รายงานวิจัยฉบับสมบูรณ์

การศึกษาบทบาทของ CD147 ต่อการชักนำให้เกิดการตายของเซลล์แบบอะพอพโทซิสโดยผ่านกระบวนการตอบสนองของเซลล์ต่อภาวะเครียดในเซลล์มะเร็งปอดชนิด A549

นาย ภัทรพล ลีธนัชอุดม

พฤษภาคม 2559

สัญญาเลขที่ MRG5680080

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นาย ภัทรพล ลีธนัชอุดม
คณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกว. ไม่จำเป็นต้องเห็นด้วยเสมอไป)

รูปแบบ **Abstract (บทคัดย่อ)**

Project Code: MRG5680080

Project Title: The role of CD147 transmembrane glycoprotein on major stress adaptive mechanisms leading to apoptotic cell death in lung cancer cell line (A549)

Investigator: Mr.Pathrapol Lithanatudom, Department of Biology, Faculty of Science, Chiang Mai University

E-mail Address: pathrapol_li@hotmail.com

Project Period: 2 years and 6 months

เนื้อหางานวิจัยประกอบด้วย วัตถุประสงค์ วิธีทดลอง ผลการทดลอง สรุปและวิจารณ์ผลการทดลอง และข้อเสนอแนะสำหรับงานวิจัยในอนาคต

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Project Code: MRG5680080

Project Title: The role of CD147 transmembrane glycoprotein on major stress adaptive mechanisms leading to apoptotic cell death in lung cancer cell line (A549)

Investigator: Mr. Pathrapol Lithanatudom, Department of Biology, Faculty of Science, Chiang Mai University

E-mail Address: pathrapol_li@hotmail.com

Project Period: 3 years

Abstract

CD147, also known as basigin or EMMPRIN, is an immunoglobulin transmembrane glycoprotein which has been reported as a promising target for certain diseases including cancer. Until recently, the monoclonal antibody (mAb) directly against several epitopes of CD147 has been developed and tested in a number of human cancer cell lines in hope for immunotherapy. Here we exploited the anti-human CD147 mouse mAb produced by Kasinrerk, et al namely M6-1D4, M6-2F9 and M6-1F3 as IgM and M6-1E9 as IgG to screen the potential use of these antibodies against certain cancer cell lines. Results showed that after stimulation of several cancer cell lines by anti-CD147 mAb, M6-1D4 induced SupT1 homotypic cell aggregation and cell death (72h post stimulation) while only IgM not IgG stimulation strongly induced apoptotic cell death in HepG2 cell lines. These results suggested the monoclonal antibodies functioned as cancer cell type specific. In particular, M6-1D4 showed a more profound effect on HepG2 cell line as compared to other mAb. Furthermore, we also addressed the mechanistic role of M6-1D4 in cell death induction and found that M6-1D4 induced apoptotic cell death through the endoplasmic reticulum (ER) stress, the accumulation of autophagosome (the sign of autophagy) and the cleavage of caspase 9. Therefore, our study provides for the first time that M6-1D4 might be served as a promising therapeutic agent for hepatocellular carcinoma. However, *in vivo* study is needed to ensure the clinical use of this agent in the patients.

บทคัดย่อ

CD147 หรือ Basigin หรือ EMMPRIN คือไกลโคโปรตีนที่อยู่บนผิวเซลล์ และมีความสัมพันธ์กับโรคหลายชนิด โดยเฉพาะโรคมะเร็ง ปัจจุบันนี้ได้มีการพัฒนาและศึกษา โมโนโคลนอลแอนติบอดี้ (monoclonal antibody (mAb)) ที่จับอย่างจำเพาะกับตำแหน่ง หรือ epitope ต่างๆ ของโปรตีน CD147 บนผิวเซลล์มะเร็งเพื่อการรักษา ในงานวิจัยนี้ได้นำโมโนโคลนอลแอนติบอดี้ต่อโปรตีนชนิด CD147 ที่ผลิตโดย ศ.ดร.วัชระ กสิณฤกษ์ ได้แก่ โมโนโคลนอลแอนติบอดี้โคลน M6-1D4 M6-2F9 และ M6-1F3 (แอนติบอดี้ชนิด IgM) และ M6-1E9 (แอนติบอดี้ชนิด IgG) ที่มีความสามารถในการจับกับโปรตีน CD147 บนผิวเซลล์มะเร็งเพาะเลี้ยงหลายๆ ชนิด โดยพบว่า โมโนโคลนอลแอนติบอดี้ต่อโปรตีนชนิด CD147 โคลน M6-1D4 ทำให้เกิดการเกาะกลุ่มกันของเซลล์และสามารถกระตุ้นการตายของเซลล์มะเร็งเลือดขาวชนิด SupT1 หลังจากเติมแอนติบอดี้ 72 ชั่วโมง ในขณะที่กลุ่มของโมโนแอนติบอดี้ชนิด IgM (ทั้ง 3 โคลน) สามารถกระตุ้นการตายของเซลล์มะเร็งตับชนิด HepG2 เป็นอย่างมาก ซึ่งผลการทดลองทำให้ทราบว่าโมโนโคลนอลแอนติบอดี้ที่นำในศึกษาในครั้งนี้เป็นโมโนโคลนอลแอนติบอดี้ที่จำเพาะต่อเซลล์มะเร็งบางชนิดเท่านั้น และที่น่าสนใจอย่างยิ่งคือ โมโนโคลนอลแอนติบอดี้ต่อโปรตีนชนิด CD147 โคลน M6-1D4 นั้นเป็นโคลนที่มีผลต่อการตายของเซลล์มะเร็งตับชนิด HepG2 มากที่สุด ซึ่งการทดลองพบว่า M6-1D4 จะกระตุ้นการตายของเซลล์มะเร็งโดยผ่านกระบวนการการตอบสนองต่อภาวะเครียดผ่าน endoplasmic reticulum (ER) การกระตุ้นการเกิดออโทฟาโกโซม (Autophagosome) ซึ่งเป็นสัญญาณของกระบวนการการกินตัวเองของเซลล์ (Autophagy) และการย่อยของโปรตีนชนิด caspase 9 ดังนั้นงานวิจัยนี้จึงเป็นงานวิจัยแรกที่แสดงให้เห็นว่าโมโนโคลนอลแอนติบอดี้ต่อโปรตีนชนิด CD147 โคลน M6-1D4 นั้นน่าจะเป็นโมโนโคลนอลแอนติบอดี้ที่สำคัญที่สามารถนำมาใช้เพื่อการรักษาโรคมะเร็งตับได้ ทั้งนี้จะต้องมีการศึกษาเพิ่มเติมต่อไปเพื่อยืนยันก่อนนำไปใช้สำหรับการรักษาจริง

Executive Summary

Introduction to the research problem and its significance

Lung cancer is one of the most common cancers and results in approximately a million deaths annually worldwide. In Thailand, lung cancer is the second most common cancer after liver cancer in males and the fourth most common in females and despite major advances in cancer treatment and therapy, new approaches are urgently require to reduce the mortality burden.

CD147 is the multifunctional transmembrane glycoprotein with two extracellular Ig-like domains and a cytoplasmic tail of 40 amino acids (1, 2). This glycoprotein is implicated in a wide range of cellular functions including fetal development, retinal function, however, one of the most crucial functions of this CD147 glycoprotein implicating on cancer cell lines is the Extracellular Matrix Metalloproteinase-Inducer (EMMPRIN) which plays an important role in the malignant invasion (3). The interaction of CD147 with fibroblast leads to the production of various matrix metalloproteinase (MMPs) resulting in the degradation of the extracellular matrix, tumor invasion and finally, metastasis (2). This is supported by recent studies which showed CD147 is overexpressed on many cancer cells derived from liver, skin, bladder, lung and breast (4, 5). Moreover, certain studies in cancer cell lines have also shown CD147 induces ER stress response through the activation of the unfolded protein response (UPR) responsive mRNAs and proteins to inhibit apoptosis and chemosensitity (6). Additionally, in the other independent study revealed CD147 is also involved in suppression of macroautophagy herein referred to as autophagy in mammalian cells leading to the reduction of cell death in human hepatoma cell (smmc7721) (5). Interestingly, the blockade of CD147 activity by siRNA and anti-CD147 monoclonal antibody results in the impairment of glycolytic energy metabolism leading to cell death in certain cancer cell line (1, 5).

Currently, the UPR and autophagy pathway are poorly characterized in lung cancer given the fact that this type of cancer is one of the most common mortality cancer in Thailand. More interestingly, CD147 overexpression has been reported on lung cancer cell lines therefore, this study is proposed to dissect the effects of CD147 modulation using the monoclonal antibody and/or insulin-like growth factor-I (IGF-I) on cellular stress responses contributing to apoptotic cell death in lung cancer cell line (A549). Hopefully this project might shed new light on the application of using CD147 monoclonal antibody as therapeutic purpose on lung cancer.

Objective

To evaluate the apoptotic effects and the possible induction of cellular stress responses in cancer cell lines through CD147 stimulation by monoclonal antibody.

Methodology

Year 1: Investigation of the UPR pathway in lung cancer cell line (A549)

1. Characterization of the UPR induction in lung cancer cell line (A549) with and without tunicamycin treatment (UPR inducer).

2. Modulation of CD147 protein effect on the UPR pathway either by CD147 monoclonal antibody (mAbs) blocking and the insulin-like growth factor-I (IGF-I) (CD147 inducer).

Year 2: Investigation of the autophagy in lung cancer cell line (A549)

1. Characterization of the autophagy induction in lung cancer cell line (A549) with and without starvation (autophagy inducer).

2. Modulation of CD147 protein effect on the autophagy either by CD147 monoclonal antibody blocking or the insulin-like growth factor-I (IGF-I) (CD147 inducer).

Methods

1. Treatment of A549 cell culture

1.1 Induction of the UPR pathway by tunicamycin treatment

Cells will be resuspended with complete medium for control and tunicamycin treated medium for the UPR induction.

1.2 Induction of autophagy and determination of autophagic flux in A549 cell line

Cells will be resuspended with serum-free medium for autophagy induction as the positive control (serum starvation). To examine autophagic flux activity in A549, cells will be pre-treated with 35 mM L-asparagine for 1 hr before further incubation with 35 mM L-asparagine in the presence or absence of 1 mM EGTA for 72 hr and then cells will be harvested for western blot analysis of autophagy related proteins.

1.3 Modulation of CD147 protein by CD147 mAbs blocking and IGF-I CD147 inducer

For the CD147 inhibition study, A549 will be cultured in the presence or absence of purified CD147 mAbs or isotype-matched control mAbs. For the induction of CD147 protein expression, A549 will be grown in the culture medium with the addition of IGF-I.

1.4 Analysis of the UPR and autophagy responsive mRNAs and proteins.

The stress-inducible mRNAs will be analysed by RT-PCR or real-time RT-PCR whereas the protein level will be examined by western blot and immunofluorescence.

1.5 Detection of apoptotic cell

A549 in the presence or absence of either the UPR or autophagy modulators will be harvested for the detection of the apoptosis level by Annexin V and propidium iodide (PI) analyzed by flow cytometry.

1.6 Statistical analysis

All data obtained from each experiment including semi-quantitative and quantitative RT-PCR, western blot and immunofluoresence will be statistically determined by Quantity one 1-D Analysis Software, ImageJ 1.41 program.

Schedule for the entire project and expected outputs

Experiment	Year 1				Year 2			
	Month 1-3	Month 4-6	Month 7-9	Month 10-12	Month 1-3	Month 4-6	Month 7-12	Month10-12
Study of the UPR pathway								
- Establishment of A549 lung cancer cell line.	←	→						
- Transcriptional analysis of UPR responsive mRNA in A549 with and without the modulation of CD147 protein expression.		←	—	→				
-Translational analysis of UPR responsive protein in A549 with and without the modulation of CD147 protein expression.			←	—	→			
-Statistical analysis and writing the progression report.				←	→			
Study of the Autophagy								
- Transcriptional analysis of autophagy responsive mRNA in A549 with and without the modulation of CD147 protein expression.					←	—	→	
- Translational analysis of UPR responsive protein in A549 with and without the modulation of CD147 protein expression.					←	—	—	→
- Statistical analysis and writing the progression report and manuscript for paper publication.							←	→

Objective

To evaluate the apoptotic effects and the possible induction of cellular stress responses in cancer cell lines through CD147 stimulation by monoclonal antibody.

Material and Method

2. Screening for positive anti-CD147 antibodies against cell surface of lung cancer cell line (A549).

The reactivity of five monoclonal antibodies (mAb) specific for certain epitopes of CD147 namely M6-1D4, M6-1F3, M6-2F9, M6-1E9 and M6-2B1 were tested in lung cancer cell line (A549). The experimental controls consist of isotype-matched control mAbs (Hb1A) and 13M). Details of four mAbs and isotype control mAbs using in this study were shown in table 1. In parallel, the positive reactivity of mAb to the human monocytic cell line, U937 was evaluated as positive control (7).

Indirect immunofluorescence was employed to analyze the reactivity of anti-CD147 mAbs using FITC-conjugated sheep anti-mouse immunoglobulin antibodies. A hundred thousand (1×10^5) of cells were collected and pre-incubated with 10% human AB serum for 30 minutes at 4°C to block non-specific FcR-mediated binding of mAb. Cells were then plated onto 96 well culture plate and incubated with anti-CD147 mAbs (10 ug/ml) for 30 minutes. After appropriate washing, membrane fluorescence was analyzed using flow cytometry.

Table 1 List of Monoclonal antibody used in this study

Name	Type of Antibody
M6-1D4	IgM
M6-1F3	IgM
M6-2F9	IgM
M6-1E9	IgG
M6-2B1	IgG
Hb1A*1	IgM
13M*2	IgG

*1 and *2 mAb are the IgM and IgG isotype-matched control mAbs, respectively.

3. Screening for positive anti-CD147 antibodies for cancer cell line.

The positive mAb resulting from the previous experiment with A549 cell line were further studied in other types of cancer cell line including K562, RAJI and SUP-T1 (human hematopoietic cell lines), Hela (cervical cancer cells) and HepG2 (liver hepatocellular cells). The reactivity of anti-CD147 mAbs on theses cancer cell lines were analyzed using the flow cytometry as previously done in the above experiment.

Moreover, the morphology of cell at 24 hours post anti-CD147 mAbs treatment was also observed.

4. The engagement of anti-CD147 monoclonal antibody to the apoptotic human cancer cell line.

The positive reactivity mAbs were selected to test for the possible induction of cell death in cancer cell lines. U937, K562, RAJI, SUP-T1, A549, Hela and HepG2 were included in this study. A hundred thousand (1×10^5) of cells incubated with anti-CD147 mAbs in various time points were collected and washed with 1 ml of 1X PBS before centrifugation at 5000 rpm for 5 min. After discarding the supernatant, the pellet was resuspended with 200 µl of 1X PBS followed by staining with 1 µl of FITC-conjugated Annexin V and 5 µl of propidium iodide (1) for 15 min. The signal from fluorescence emission was detected by FACSCAlibur flow cytometry (Becton Dickinson, Sunnyvale, CA) which was warmed up before use while incubation. The cell populations were gated according to their forward and side scatter characteristics. The analysis was performed paralleled with the experimental control which were isotype-matched control mAbs, negative control (non treated cell), positive control (hydrogen peroxide (H_2O_2) induction). The percentage of late apoptosis or necrosis and the early apoptosis cell population in either separation or in combination was analyzed from the Annexin V FITC versus PI contour plots with quadrant gates at the upper right (8) and the lower right (LR), respectively. The experiment was performed in triplicates, independent and then the average of the apoptotic cell population was calculated.

5. Analysis of gene expression of the unfolded protein response to stress.

5.1 Total RNA extraction and cDNA synthesis

Total RNA was extracted from studied cancer cell lines collected at 1, 2, 4 and 24 hours after treatment with mAb using TRI Reagent® following the manufacturer's guidelines. Harvested cells were resuspended with 1 ml of TRI Reagent® until the cell pellet was completely lysed and homogenized. After incubating the homogenate for 5

min at room temperature, 0.2 ml of chloroform was added followed by vortexing vigorously for 15 sec and then keeping at room temperature for 15 min. To ensure the complete separation of RNA aqueous phase from DNA and protein, the mixture was centrifuged at 12,000 x *g* for 15 min. The solution from aqueous phase was then transferred to a new tube followed by addition of 0.5 ml of iced-cold isopropanol and mixing until the solution was clear. The mixing aqueous solution was incubated at -20°C overnight and then after centrifugation at 12,000 x *g* for 10 min at 4°C, the RNA pellet was washed with 1 ml of iced-cold 75% methanol. The collected RNA pellet after centrifugation at 7,500 x *g* for 5 min was dried and then dissolved by pipetting in DNase and RNase water. Total RNA concentration was determined by $OD_{260/280}$ using a spectrophotometer. Prior to cDNA synthesis, RNA was heated at 55°C for 10 min.

The extracted RNA sample was used as templates for synthesizing first-strand cDNA using oligo-$(dT)_{15}$ together with ImpromII™ reverse transcriptase. The RT-PCR reaction was performed in a total volume 20 µl consisted of 1 µg of RNA template, 1 mM of oligo-$(dT)_{15}$ and RNase free water was added to 11.6 µl. The solutions were mixed, incubated in the tube at 70ºC for 10 min and immediately placed on ice for 5 min. 8.4 microliters of the master mix reagent was added into the each PCR tube with 1X Improme-II™ buffer, 2.4 µl of 25 mM of $MgCl_2$, 1 µl of 10 mM dNTPs (each) and 1 µl of Improme-II™ Reverse transcriptase. The tube was mixed, incubated at 25ºC for 5 min, 42ºC for 60 min and the finally heated at 70ºC for 15 min. The first-strand cDNA was generated and used as the template for adjusting the optimal PCR condition.

5.2 Semi-qualitative RT-PCR for UPR responsive

The cDNA targets were then amplified for actin (as the internal control) and XBP1 mRNA by *Taq* DNA polymerase enzyme in PCR reactions. The PCR reaction constituted a total of 20 µl reaction. The reagent of PCR reaction consisted of the first-strand cDNA template, 1x HOT FIREPol® Blend Master Mix (with 2 mM $MgCl_2$, 200 uM dNTPs of each) (Solis Biodyne), 2 uM of each forward and reverse primer. The PCR temperature cycle included 1 cycle of 95°C for 10 min, followed by 35 cycles of 95°C for 30 second, 56°C (for actin) 55°C (for XBP1) for 45 sec and 72°C for 1 min, and 1 cycles of 72°C for 10 min. All amplified cDNA targets was separated by electrophoresis on 1.5% gel for actin and 3.0% gel for XBP1, and then visualized by staining with SYBR SAFE DNA Gel Stain (Invitrogen, U.S.A) (9).

5.3 The stress-inducible mRNAs analysis by real-time RT-PCR

The quantitative real time RT-PCR (qRT-PCR) was performed based on the SYBR green technique using SsoAdvanced™ Universal SYBR® Green Supermix

(BioRad). PCR amplification reaction was performed using eppendorf mastercycler realplex 4. All amplified cDNA targets were initially activated at 95°C, 12 min, followed by 40 cycles of denaturation at 95°C, 30 sec, annealed at 56°C, 45 sec and extended at 72°C, 60 sec. The expression levels of transcripts were normalized against actin. The $2^{\Delta\Delta CT}$ method was used to calculate relative changes in gene expression. The efficiency of the amplification was controlled by a standard curve of each gene using the cycle threshold (CT) against two-fold serial dilutions of cDNA (1:2, 1:4, and 1:8). The specificity of the reaction is shown by the melting curve of the amplification products immediately after the last reaction cycle. The statistical significance of mean differences between experiments was tested by non-parametric t-test with observed *P* values less than either 0.01 or 0.05 and all analyzed data were represented as mean ± SEM using the GraphPad Prism 6 software. Sequence of primers used for this study was shown in table 2 (9).

Table 2 Sequence of primers used for stress responsive mRNA expression analysis

Primers	**Sequence (5'-3')**	**Annealing temperature (^{O}C)**	**Product size (bp)**
Actin_F	GAAGATGACCCAGATCATGT	56	330
Actin_R	ATCTCTTGCTCGAAGTCCAG		
CHOP-F	CAGAACCAGCAGAGGTCACA	55	316
CHOP-R	CCAATTGTTCATGCTTGGTG		
GRP78_F	GTATTGAAACTGTAGGAGGTGTC	55	249
GRP78_R	TATTACAGCACTAGCAGATCAG		
XBPsp-F	TGCTGAGTCCGCAGCAGGTG	56	169
XBPsp-R	GCTGGCAGGCTCTGGGGAAG		
XBP1_F	CCTTGTAGTTGAGAACCAGG	55	Spliced 416 Unspliced 442
XBP1_R	GGGGCTTGGTATATATGTGG		

6. Western blot analysis

6.1 Extraction of cellular proteins

Harvested cells from each experiment were washed with cold 1X PBS twice and then lysed in lysis buffer. The mixture was vortexed and sonicated for 5 sec for 4 times before centrifugation at 16,000 rpm for 10 min at 4°C for cellular debris removal. The clear supernatant was kept at -80°C until use (10).

6.2 SDS-PAGE and semi-dry blotting

The concentration of total proteins extracted was determined by Pierce™ BCA Protein Assay Kit (Thermo fisher scientific) following manufacturer's guidelines. Forty micrograms of extracted protein was separated by SDS according to the molecular weight. After boiling for 5 min, the proteins were loaded on 12.5% denaturing polyacrylamide gels at 100 volts for 2 hr and then transferred onto polyvinylidene difluoride (PVDF) membranes using the Semi Dry Blotters at a constant current of 200 mA, for 2 hr.

6.3 Protein expression detection (immunoblotting)

The PVDF membranes were soaked with TBS for 5 min then incubated overnight at 4 $^{\circ}$C with gentle shaking in 5% non-fat dry milk in TBS with 0.05% Tween-20 (TBS-T) for 1-2 hr to prevent the non-specific binding of antibodies used and then washed with TBS-T for 5 min for 3 times. The membrane was probed with primary antibodies against the apoptosis and autophagy-inducible proteins including LC3 (Microtubule-associated protein 1 light chain), Caspase-3, Caspase-7, Caspase-9, and house-keeping protein which is actin for normalization of proteins at dilution 1:1000 in TBS-T supplemented with 5% BSA for 12-16 hr 4 $^{\circ}$C with gentle shaking. After washing off the excess primary antibodies binding with TBS-T for 5 min (3 times), membranes were incubated further with HRP-conjugated secondary antibodies at dilutions of 1:2000 in 5% non-fat dry milk in TBS-T for 1 hr at room temperature. Before detection of protein signal (chemiluminescence), membranes were washed three times with TBS-T for 5 min each. The chemiluminescence signal from membranes probed with primary antibody against interesting protein was developed by SuperSignal™ West Pico Chemiluminescent Substrate (Thermo fisher scientific) following strictly to the manufacturer's guidelines.

7. Indirect immunofluorescence

Approximately, 2.5 x 10^4 cells were collected at 4 hr after treatment with mAb. Cells attached to the coverslip were washed with 1X PBS and then fixed with ice-cold absolute methanol for 20 min. Cells were washed twice for 5 min each with 1X PBS before the permeabilization was performed by incubation with 0.3% triton-X100/1X PBS for 10 min. After washing 5 min for 2 times with 0.03% triton-X100/1X PBS, cells were incubated in 10% human AB serum for 30 min to block the non-specific interaction and cells were then incubated with one or two primary antibody at 4°C overnight. Cells were washed with 0.03% triton-X100/1X PBS for 5 min 4 times to prevent the over fluorescent signals and then were incubated with one or two secondary antibodies for one hr at room temperature followed by 5 min washing with 0.03% triton-X100/1X PBS for 6 times. Cell-attached cover slips were carefully mounted onto glass slides by Prolong® Gold Antifade reagent. The signal from fluorescence was observed and captured under an Olympus FluoView 1000 confocal microscope using Olympus FluoView software v. 1.6.

Results

1. Screening for positive anti-CD147 antibodies for lung cancer cell line (A549)

The reactivity of each anti-CD147 mAb to the cellular surface of several cancer cell lines was analyzed by flow cytometry compared with isotype-matched control mAbs (using Hb1A (IgM) as isotype-matched control mAbs for M6-1D4, M6-1F3 and M6-2F9 and 13M (IgG) as isotype-matched control mAbs for M6-1E9 and M6-2B1). The result showed that four mAbs (M6-1D4, M6-1F3, M6-2F9 and M6-1E9) strongly bound to CD147 transmembrane protein on A549 lung cancer cell while no reactivity of M6-2B1 to the cell was observed (figure 1). The experiment was performed paralleled with the human monocytic cell line, U937 which have been study previously as the positive control (7).

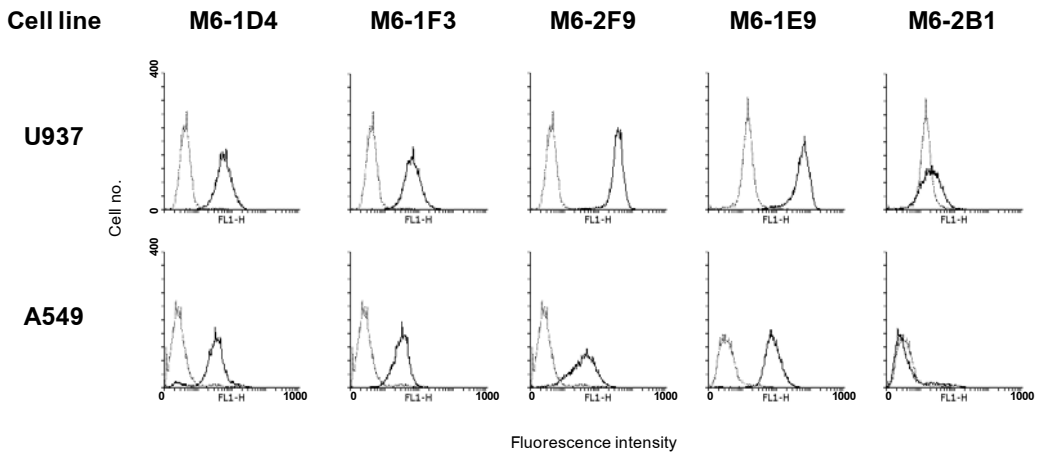


Figure 1 The immunofluorescence analysis of anti- CD147 mAbs reactivity with U937 and A549 cancer cells line. Solid line represents anti-CD147 mAbs. Dotted line represents the isotype-matched control mAbs.

2. Screening for positive anti-CD147 antibodies for certain human cancer cell lines

Anti-CD147 mAbs, M6-1D4, M6-1F3, M6-2F9 and M6-1E9, showing a strong binding to A549 cell line were further used to screen the reactivity of these mAb to various types of cancer cell line. The result showed all four mAbs positively reacted to all cancer cell lines examined (figure 2). The aggregation of hematopoietic cancer cell lines which were U937, K562, Raji and SupT1 cell lines were observed after treatment with M6-1D4, M6-1F3, M6-2F9 and M6-1E9 at 24, 48 and 72 hr. The aggregation of Raji and SupT1 were observed after treatment with M6-1D4, M6-1F3 and M6-2F9 whereas no aggregation was seen in those cells treated with M6-1E9 mAb at every time point investigated. In addition, the aggregation of K562 cell line was also observed only with M6-2F9 treatment. The aggregation results were shown in figure 3 (24 hr), 4 (48 hr) and 5 (72 hr).

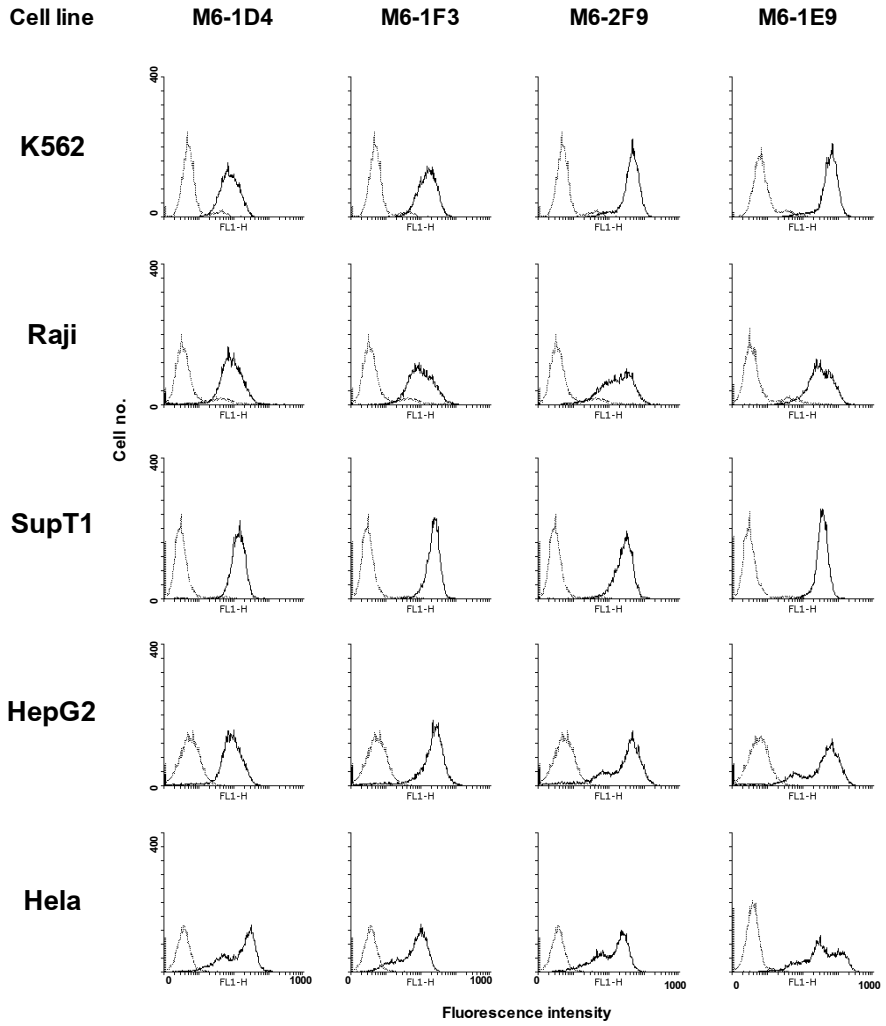


Figure 2 The immunofluorescence analysis of the reactivity of CD147 mAbs with K562, Raji, SupT1, HepG2 and Hela cell lines. Solid line represents anti-CD147 mAbs. Dotted line represents the isotype-matched control mAbs.

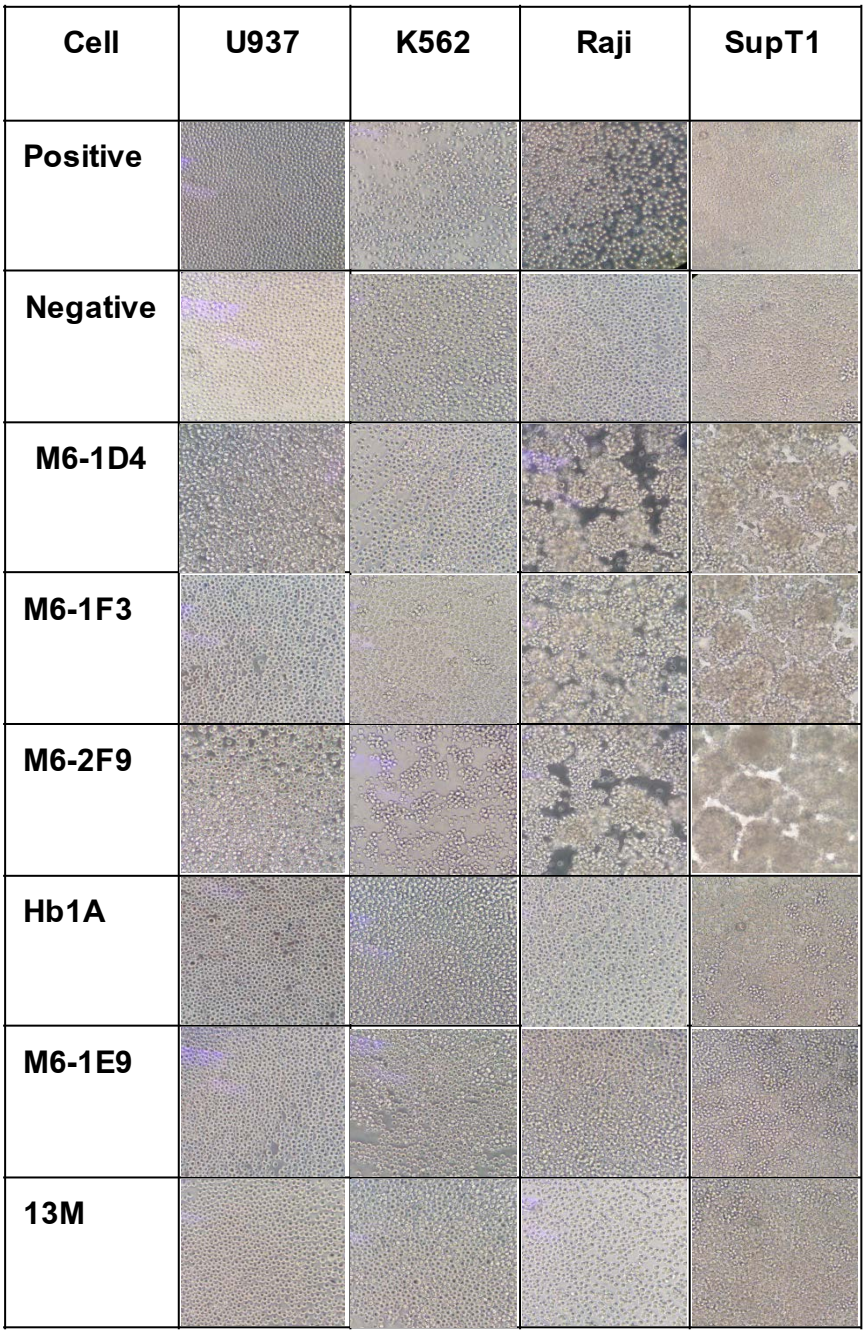


Figure 3 Morphological observation of hematopoietic cell lines treated with 24 hr post anti-CD147 mAbs treatment.

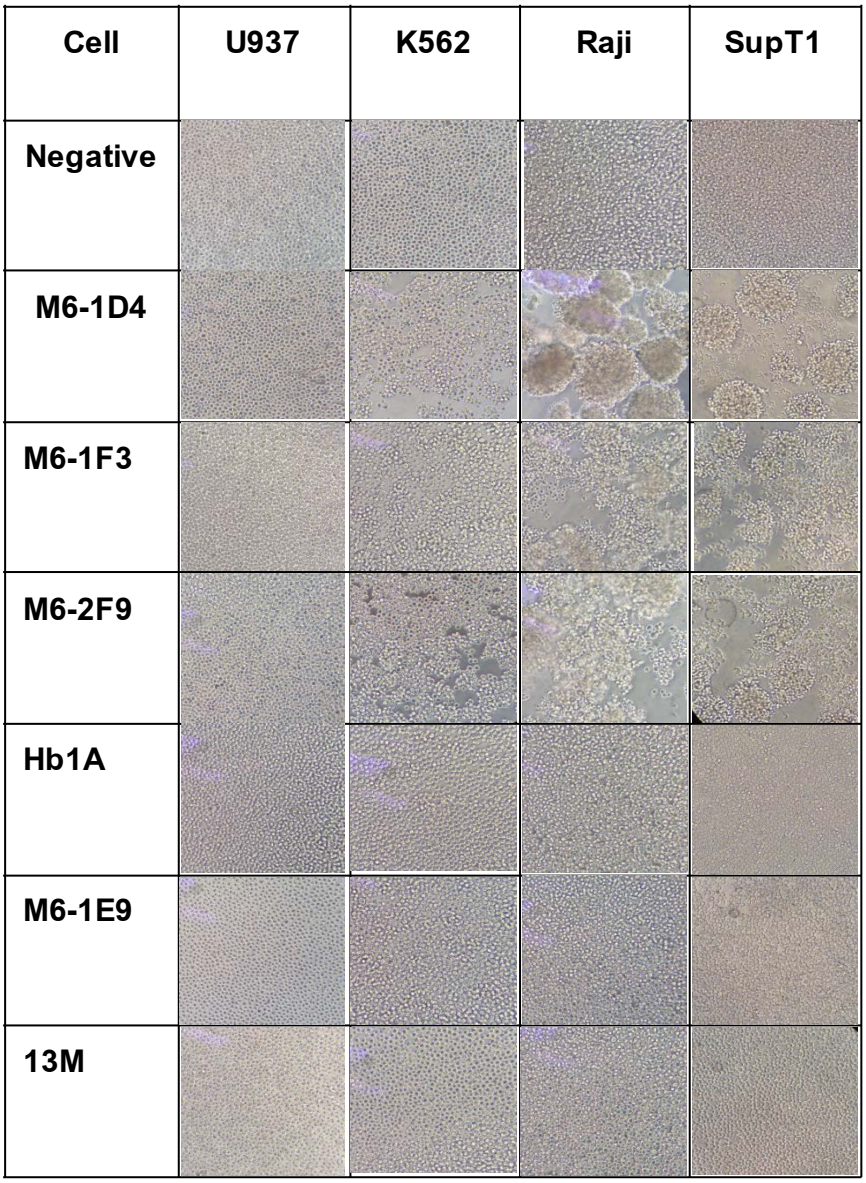


Figure 4 Morphological observation of hematopoietic cell lines treated with anti-CD147 mAbs at 48 hr.

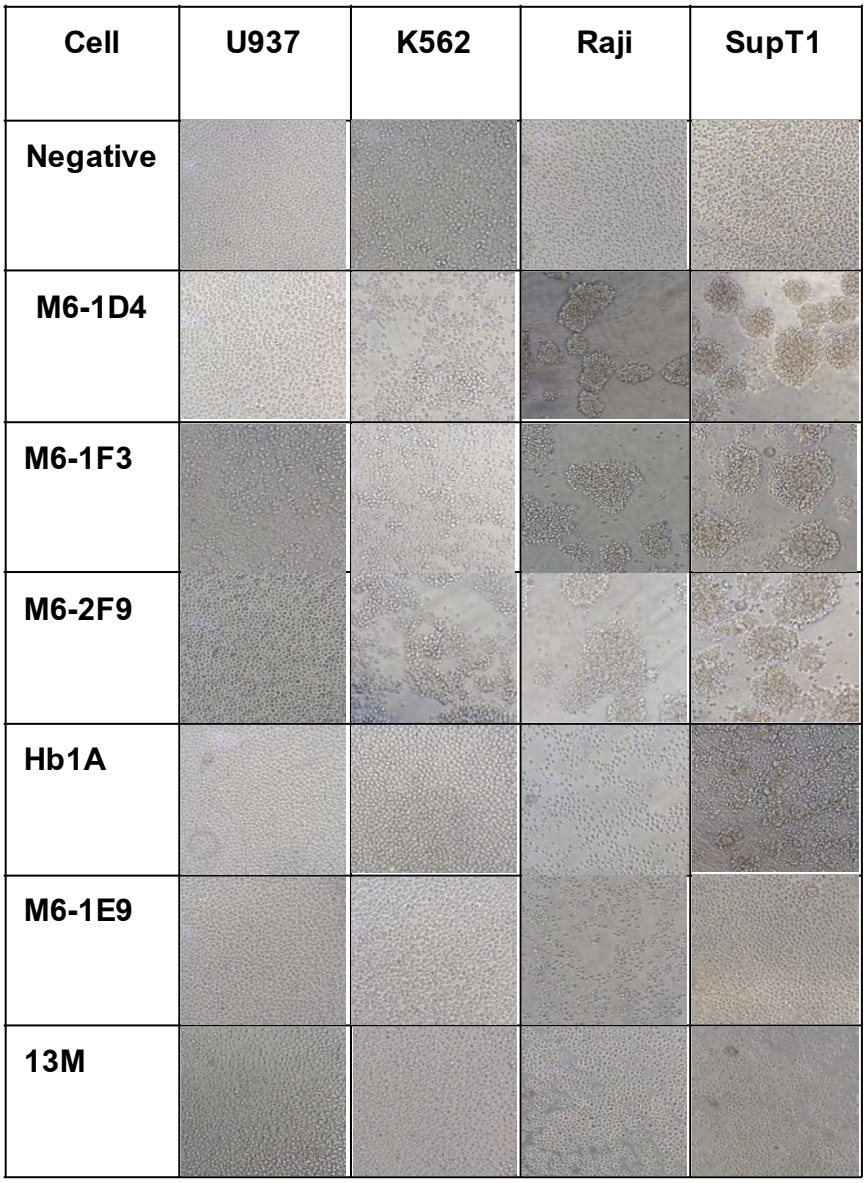


Figure 5 Morphological observation of hematoietic cell lines treated with anti-CD147 mAbs at 72 hr.

3. The engagement of anti-CD147 monoclonal antibody to the apoptotic human cancer cell line.

The possible induction of apoptotic cells was studied in U937, K562, Raji and SupT1 hematopoietic cancer cell lines by the treatment of cells with 4 clones of anti-CD147 mAb at 24, 48 and 72 hr. The percentage of late apoptosis or necrosis and the early apoptotic cell population combined was analyzed by Annexin V FITC versus PI contour plots with quadrant gates at the upper right (8) and the lower right (LR), respectively. Results showed a high percentage of apoptotic cell in SupT1 treated with M6-1D4 at 72 hr after treatment as compared to other cell lines with other mAbs. Figure

6 (A-C) showed representative analyses of quadrant gates from flow cytometry and figure 7 showed graphical representation of late apoptosis or necrosis (A) and the early apoptosis cell population (B) comparing among hematopoietic cancer cell lines.

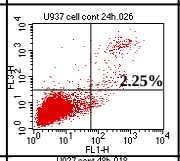
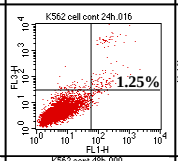
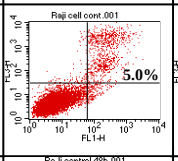
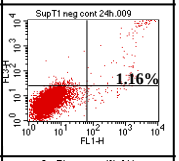
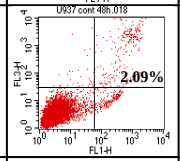
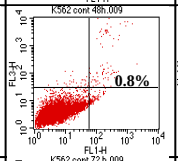
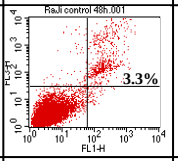
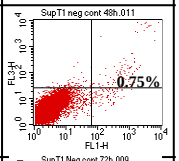
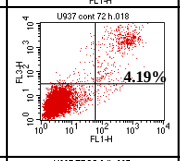
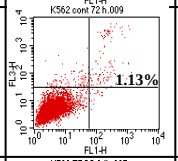
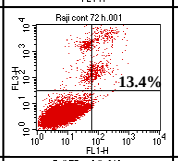
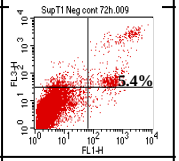
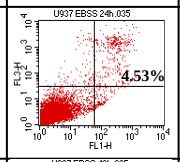
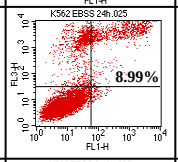
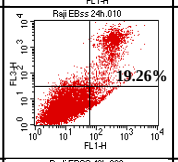
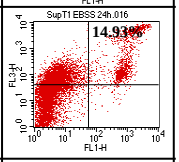
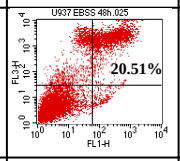
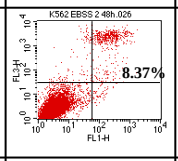
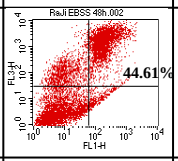
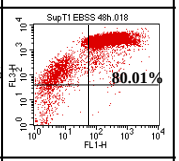
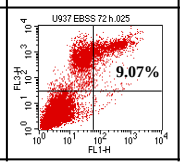
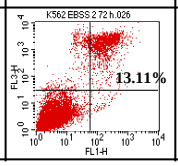
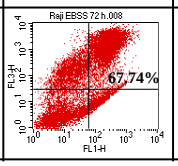
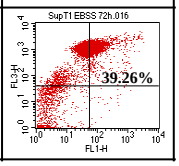
Condition	Time (hr)	Cell Line			
		U937	K562	Raji	SupT1
Negative	24				
	48				
	72				
Positive	24				
	48				
	72				

Figure 6 (A) The apoptotic cell induction in U937, K562, Raji and SupT1 cancer cell lines treated with 4 clone of anti-CD147 mAb analyzed by flow cytometry at 24 48 and 72 hr after treatment. Negative = non treated cell, positive= H_2O_2 treated cell. (A) negative and positive control.

Condition	Time (hr)	Cell Line			
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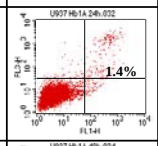
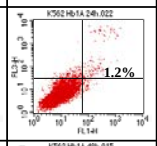
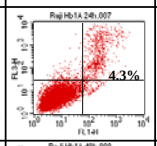
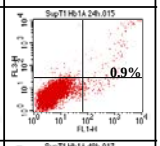
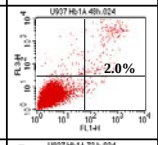
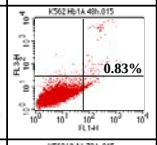
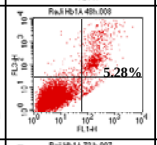
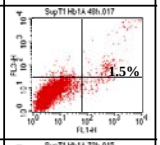
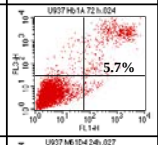
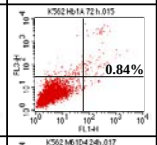
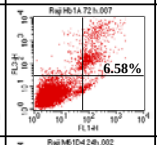
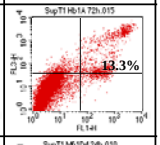
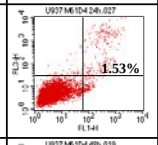
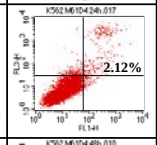
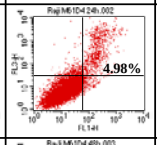
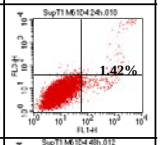
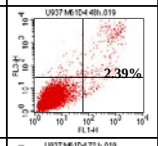
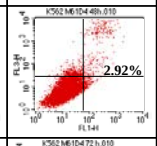
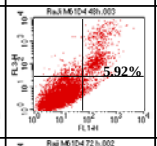
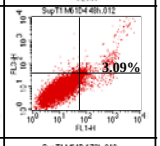
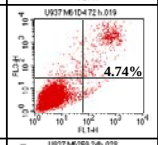
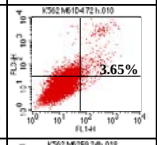
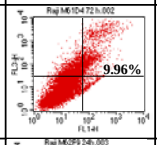
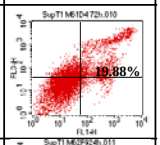
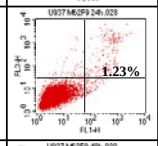
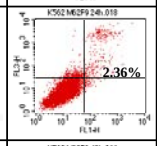
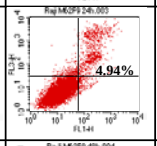
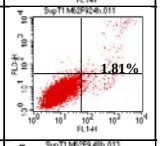
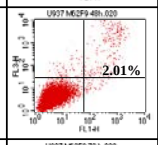
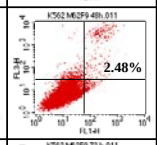
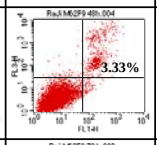
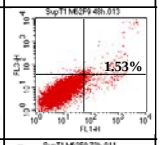
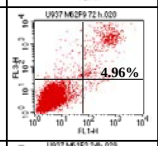
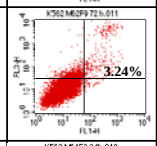
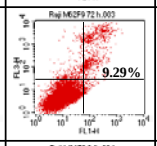
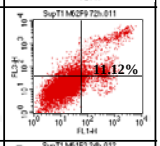
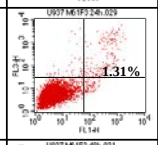
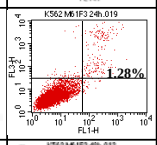
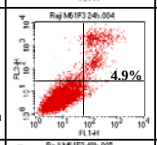
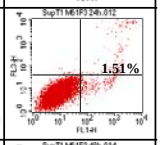
Hb1A	24				
	48				
	72				
M6-1D4	24				
	48				
	72				
M6-2F9	24				
	48				
	72				
M6-1F3	24				
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Figure 6 (B)

Condition	Time (hr)	Cell Line			
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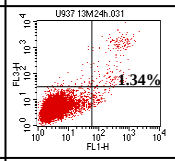
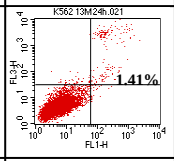
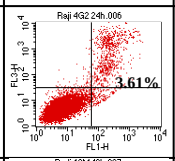
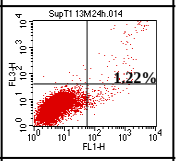
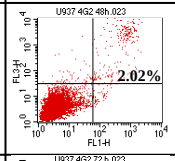
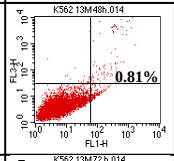
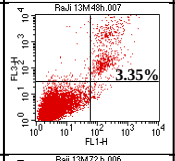
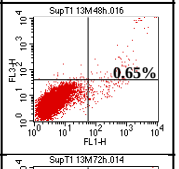
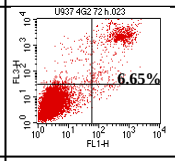
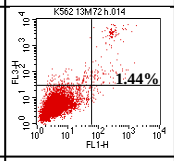
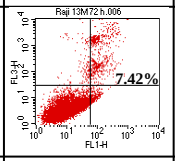
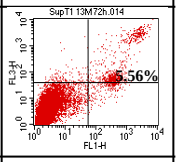
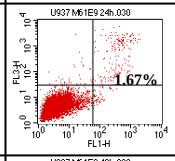
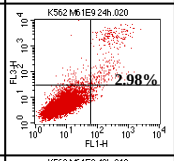
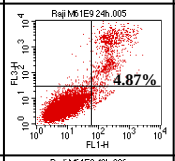
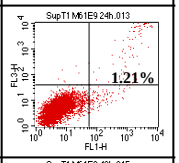
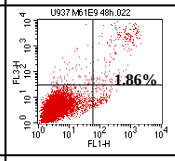
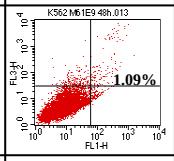
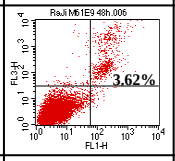
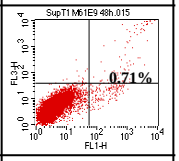
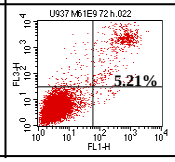
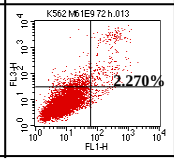
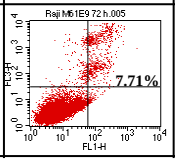
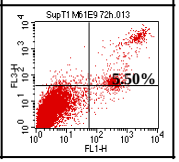
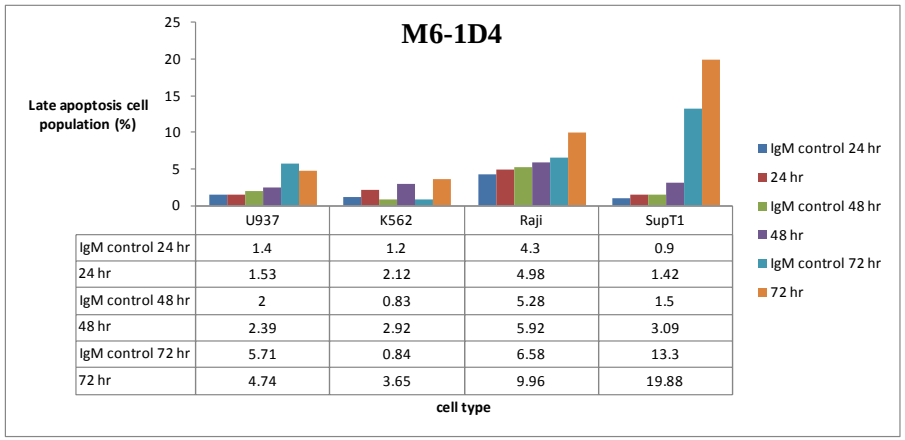
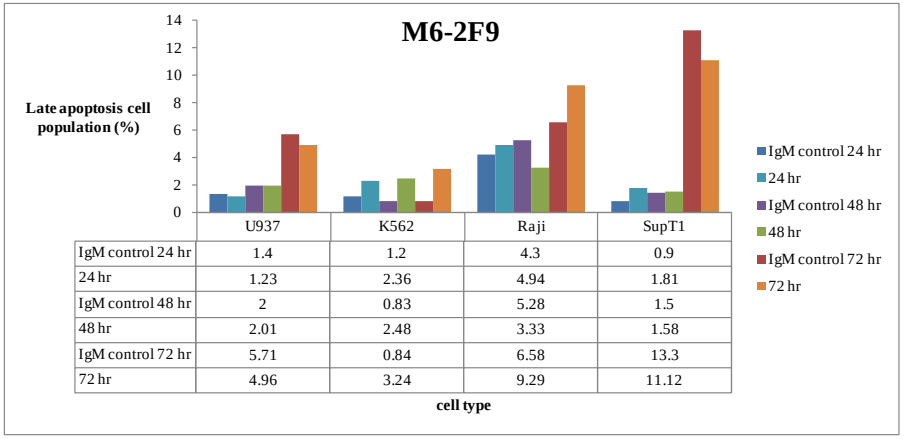
13M	24				
	48				
	72				
M6-1E9	24				
	48				
	72				

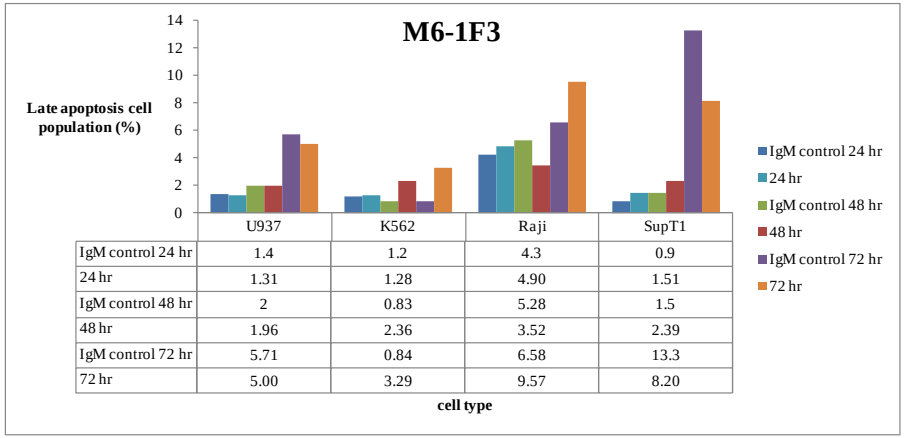
Figure 6 (C)



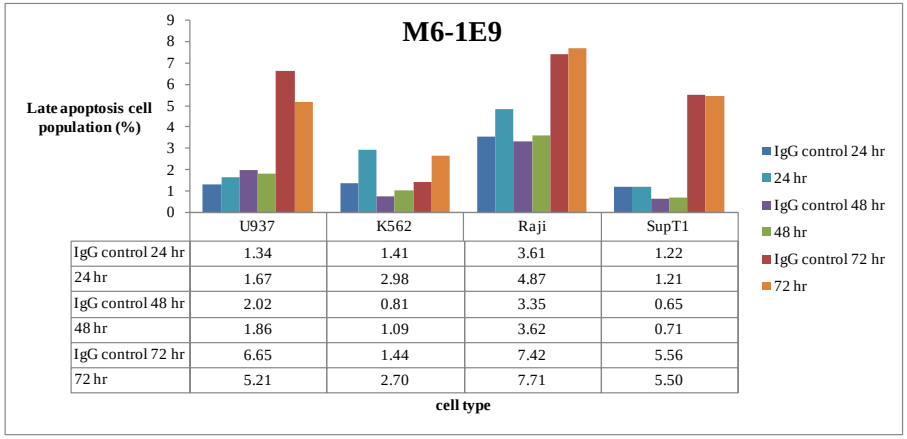
	U937	K562	Raji	SupT1
IgM control 24 hr	1.4	1.2	4.3	0.9
24 hr	1.53	2.12	4.98	1.42
IgM control 48 hr	2	0.83	5.28	1.5
48 hr	2.39	2.92	5.92	3.09
IgM control 72 hr	5.71	0.84	6.58	13.3
72 hr	4.74	3.65	9.96	19.88



	U937	K562	Raji	SupT1
IgM control 24 hr	1.4	1.2	4.3	0.9
24 hr	1.23	2.36	4.94	1.81
IgM control 48 hr	2	0.83	5.28	1.5
48 hr	2.01	2.48	3.33	1.58
IgM control 72 hr	5.71	0.84	6.58	13.3
72 hr	4.96	3.24	9.29	11.12

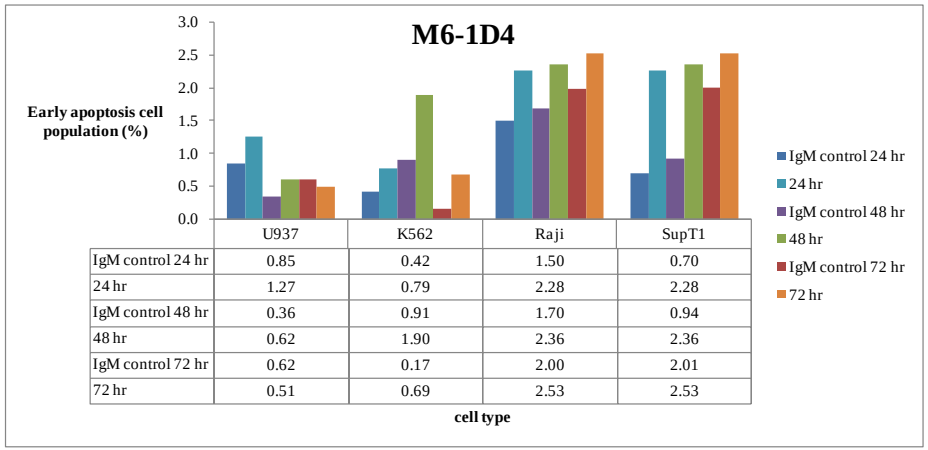


	U937	K562	Raji	SupT1
IgM control 24 hr	1.4	1.2	4.3	0.9
24 hr	1.31	1.28	4.90	1.51
IgM control 48 hr	2	0.83	5.28	1.5
48 hr	1.96	2.36	3.52	2.39
IgM control 72 hr	5.71	0.84	6.58	13.3
72 hr	5.00	3.29	9.57	8.20

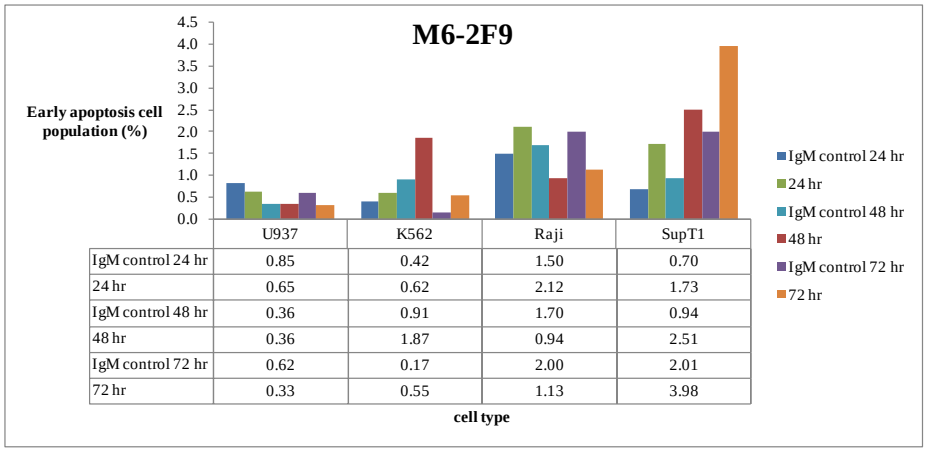


	U937	K562	Raji	SupT1
IgG control 24 hr	1.34	1.41	3.61	1.22
24 hr	1.67	2.98	4.87	1.21
IgG control 48 hr	2.02	0.81	3.35	0.65
48 hr	1.86	1.09	3.62	0.71
IgG control 72 hr	6.65	1.44	7.42	5.56
72 hr	5.21	2.70	7.71	5.50

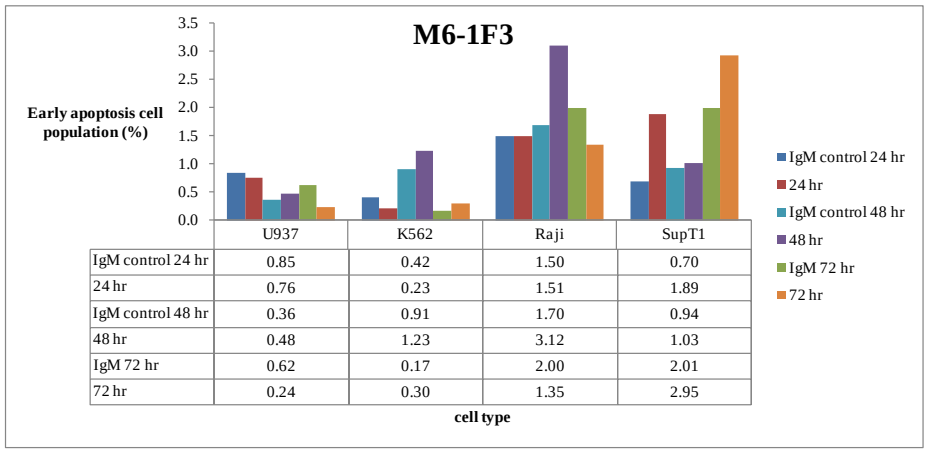
Figure 7 (A) The percentage of late apoptotic cell induction in cancer cell lines treated with 4 clone of CD147 mAbs at 24, 48, and 72 hr. Hb1A and 13M mAb are the IgM and IgG isotype-matched control mAbs, respectively. (A) Late apoptosis or necrosis (AnnexinV+, PI+) and (B) Early apoptosis (AnnexinV+, PI-).



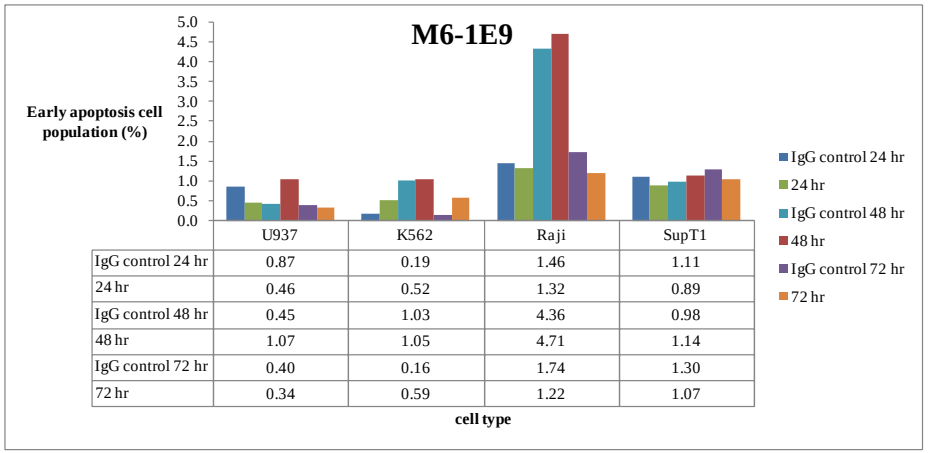
	U937	K562	Raji	SupT1
IgM control 24 hr	0.85	0.42	1.50	0.70
24 hr	1.27	0.79	2.28	2.28
IgM control 48 hr	0.36	0.91	1.70	0.94
48 hr	0.62	1.90	2.36	2.36
IgM control 72 hr	0.62	0.17	2.00	2.01
72 hr	0.51	0.69	2.53	2.53



	U937	K562	Raji	SupT1
IgM control 24 hr	0.85	0.42	1.50	0.70
24 hr	0.65	0.62	2.12	1.73
IgM control 48 hr	0.36	0.91	1.70	0.94
48 hr	0.36	1.87	0.94	2.51
IgM control 72 hr	0.62	0.17	2.00	2.01
72 hr	0.33	0.55	1.13	3.98



	U937	K562	Raji	SupT1
IgM control 24 hr	0.85	0.42	1.50	0.70
24 hr	0.76	0.23	1.51	1.89
IgM control 48 hr	0.36	0.91	1.70	0.94
48 hr	0.48	1.23	3.12	1.03
IgM 72 hr	0.62	0.17	2.00	2.01
72 hr	0.24	0.30	1.35	2.95



	U937	K562	Raji	SupT1
IgG control 24 hr	0.87	0.19	1.46	1.11
24 hr	0.46	0.52	1.32	0.89
IgG control 48 hr	0.45	1.03	4.36	0.98
48 hr	1.07	1.05	4.71	1.14
IgG control 72 hr	0.40	0.16	1.74	1.30
72 hr	0.34	0.59	1.22	1.07

Figure 7 (B) The percentage of early apoptotic cell induction in cancer cell lines treated with 4 clone of CD147 mAbs at 24, 48, and 72 hr. Hb1A and 13M mAb are the IgM and IgG isotype-matched control mAbs, respectively. (B) Early apoptosis (AnnexinV+, PI-).

Due to the induction of apoptosis was not significantly observed in U937 and K562 cell lines and only low percentage was seen in Raji and SupT1 cell lines treated with anti-CD147 mAbs at 24, 48 and 72 hr. Therefore, the investigation was changed to study in Raji, SupT1, HepG2, Hela and A549 at the earlier time points which were 1, 2 and 4 hr after treatment with anti-CD147 mAbs. Interestingly, anti-CD147 mAb clone M6-1D4, M6-2F9 and M6-1F3 induced cell death in HepG2 cell line (30-50%), whereas the induction of cell death by M6-1E9 showed the lowest percentage of HepG2 cell death with 9-14% (Figure 8 and 9(A)). The early apoptotic cell was also found in HepG2 cell line treated with M6-1D4, M6-2F9, M6-1F3 and M6-1E9 mAb as compared with IgM (Hb1A) and IgG (13M) isotype-matched control mAbs with the percentage lower than the late apoptosis. Moreover, the early apoptosis was also found in Hela cell lines treated with M6-2F9 and M6-1F3 with low level about 3-12 % (Figure 8 and 9(B)). Due to the highest percentage of apoptosis cell was found in HepG2 cell line as compared with other cell lines. Therefore, the analysis of gene expression, protein and co-localization of sensor proteins of the unfolded protein response to stress were further studied by using HepG2 cell line treated with 4 clone of anti-CD147 mAbs at 15 min, 1, 2, 4 and 24 hr.

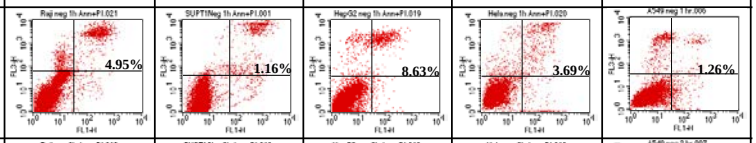
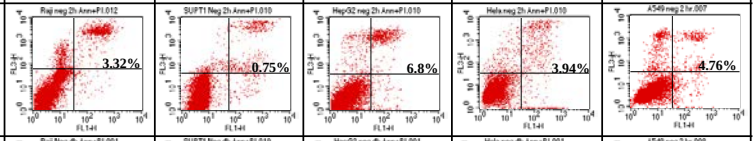
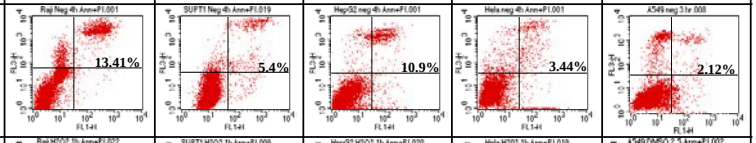
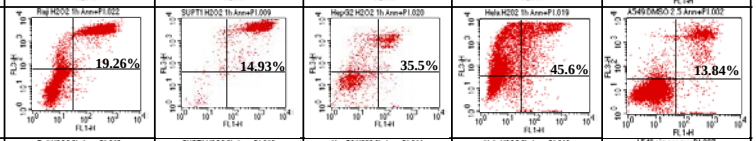
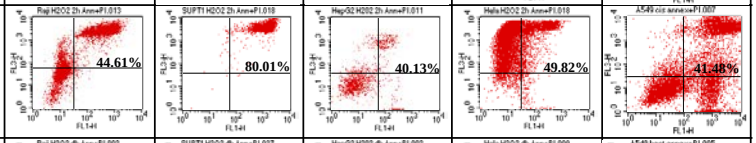
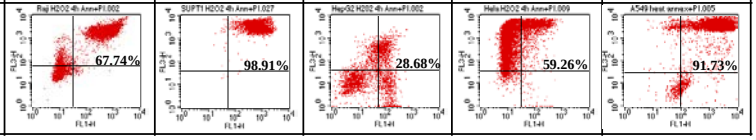
Condition	Time(hr)	Cell Line				
		Raji	SupT1	HepG2	Hela	A549
Negative	1					
	2					
	4					
Positive	1					
	2					
	4					

Figure 8 (A) The apoptotic cell induction in Raji, SupT1, HepG2, Hela and A549 cancer cell lines treated with 4 clone of anti-CD147 mAb analyzed by flow cytometry at 1, 2 and 4 hr after treatment. Negative = non treated cell, positive= H_2O_2 treated cell. Hb1A and 13M mAb are the IgM and IgG isotype-matched control mAbs, respectively. (A) Negative and positive, (B) Hb1A, M6-1D4, M6-2F9 and M61-F3 (C) 13M and M6-1E9.

Condition	Time(hr)	Cell Line				
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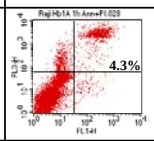
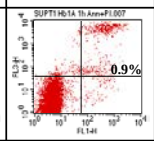
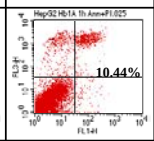
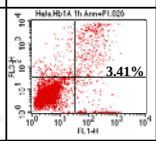
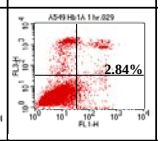
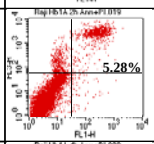
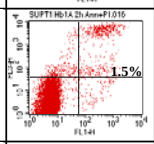
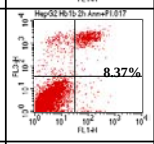
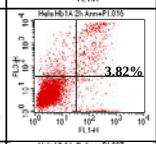
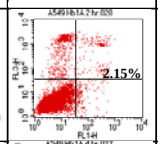
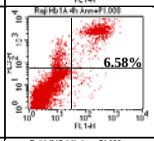
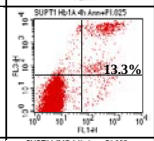
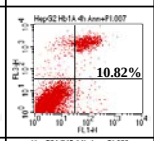
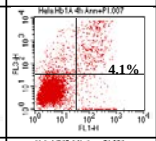
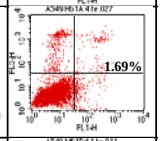
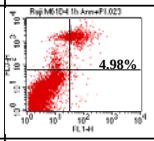
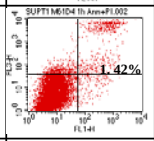
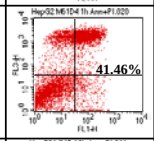
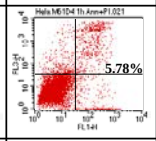
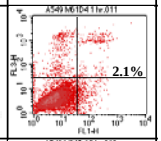
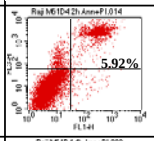
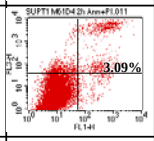
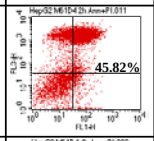
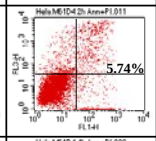
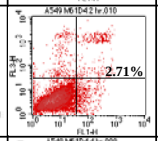
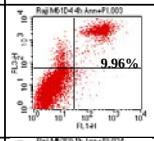
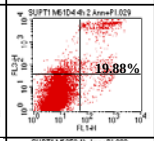
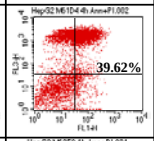
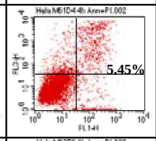
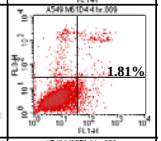
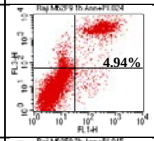
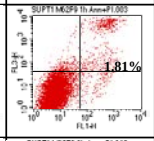
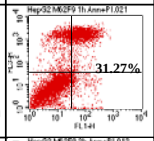
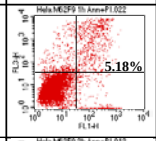
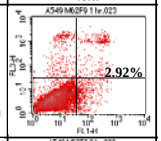
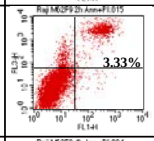
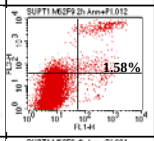
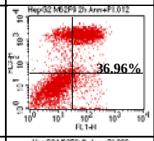
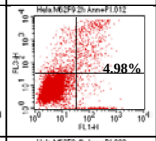
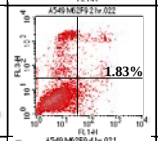
Hb1A	1					
	2					
	4					
M6-1D4	1					
	2					
	4					
M6-2F9	1					
	2					
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Figure 8 (B)

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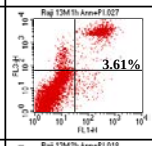
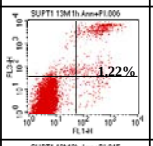
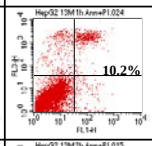
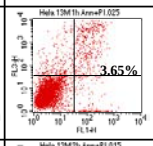
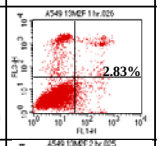
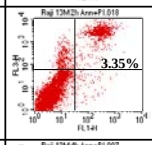
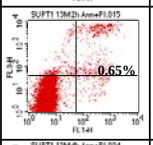
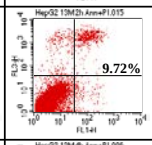
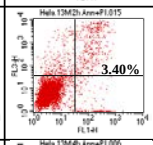
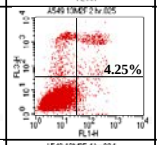
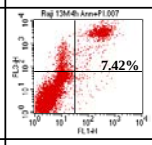
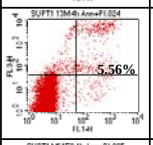
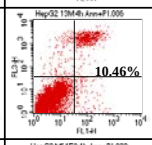
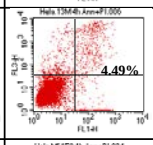
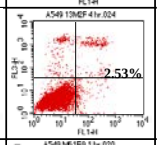
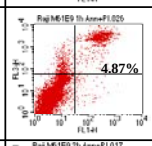
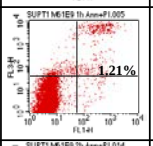
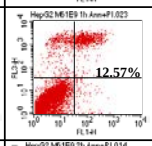
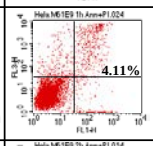
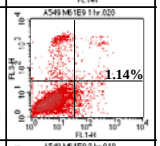
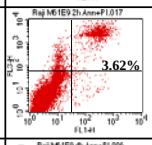
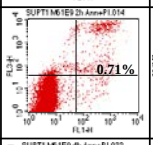
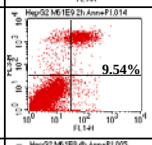
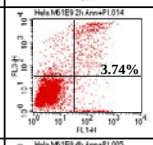
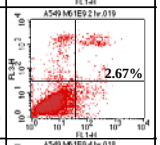
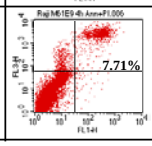
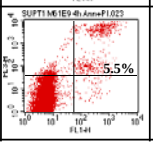
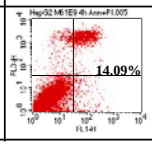
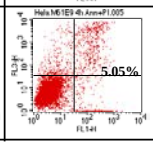
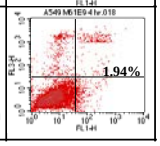
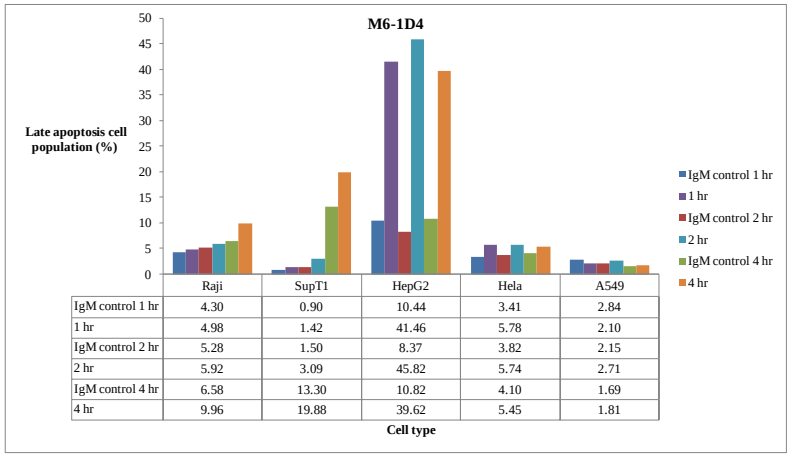
13M	1					
	2					
	4					
M6-1E9	1					
	2					
	4					

Figure 8 (C)



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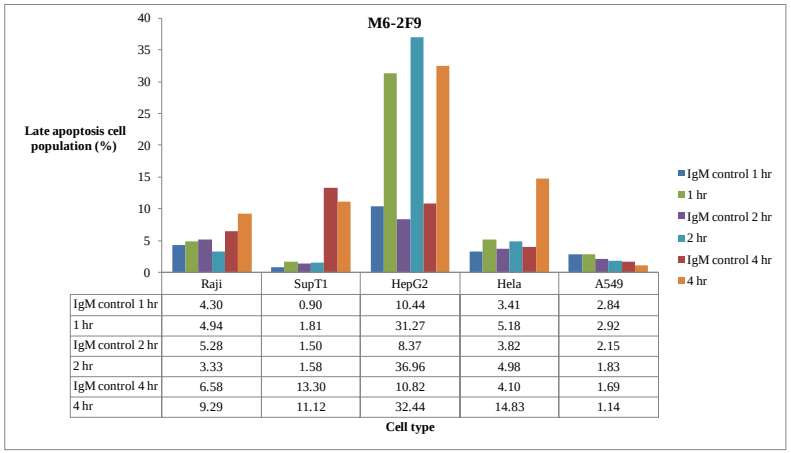
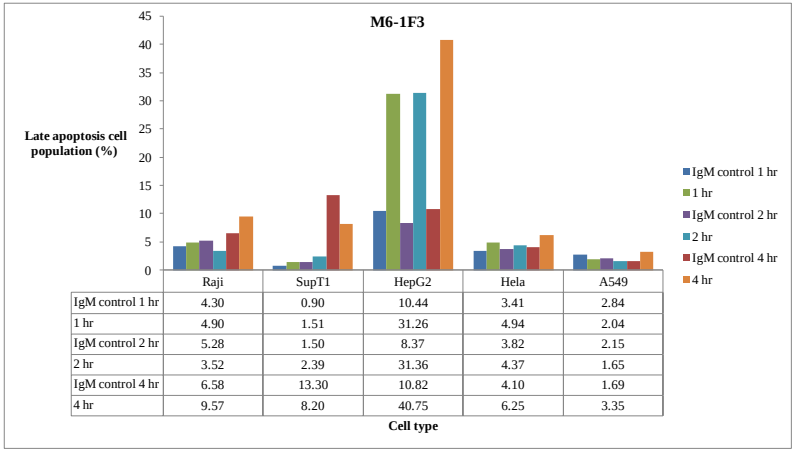
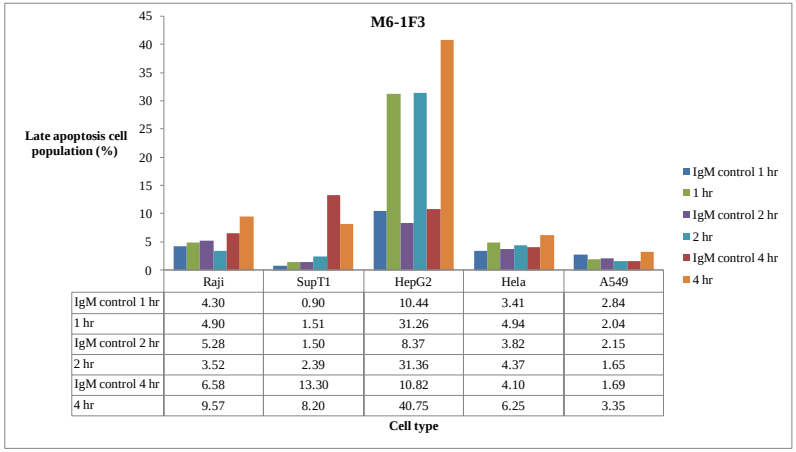
IgM control 1 hr	4.30	0.90	10.44	3.41	2.84
1 hr	4.98	1.42	41.46	5.78	2.10
IgM control 2 hr	5.28	1.50	8.37	3.82	2.15
2 hr	5.92	3.09	45.82	5.74	2.71
IgM control 4 hr	6.58	13.30	10.82	4.10	1.69
4 hr	9.96	19.88	39.62	5.45	1.81



	Raji	SupT1	HepG2	Hela	A549
IgM control 1 hr	4.30	0.90	10.44	3.41	2.84
1 hr	4.94	1.81	31.27	5.18	2.92
IgM control 2 hr	5.28	1.50	8.37	3.82	2.15
2 hr	3.33	1.58	36.96	4.98	1.83
IgM control 4 hr	6.58	13.30	10.82	4.10	1.69
4 hr	9.29	11.12	32.44	14.83	1.14

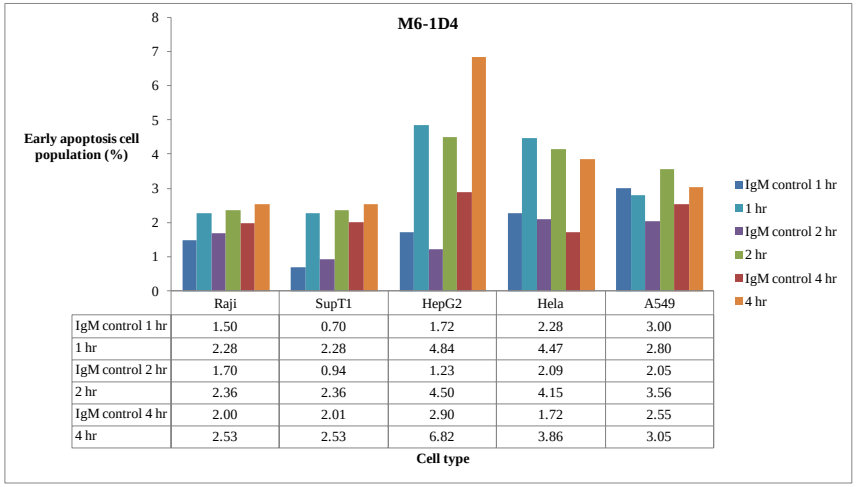


	Raji	SupT1	HepG2	Hela	A549
IgM control 1 hr	4.30	0.90	10.44	3.41	2.84
1 hr	4.90	1.51	31.26	4.94	2.04
IgM control 2 hr	5.28	1.50	8.37	3.82	2.15
2 hr	3.52	2.39	31.36	4.37	1.65
IgM control 4 hr	6.58	13.30	10.82	4.10	1.69
4 hr	9.57	8.20	40.75	6.25	3.35

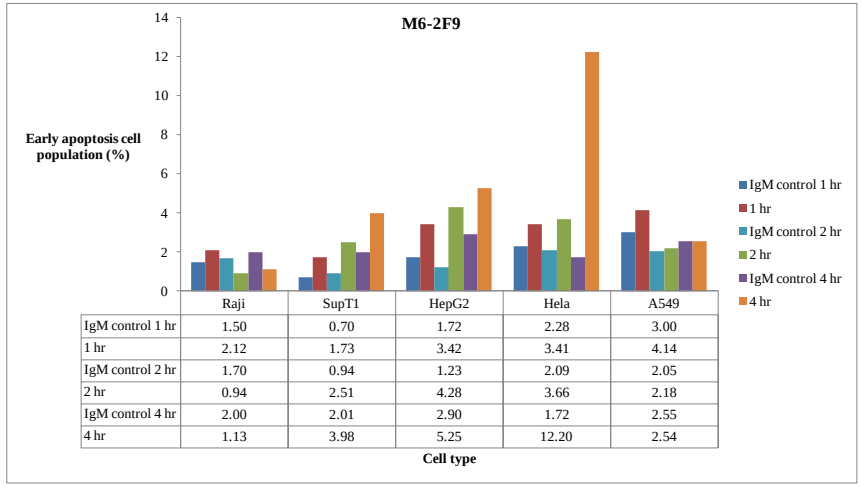


	Raji	SupT1	HepG2	Hela	A549
IgM control 1 hr	4.30	0.90	10.44	3.41	2.84
1 hr	4.90	1.51	31.26	4.94	2.04
IgM control 2 hr	5.28	1.50	8.37	3.82	2.15
2 hr	3.52	2.39	31.36	4.37	1.65
IgM control 4 hr	6.58	13.30	10.82	4.10	1.69
4 hr	9.57	8.20	40.75	6.25	3.35

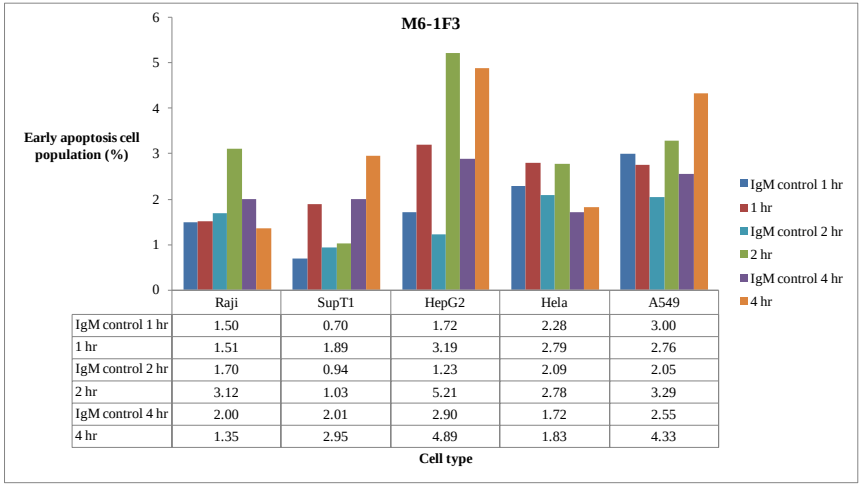
Figure 9 (A) The percentage of apoptotic cell induction in cancer cell lines treated with 4 clones of anti-CD147 mAb at 1, 2, and 4 hr. Hb1A and 13M mAb are the IgM and IgG isotype-matched control mAbs, respectively. (A) Late apoptosis or necrosis and (B) Early apoptosis.



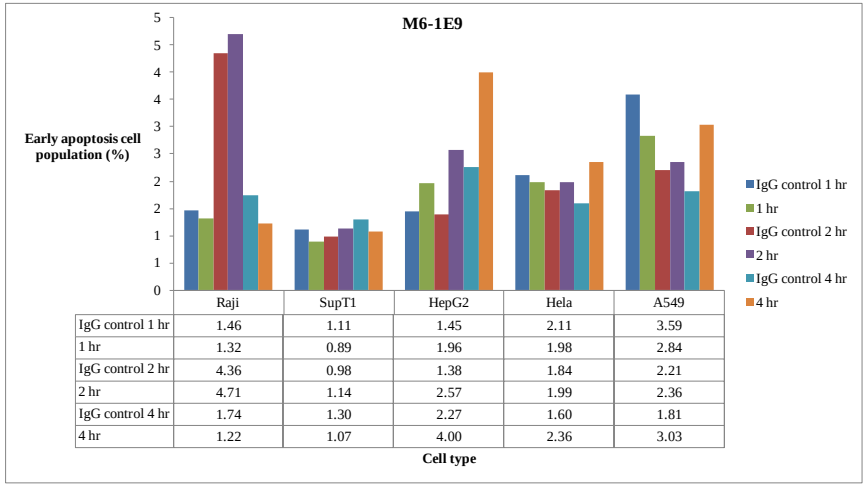
	Raji	SupT1	HepG2	Hela	A549
IgM control 1 hr	1.50	0.70	1.72	2.28	3.00
1 hr	2.28	2.28	4.84	4.47	2.80
IgM control 2 hr	1.70	0.94	1.23	2.09	2.05
2 hr	2.36	2.36	4.50	4.15	3.56
IgM control 4 hr	2.00	2.01	2.90	1.72	2.55
4 hr	2.53	2.53	6.82	3.86	3.05



	Raji	SupT1	HepG2	Hela	A549
IgM control 1 hr	1.50	0.70	1.72	2.28	3.00
1 hr	2.12	1.73	3.42	3.41	4.14
IgM control 2 hr	1.70	0.94	1.23	2.09	2.05
2 hr	0.94	2.51	4.28	3.66	2.18
IgM control 4 hr	2.00	2.01	2.90	1.72	2.55
4 hr	1.13	3.98	5.25	12.20	2.54



	Raji	SupT1	HepG2	Hela	A549
IgM control 1 hr	1.50	0.70	1.72	2.28	3.00
1 hr	1.51	1.89	3.19	2.79	2.76
IgM control 2 hr	1.70	0.94	1.23	2.09	2.05
2 hr	3.12	1.03	5.21	2.78	3.29
IgM control 4 hr	2.00	2.01	2.90	1.72	2.55
4 hr	1.35	2.95	4.89	1.83	4.33



	Raji	SupT1	HepG2	Hela	A549
IgG control 1 hr	1.46	1.11	1.45	2.11	3.59
1 hr	1.32	0.89	1.96	1.98	2.84
IgG control 2 hr	4.36	0.98	1.38	1.84	2.21
2 hr	4.71	1.14	2.57	1.99	2.36
IgG control 4 hr	1.74	1.30	2.27	1.60	1.81
4 hr	1.22	1.07	4.00	2.36	3.03

Figure 9 (B)

4. Semi-qualitative RT-PCR for UPR and autophagy responsive mRNA

The UPR induction was observed by alternative splicing of XBP1 mediated by IRE1 transducer. The detection of XBP1 transcripts was carried out by RT-PCR with specific primers designed for the simultaneous amplification of both unspliced (442 bp) and spliced (416 bp) PCR products. Result showed that the XBP1 mRNA was spliced in response to thapsigargin treatment (Positive control). However, no induction of XBP1 splicing can be seen in negative control sample and the IgM and IgG isotype-matched control mAbs. The induction of XBP1 splicing form was observed in HepG2 cell treated with M6-1D4 at 4 and 24 hr, M6-2F9 at 24 hr, M6-1F3 slightly induction at 24 hr and undetectable of in HepG2 cell treated with M6-1E9 (figure 10). The presence of larger PCR product was also observed which is believed to be the heteroduplex formed by the partially complementary sequence in spliced and unspliced transcripts.

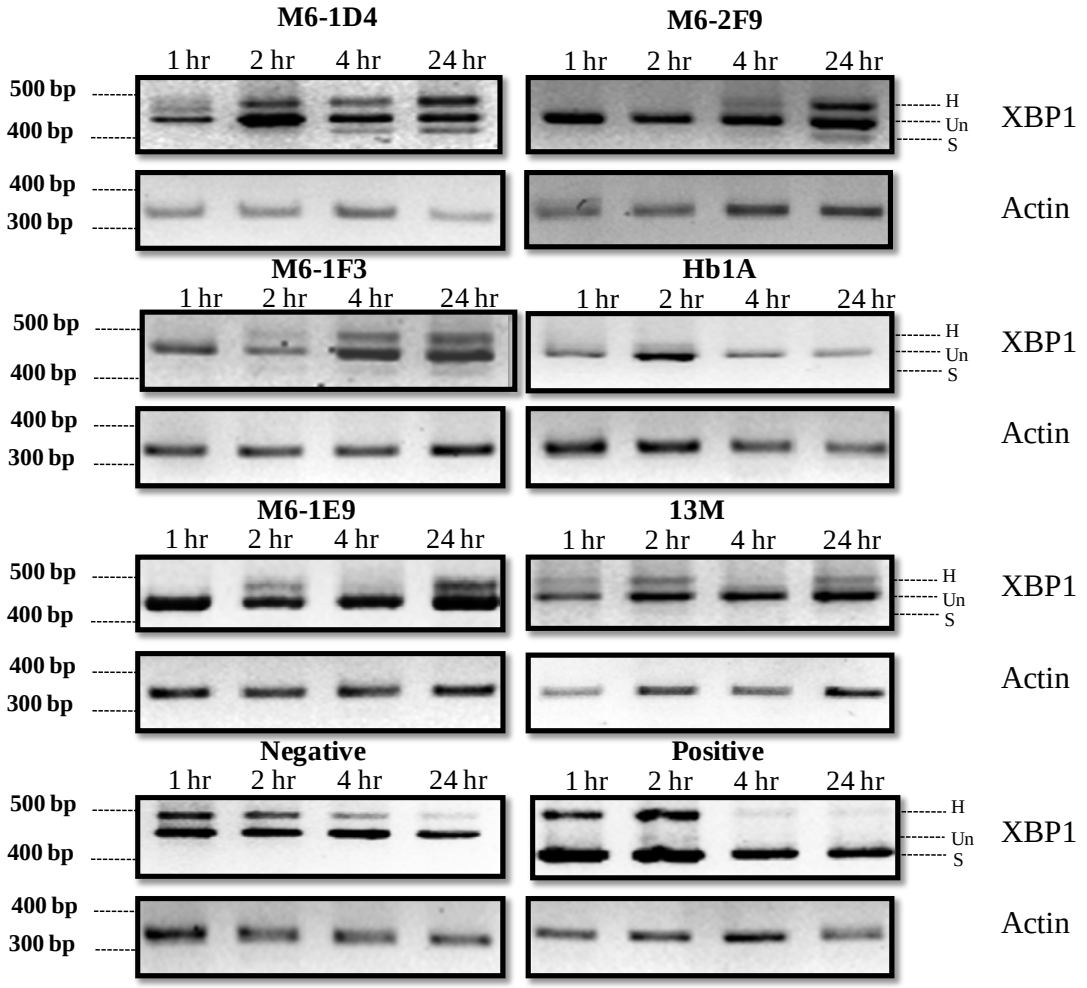


Figure 10 RT-PCR analysis of induction of IRE-1 mediated alternative splicing of XBP1 in HepG2 cell line treated with CD147 mAbs at 1, 2, 4 and 24 hr. (H= heteroduplex formed, Un= unspliced formed, S= spliced formed)

5. The stress-inducible mRNAs analysis by real-time RT-PCR

The XBPsp which is the spliced formed of XBP-1 and other stress-inducible genes downstream are activated including GRP78 and CHOP were selected to analyze. The mRNA expression of those genes was examined by quantitative real time RT-PCR. Figure 11 showed a graphical analysis of relative gene expression of 3 stress-inducible mRNAs normalized against actin expression after performing quantitative RT-PCR.

Thapsigargin treatment was clearly shown as the potent inducer of the transcriptional activation of the UPR (positive control). In response to anti-CD147 mAbs, the expression of XBPsp and GRP78 was up-regulation when treated with M6-1D4, M6-2F9 and M6-1F3 but not with M6-1E9. However, up-regulation of CHOP mRNA expression was not observed with all four anti-CD147 mAbs (Figure 11).

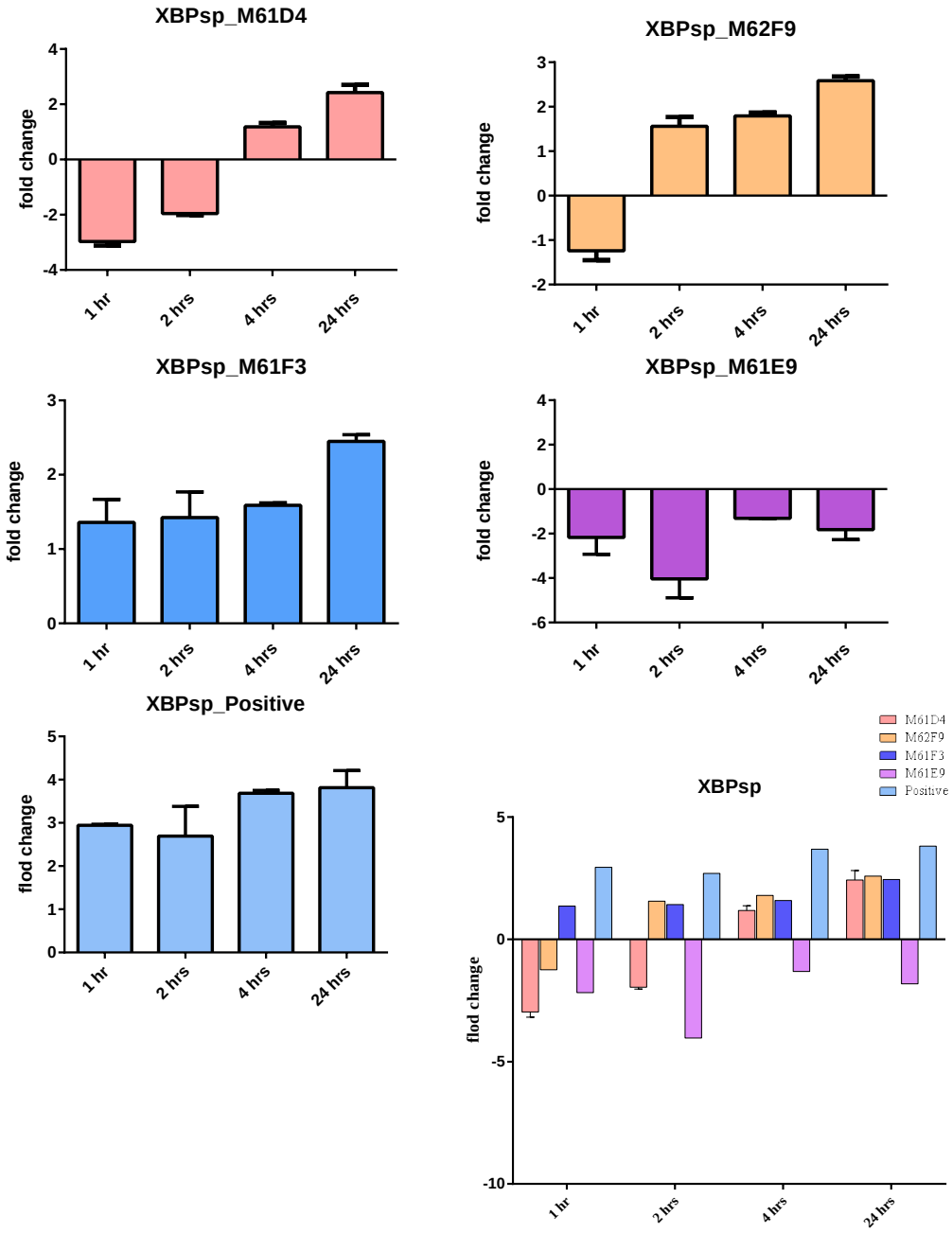


Figure 11 (A) Quantitative real time RT-PCR of transcriptional profiling of XBPsp, GRP78, and CHOP in HepG2 cell line treated with 4 clones of anti-CD147 mAbs. (**A) XBPsp, (B) GRP78 and (C) CHOP.**

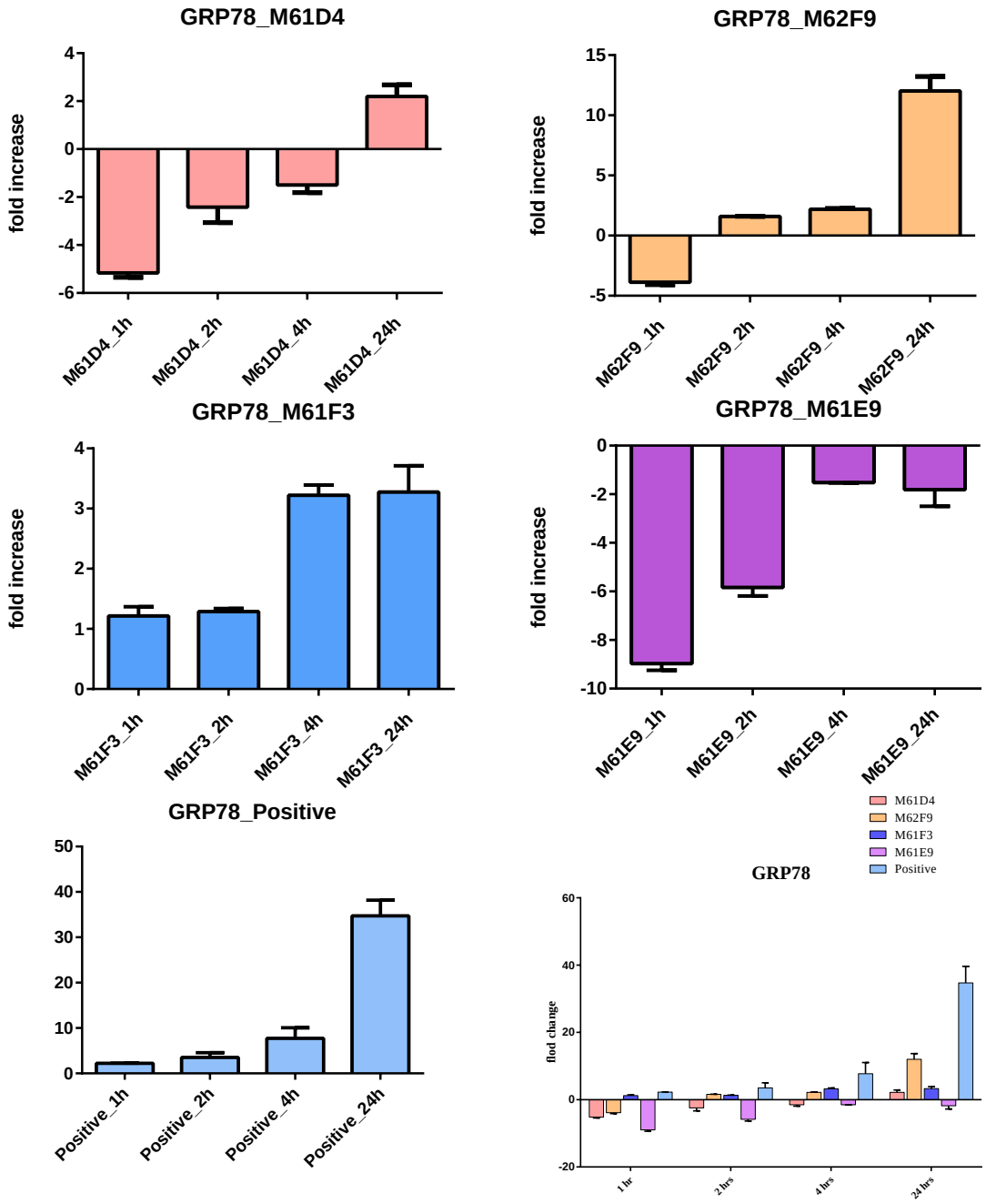

GRP78_M61D4
fold increase
4
2
0
-2
-4
-6
M61D4_1h
M61D4_2h
M61D4_4h
M61D4_24h
GRP78_M62F9
fold increase
15
10
5
0
-5
M62F9_1h
M62F9_2h
M62F9_4h
M62F9_24h
GRP78_M61F3
fold increase
4
3
2
1
0
M61F3_1h
M61F3_2h
M61F3_4h
M61F3_24h
GRP78_M61E9
fold increase
0
-2
-4
-6
-8
-10
M61E9_1h
M61E9_2h
M61E9_4h
M61E9_24h
GRP78_Positive
50
40
30
20
10
0
Positive_1h
Positive_2h
Positive_4h
Positive_24h
M61D4
M62F9
M61F3
M61E9
Positive
GRP78
flod change
60
40
20
0
-20
1 hr
2 hrs
4 hrs
24 hrs

Figure 11 (B)

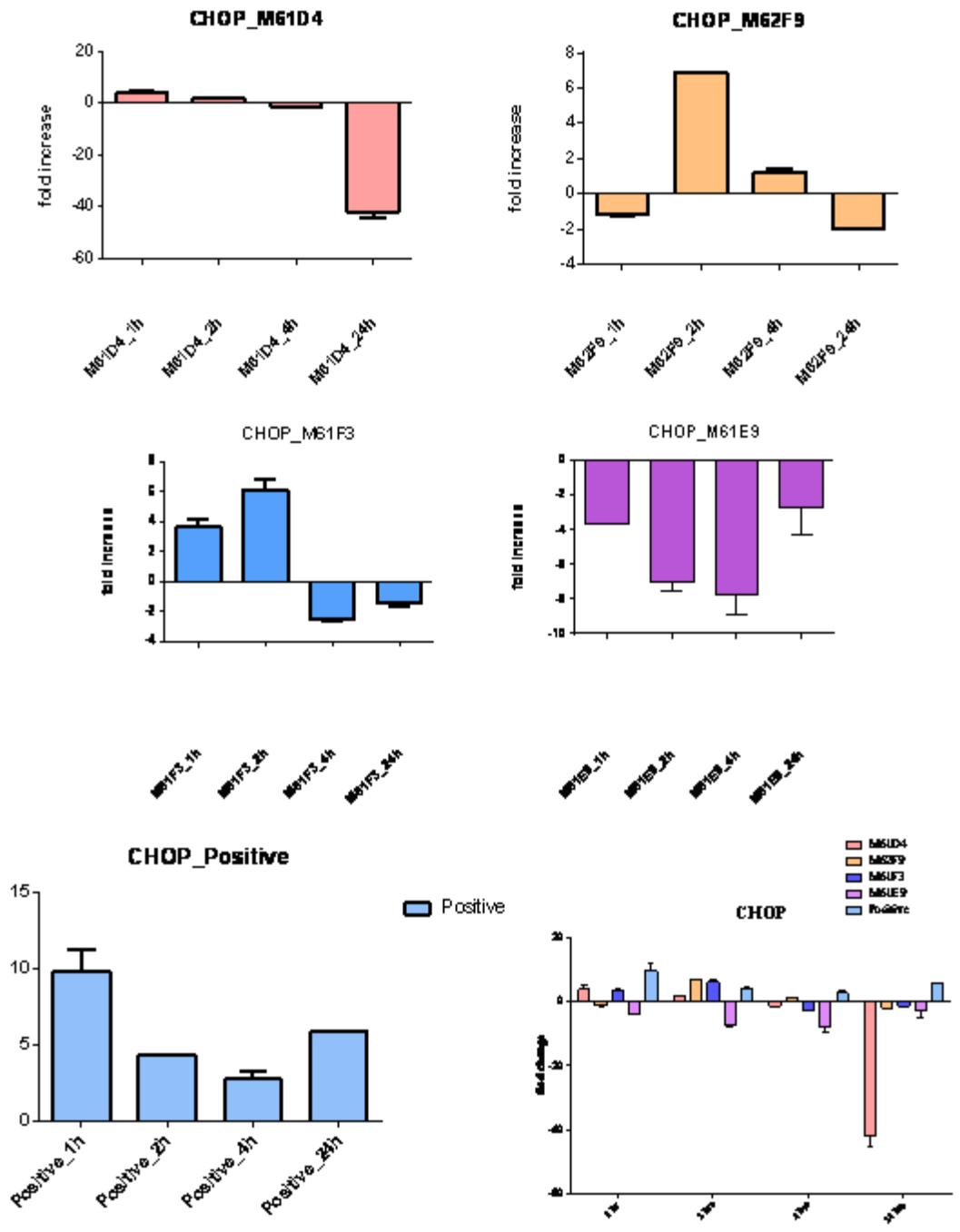

CHOP_M61D4
fold increase
20
0
-20
-40
-60
M61D4_1h
M61D4_2h
M61D4_4h
M61D4_24h
CHOP_M62F9
fold increase
8
6
4
2
0
-2
-4
M62F9_1h
M62F9_2h
M62F9_4h
M62F9_24h
CHOP_M61F3
CHOP_M61E9
CHOP_Positive
Positive
15
10
5
0
Positive_1h
Positive_2h
Positive_4h
Positive_24h
CHOP

Figure 11 (C)

6. Western blot analysis

The increased level of LC3A/B-II expression in HepG2 cell treated with M6-1D4, M6-2F9 and M6-1F3 was observed at 4 and 24 hr as compared with isotype-matched control mAbs whereas no enhanced induction of LC3 protein expression was found with M6-1E9. The induction of LC3 implied the associated of between these anti-C147 mAbs and the accumulation of autophagosome. The activation of apoptotic cell by anti-CD147 mAbs was confirmed by western blot analysis of cleaved caspase-9 and caspase-3 protein expression. The result showed cleaved caspase-9 was detected in HepG2 treated with M6-1D4, M6-2F9 and M6-1F3 at 4 and 24 hr as compared with isotype-matched control mAbs but no with M6-1E9 (figure 12).

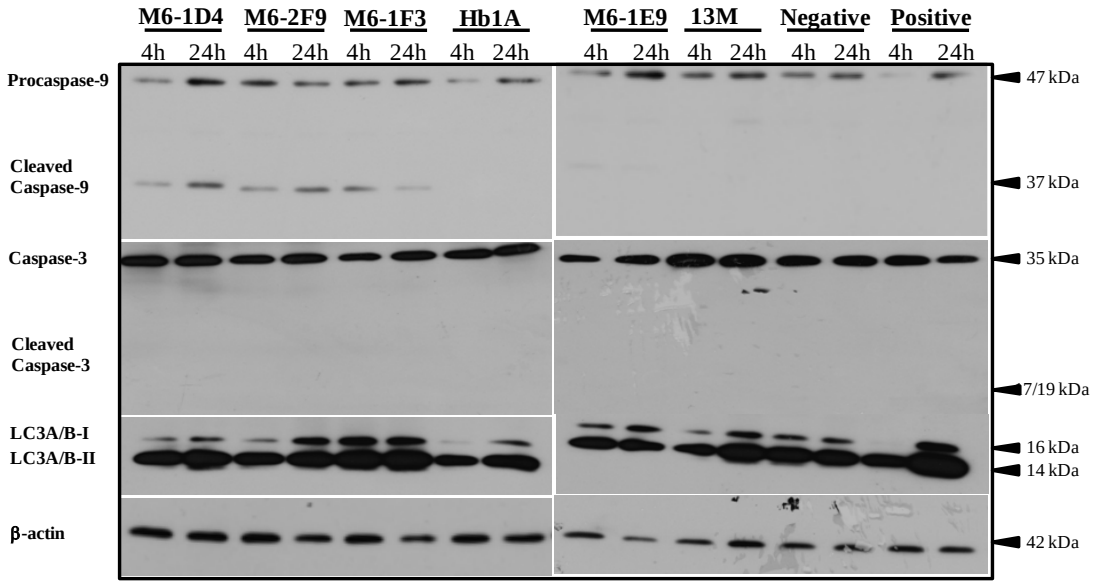


Figure 12 Western blot analysis of stress and apoptotic inducible proteins.

7. Colocalization analysis of UPR interacting proteins in HepG2 cells after anti-CD147 monoclonal antibody treatment.

The UPR response in cells is primarily mediated by the interaction between LC3-LAMP1 (Accumulation of autophagosome), and GRP78 and PERK (the induction of unfolded protein response). Using confocal microscopy, the colocalization of these protein was investigated in all 8 treatment samples consisted of 4 monoclonal antibody treated cell (M6-1D4, M6-2F9, M6-1F3 and M6-1E9), IgG (Hb1A) and IgM (13M) isotype-matched control mAbs, negative and positive (thapsigargin treated) control. The HepG2 cell treated with M6-1D4, M6-2F9, M6-1F3 showed an increased colocalization of LC3 and LAMP1 (yellow color) indicating the accumulation of autophagosome (the

sign of autophagy). Moreover, the stimulation of HepG2 by anti-CD147 mAbs led to the dissociation of GRP78 from PERK which triggered the induction of UPR-responsive proteins. The colocalization of UPR responsive protein from HepG2 cell treated with M6-1D4 mAb compared with isotype-matched control mAbs (Hb1A), negative and positive control were shown in figure 12.

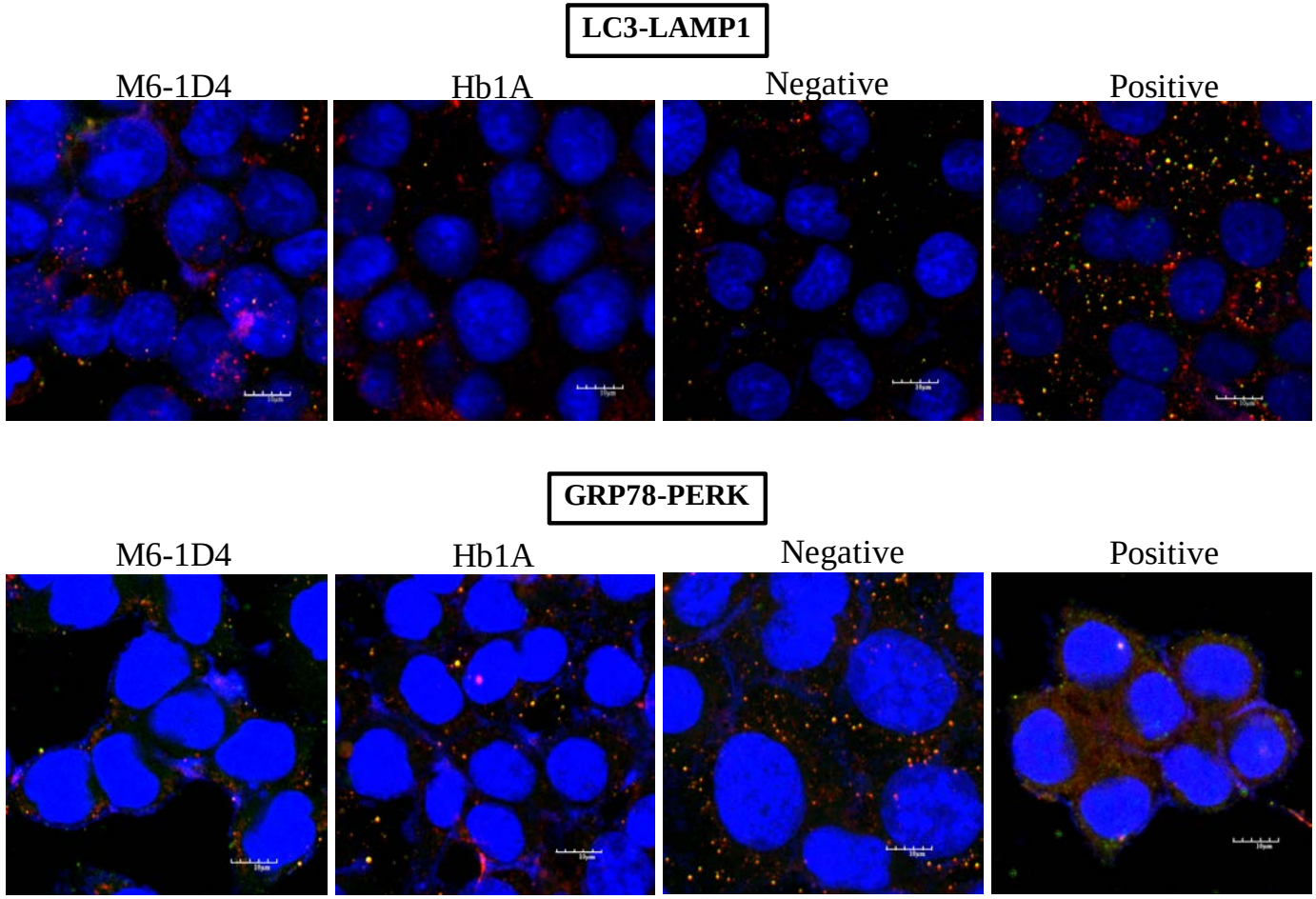


Figure 13 The UPR induction and the accumulation of autophagosone were investigated in HepG2 cells in response to anti-CD147 mAb (M61D4), can be analysed by the interaction between LC3-LAMP1 (Accumulation of autophagosome) (above panel), and GRP78 and PERK (the induction of unfolded protein response) (below panel).

Discussion and conclusion

Four clones of anti-CD147 mAbs which are M6-1D4, M6-1F3, M6-2F9 and M6-1E9 strongly bound to CD147 transmembrane protein on A549 lung cancer cell line but the induction of apoptosis cannot be detected. However, the experiment was further performed in other cell lines and the HepG2 cell line showed promising results with the highest percentage of apoptotic induction after stimulation by anti-CD147 mAbs. The results of flow cytrometry suggested the apoptotic cell induction occurred at the early phase of treatment. The percentage of apoptotic cell population was stable after 24 hr of anti-CD147 mAbs treatment. This might be caused by cell division.

The semi-quantitative RT-PCR analysis of XBP-1 mRNA splicing is comparatively simple methodology and is commonly used to initially examine the UPR pathway in a number of studies with simultaneous amplification of both unpliced and spliced XBP-1 indicating the activation of the UPR (11). The splicing of XBP-1 mRNA was investigated in HepG2 cell treated with each anti-CD147 mAbs comparing with isotype-matched control mAbs control at 1, 2, 4 and 24 hr after treatment. XBP-1 mRNA was clearly spliced in positive control (thapsigargin treated) while no splicing of XBP-1 was observed in negative control. The spliced form of XBP-1 was detected in samples treated with M6-1D4 at 4 and 24 hr, and was slightly expressed with M6-2F9 and M6-1F3 at 24 hr (figure 10). In addition, this was further confirmed by the investigation in other several UPR-responsive mRNA including GRP78, CHOP and XBPsp mRNAs (12) by quantitative real-time PCR analysis. XBPsp mRNA in the sample treated with M6-1D4 mAb was shown to be up-regulated. This was correlated well with the highest induction of XBP-1 mRNA splicing observed in semi-quatitative RT-PCR analysis as described above. Moreover, GRP78 was also shown to be up-regulated in sample treated with M6-1D4, M6-2F9 and M6-1F3 at 24 hr after treatment. However, no significant change was observed in CHOP mRNA expresseion as compared to controls

The autophagosome formation and the activation of apoptotic cell following anti-C147 mAbs stimulation were analyzed by the LC3A/B-II caspase-9 and caspase-3 protein expression using western blot technique (13-15). Again the result shows these proteins were upregulated upon M6-1D4, M6-2F9 and M6-1F3 anti-CD147 mAbs stimulation except for caspase-3.

Colocalization study from immunofluorescence showed the increased colocalization between LC3-LAMP1 (Accumulation of autophagosome) (16) and dissociation of GRP78 from and PERK (the induction of unfolded protein response) (17, 18) of samples treated with M6-1D4, M6-2F9 and M6-1F3 anti-CD147 mAbs.

The results from this study revealed that the anti-CD147 mAb clone M6-1D4 is the most effective monoclonal antibody which can induced the apoptotic cell death of HepG2 (liver hepatocellular cells) and might be useful for liver cancer treatment. However, the other study should be under taken such as normal cell treatment (toxicity test with primary human hepatocyte) and *in vivo* study in animal study before clinical trial can be achieved.

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