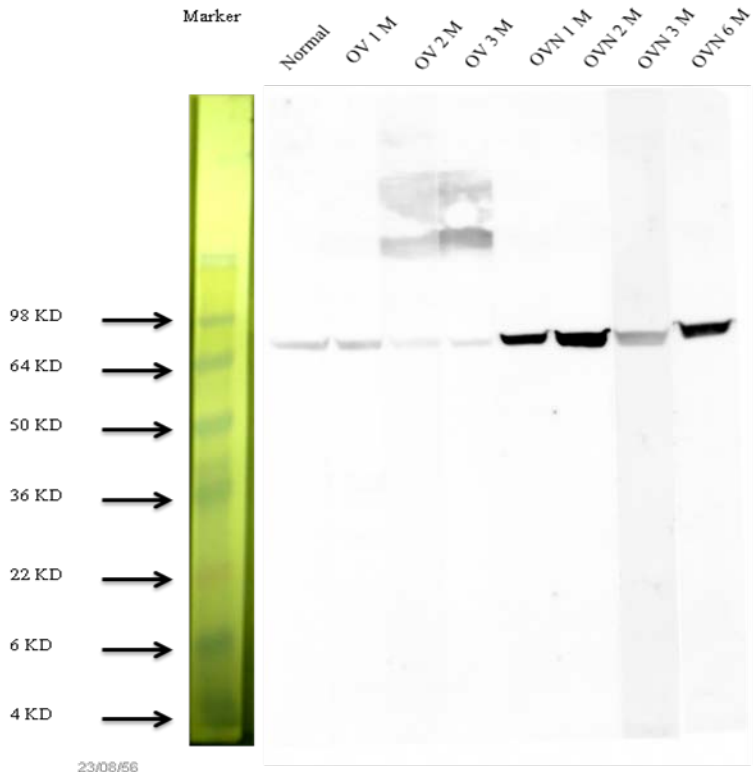


Figure 2 Immunowestern blotting of normal hamster (Normal), *Opisthorchis viverrini* infected for 1 month (OV1M), *Opisthorchis viverrini* infected for 2 months (OV2M), *Opisthorchis viverrini* infected for 3 months (OV3M), *Opisthorchis viverrini* infected and NDMA administered for 1 month (OVN1M), *Opisthorchis viverrini* infected NDMA administered for 2 months (OVN2M), *Opisthorchis viverrini* infected NDMA administered for 3 months (OVN3M) and *Opisthorchis viverrini* infected NDMA administered for 6 months (OVN 6M).

Protein sequence coverage: 48%

Matched peptides shown in ***bold red***.

1	MPEETQTQDQ	PMEEEEVETF	AFQAEIAQLM	SLIINTFYSN
51	NSSDALDKIR	YESLTDPSKL	DSGKELHINI	IPNKQDRTLT
101	ADLINNLGTI	AKSGTKAFME	ALQAGADISM	IGQFGVGFYT
151	ITKHNDDEQY	AWESSAGGSF	TVRTDTGEPM	GRGTKVILHL
201	RRIKEIVKKH	SQFIGYPITL	FVEKERDKEV	SDDEAEEKED
251	GIDDKPEIED	VGSDEEEEEK	KDGDKKKKKK	IKEKYIDQEE
301	NPDDITNEEY	GEFYKSLTND	WEEHLAVKHF	SVEGQLEFRA
351	DLFENRKKKN	NIKLYVRRVF	IMDNCEELFP	EYLNFIRGVV
401	REILQQSKIL	KVIRKNLVRK	CLELFHELAE	DKENYKKFYE
451	HEDSQNRKKL	SELLRYYTSA	SGDEMVSLKD	YCTRMKENQK
501	DQVANSAFVE	RLRKHGLEVI	YMIEPIDEYC	VQQLKEFEGK
551	ELPEDEEEKK	KQEEKKTKFE	NLCKIMKDIL	EKKVEKVVVS
601	VTSTYGWTAN	MERIIKAQAL	RDNSTMGYMA	AKKHLEINPD
651	AEADKNDKSV	KDLVILLYET	ALLSSGFSLE	DPQTHANRIY
701	EDDPTVDDTS	AAVTEEMPPL	EGDDDTSRME	EVD

Figure 3 Amino acid sequences of acryl amide gel protein sequence coverage 48% matched peptide shown in bold red

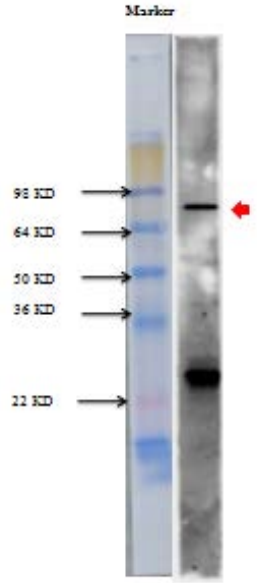
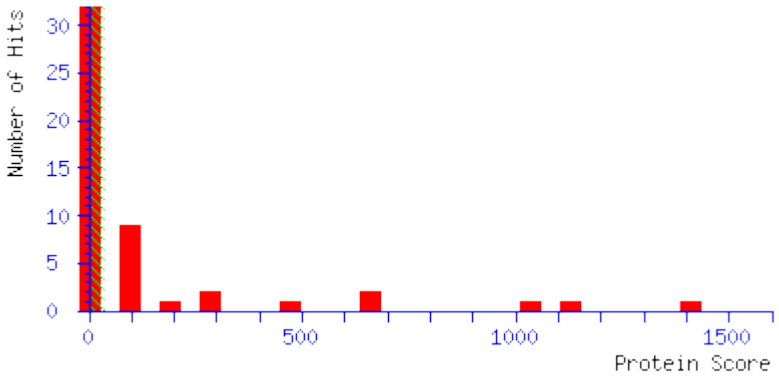


Figure 4 Gel electrophoresis and western blotting analysis to confirming the α HSP90

Protein identification

The coomassie blue-stained gels was estimated band with nitrocellulose membrane band and cut for protein analysis. Protein identification as differentially expressed in hamster CCA cells



Ions score is -10*Log(P), where P is the probability that the observed match is a random event.

HSP90 α expression profile in hamster opisthithorchiasis and cholangiocarcinogenesis

The expression of HSP 90 α was observed in all groups, uninfected normal control, OV infected (OV) and OV infected and NDMA administered (OVND) groups in different level. Regarding to uninfected control, the HSP90 α in OV infected was 2- 5 fold and OV infected and NDMA administered groups was 2-14 fold. The highest expression was observed at 2 M of both OV and OVND groups but in OVND group was higher than OV group with statistic significant difference ($P<0.000$). At 6 M, the α HSP90 expression was lower than at 2 M in both groups but still higher than uninfected normal control . In OVND group was higher than OV group with statistic significant difference ($P<0.000$).

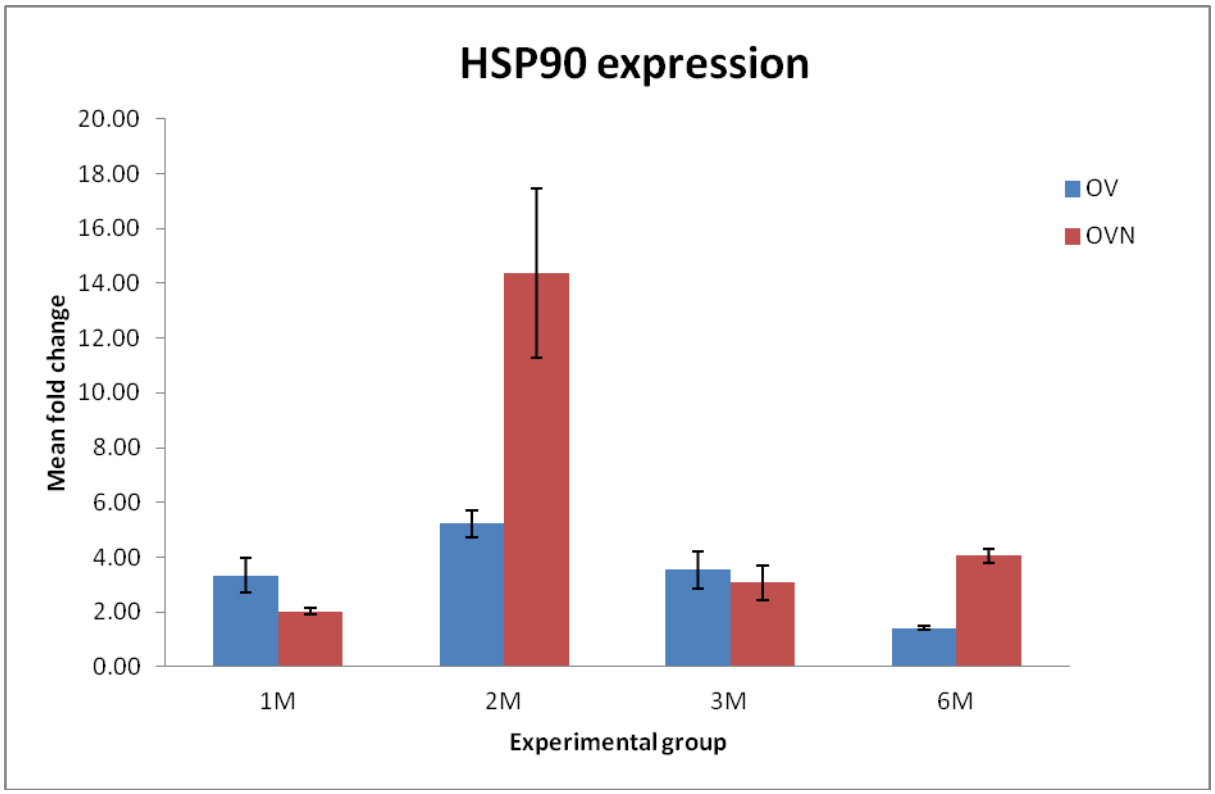


Figure 5 HSP 90 gene expression. Red star, statistic significant difference ($P<0.05$).

Discussion

The present study is the first report which showed the serum marker, anti-HSP90α, was significantly increased with the time manner of CCA development which suggesting that anti-HSP90α is a promising for hamster CCA using 1D gel electrophoresis, immune-western blotting and identification of protein using Mass spectrophotometer.

HSPs were identified as a group of proteins induced during heat shock and by other stimuli, such as growth factors, inflammation, oxidative stress and infections (Welch, 1993). The HSPs have been characterized as molecular chaperones, proteins, which have in common the property of modifying the structures and interactions of other proteins (Netzer, Hartl, 1998; Freeman, Yamamoto, 2002). HSPs are classified in different families and named

according to their molecular weight. HSPs share chaperone activity, contributing to cell survival under stress conditions and facilitating the proper assembly and folding of denatured proteins (Kaufmann, 1990). The high expression of HSPs within physiological levels in advanced cancer could simply reflect an adaptation of the cancer cells to stress (Santarosa et al., 1997; Conroy et al., 1998). These cells have to survive in a hostile microenvironment of hypoxia, acidosis and nutrients starvation. HSPs protect tumor cells from the action of oxygen peroxide and oxyradicals generated by anti-cancer drugs (Jaattela, 1999). In normal cells, the HSP90 interacts in a low affinity dynamic with a myriad of protein, helping them to improve their folding and functionality. But in malignant cells, it establishes a tight association with client oncoproteins, supporting their aberrant state and function, essential for malignant transformation. HSP90 regulates signaling pathways needed for growth, survival and unlimited replicating potential of the tumor (Freeman, Yamamoto, 2002). The HSP90 and their co-chaperones also modulate tumor apoptosis, mainly through their effects on the AKT kinase, TNF-α receptors and the NF-kB transcriptional factor (Basso et al., 2002; Chen et al., 2002; Vanden Berghe et al., 2003).

In conclusion, anti-α HSP 90 α appears to be a potentially relevant serological biomarker for hamster CCA. This autoantibody is present related to the CCA development. The serum level of cancer biomarker could predict both stage of disease and prognosis

Acknowledgements

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10.ผลลัพธ์ที่ได้จากโครงการวิจัย

อยู่ระหว่างการรอพิจารณาตีพิมพ์วารสารนานาชาติจำนวน 1 เรื่อง คือ promising serum marker, anti- Heat Shock Protein 90 α for Syrian Hamster Cholangiocarcinoma