CHAPTER 3

RESULTS

3.1 Identification the protein reacted to CM5pAb

3.1.1 Protein purification by immunoprecipitation with CM5pAb

Cell extracts prepared from lung tumor tissues were incubated with CM5pAb immobilized protein G agarose bead for 24 hours. After incubation, the unbound proteins were washed away and the bound protein was eluted and subjected to SDS-PAGE. The gel was stained with a coomassie blue, and protein bands with apparent molecular weight around 95-100 kDa were excised and subjected to protein identification by LC-MS/MS. As shown in Figure 3.1, there are three faint bands appear next to each other around the expected area of gel; therefore, all of the protein bands were individually excised. The excised bands were referred to band A, band B and band C, respectively.

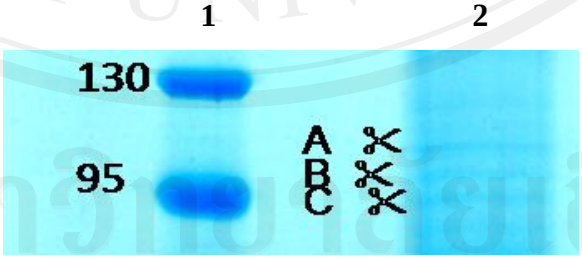


Figure 3.1 Coomassie blue stained gel showing the CM5pAb immunoprecipitated protein bands. The immunoprecipitated proteins were separated through 10% SDS-PAGE and stained with coomassie blue. Marker (lane1), immunoprecipitated proteins (lane 2)

3.1.2 Identification of immunoprecipitated proteins by LC-MS/MS (Liquid chromatography – tandem mass spectrometry)

The excised immunoprecipitated proteins from gel were collected in sterile distilled water before sending for identification as describe in section 2.2.2.2. Using mass spectrum data searched against mammalian protein database, the resulting mass spectra of band A, B, C were identified to belong to several candidate proteins as shown in Table 3.1, Table 3.2 and Table 3.3, repectively. In general, the delta Cn score of greater than 100 represents significant similarity. Taken together of all the matched profile, the potential candidates showing highest delta Cn score were mouse glucose-regulated protein, 78 kDa; GRP-78 (gi1304157, delta Cn score 184.21) and human ER glucosidase II (gi2274968, delta Cn score 174.26).

Table 3.1 LC-MS/MS result of immunoprecipitated protein band A

No.	Reference scan	Sequence	MH+	Charge	Delta Cn Score
1	gi|1304157|dbj|BAA11462.1| 78 kDa glucose-regulated protein [Mus musculus]				184.21
	2498 - 2500	-.ETAEAYLGK.-	982.0699	1	0.18
	2506 - 2508	-.M#KETAEAYLGK.-	1257.4393	2	0.21
	2798 - 2800	-.TWNDPSVQQDIK.-	1431.533	2	0.13
	2938	-.NQLTSNPENTVFDAK.-	1678.7823	2	0.24
	2970	-.TKPYIQVDIGGGQTK.-	1605.8177	2	0.32
	2972 - 2974	-SQIFSTASDNQPTVTIK.-	1838.0093	2	0.28
	2986	-.TKPYIQVDIGGGQTK.-	1605.8177	2	0.26
	3032	-.TWNDPSVQQDIK.-	1431.533	2	0.04
	3096	-.ITPSYVAFTPEGER.-	1567.7247	2	0.12
	3104	-.NQLTSNPENTVFDAK.-	1678.7823	2	0.24
	3108	-.ITPSYVAFTPEGER.-	1567.7247	2	0.07
	3118	-.NQLTSNPENTVFDAK.-	1678.7823	2	0.23
	3180	-.NELESYAYSLK.-	1317.4266	2	0.11
	3192	-.NELESYAYSLK.-	1317.4266	2	0.00
	3322	-.ELEEIVQPIISK.-	1398.6274	2	0.12
	3388	-.TFAPEEISAM#VLTK.-	1553.8029	2	0.13
	3608 - 3610	-.TFAPEEISAM#VLTK.-	1553.8029	2	0.01
	3800	-.TFAPEEISAMVLTK.-	1537.8036	2	0.09
	3806	-.TFAPEEISAMVLTK.-	1537.8036	2	0.24
	3874	-.TFAPEEISAM#VLTK.-	1553.8029	2	0.08
2	gi|2274968|emb|CAA04006.1| Glucosidase II [Homo sapiens]				174.26
	2010 - 2012	-.DPAEGDGAQPEETR.	1569.569	2	0.40
	2654	-.PAAVVLQTK.-	927.1238	2	0.17
	2660	-.PAAVVLQTK.-	927.1238	2	0.26
	2692	-.SIRPGLSPYR.-	1146.3248	2	0.11
	2720	-M#M#DYLQGSGETPQTDVR.-	1961.1214	2	0.45
	2734	-.M#M#DYLQGSGETPQTDVR.-	1961.1214	2	0.42
	2876	-.MM#DYLQGSGETPQTDVR.-	1945.122	2	0.45
	2876	-.M#MDYLQGSGETPQTDVR.-	1945.122	2	0.01
	2894	-.MM#DYLQGSGETPQTDVR.-	1945.122	2	0.00
	2894	-.M#MDYLQGSGETPQTDVR.-	1945.122	2	0.45
	3012	-.MMDYLQGSGETPQTDVR.-	1929.1227	2	0.16
	3018	-.LVAIVDPHIK.-	1105.3557	2	0.17
	3022	-.LVAIVDPHIK.-	1105.3557	2	0.12
	3028	-.MMDYLQGSGETPQTDVR.-	1929.1227	2	0.41
	3146	-.DENSVELTMAEGPYK.-	1683.8192	2	0.48
	3168	-.DENSVELTMAEGPYK.-	1683.8192	2	0.35
	3292	-.SLLLSVNAR.-	973.1526	2	0.20
	3314	-.SLLLSVNAR.-	973.1526	2	0.08

Table 3.1 (continue)

No.	Reference scan	Sequence	MH+	Charge	Delta Cn Score
3	gi|224970|prf||1205208A heat shock protein hsp70				74.23
	2096 - 2098	-.VEIIANDQGNR.-	1229.3258	2	0.00
	2326	-.VEIIANDQGNR.-	1229.3258	2	0.00
	2330	-.VEIIANDQGNR.-	1229.3258	2	0.11
	3372	-.IINEPTAAAIAYGLDK.-	1660.8933	2	0.00
	3538	-.FEELNM#DLFR.-	1330.492	2	0.28
	3550	-.FEELNM#DLFR.-	1330.492	2	0.20
	3580	-.IINEPTAAAIAYGLDK.-	1660.8933	2	0.00
	3590	-.IINEPTAAAIAYGLDK.-	1660.8933	2	0.00
4	gi|229552|prf||754920A albumin				60.19
	2902 - 2904	-.HLVDEPQNLIK.-	1306.4934	2	0.09
	2966	-.KVPQVSTPTLVEVSR.-	1640.9067	2	0.27
	2992	-.KVPQVSTPTLVEVSR.-	1640.9067	2	0.16
	3030	-.YLYEIAR.-	928.0671	2	0.00
	3128	-.VPQVSTPTLVEVSR.-	1512.7338	2	0.21
	3198	-.VPQVSTPTLVEVSR.-	1512.7338	2	0.26
5	gi|2392283|pdb|1DKG|D Chain D, Crystal Structure Of The Nucleotide Exchange Factor Grpe Bound To Th				28.23
	3372	-.IINEPTAAALAYGLDK.-	1660.8933	2	0.00
	3580	-.IINEPTAAALAYGLDK.-	1660.8933	2	0.00
	3590	-.IINEPTAAALAYGLDK.-	1660.8933	2	0.00

Table 3.2 LC-MS/MS result of immunoprecipitated protein band B

No.	Reference scan	Sequence	MH+	Charge	Delta Cn Score
1	gi|223170|prf||0602239A fibrinogen gamma				110.24
	2774	-.YLQEIYNSNNQK.-	1514.6219	2	0.05
	2778	-.YLQEIYNSNNQK.-	1514.6219	2	0.14
	2788	-.DTVQIHDITGK.-	1227.3499	2	0.31
	2796	-.DTVQIHDITGK.-	1227.3499	2	0.34
	2926	-.YEASILTHDSSIR.-	1492.6163	2	0.35
	2934	-.YEASILTHDSSIR.-	1492.6163	2	0.26
	3088	-.VELEDWNGR.-	1118.182	2	0.09
	3104	-.VELEDWNGR.-	1118.182	2	0.16
	3130	-.TSTADYAMFK.-	1135.2727	2	0.32
	3142	-.TSTADYAMFK.-	1135.2727	2	0.31
	3412	-.QSGLYFIKPLK.-	1294.5673	2	0.30
2	gi|2274968|emb|CAA04006.1| Glucosidase II [Homo sapiens]				108.17
	2990	-.M#MDYLQGSGETPQTDVR.-	1945.122	2	0.00
	2990	-.MM#DYLQGSGETPQTDVR.-	1945.122	2	0.35
	3120	-.MMDYLQGSGETPQTDVR.-	1929.1227	2	0.10
	3134	-.MMDYLQGSGETPQTDVR.-	1929.1227	2	0.07
	3144	-.VVIIGAGKPAAVVLQTK.-	1665.0577	2	0.40
	3150	-.AEKDEPGAWEETFK.-	1637.7288	2	0.24
	3156	-.AEKDEPGAWEETFK.-	1637.7288	2	0.26
	3174	-.VVIIGAGKPAAVVLQTK.-	1665.0577	2	0.29
	3274	-.FRIDELEPR.-	1175.3198	2	0.18
	3276	-.DENSVELTMAEGPYK.-	1683.8192	2	0.27
	4072	-.VLLVLELQGLQK.-	1353.6763	2	0.08
3	gi|1304157|dbj|BAA11462.1| 78 kDa glucose-regulated protein [Mus musculus]				50.17
	3184 - 3186				
	3350	-.VTHAVVTVPAYFNDAQR.-	1889.1044	2	0.28
	3372	-.IINEPTAAAIAYGLDKR.-	1817.0796	2	0.05
	3948	-.IINEPTAAAIAYGLDKR.-	1817.0796	2	0.02
	3966	-.TFAPEEISAMVLTK.-	1537.8036	2	0.13
		-.TFAPEEISAMVLTK.-	1537.8036	2	0.12
4	gi|226787|prf||1605217A Ig gamma1				36.24
	2760				
	2764	-.ALPAPIEK.-	839.015	1	0.10
	3262	-.ALPAPIEK.-	839.015	1	0.09
	3268	-.TPEVTCVVVDVSHEDPEVK.-	2140.3279	2	0.07
		-.TPEVTCVVVDVSHEDPEVK.-	2140.3279	2	0.05

Table 3.2 (continue)

No.	Reference scan	Sequence	MH+	Charge	Delta Cn Score
5	gi|30581038|sp|P23142|FBLN1_HUMAN Fibulin-1 precursor				30.26
	2598	-.YM#DGM#TVGVVR.-	1260.4667	2	0.13
	3166	-.IIEVEEEQEDPYLNDR.-	1992.087	2	0.38
	3194	-.IIEVEEEQEDPYLNDR.-	1992.087	2	0.41
6	gi|229601|prf||765044A Ig G1 H Nie				30.18
	2940	-.EPQVYTLPPSRDELTK.-	1874.0848	2	0.26
	2968	-.EPQVYTLPPSRDELTK.-	1874.0848	2	0.22
	3238 - 3240	-.EPQVYTLPPSRDELTK.-	1874.0848	2	0.03
7	gi|229552|prf||754920A albumin				28.17
	2752	-.FKDLGEEHFK.-	1250.3851	2	0.18
	3046	-.KVPQVSTPTLVEVSR.-	1640.9067	2	0.32
	3054	-.KVPQVSTPTLVEVSR.-	1640.9067	2	0.34

Table 3.3 LC-MS/MS result of immunoprecipitated protein band C

No.	Reference scan	Sequence	MH+	Charge	Delta Cn Score
1	gi|226787|prf||1605217A Ig gamma1				104.25
	2792				
	2806	-.ALPAPIEK.-	839.015	1	0.04
	2864	-.ALPAPIEK.-	839.015	1	0.11
	3294 - 3296	-.EPQVYTLPPSR.-	1287.4469	2	0.10
	3330	-.TPEVTCVVVDVSHEDPEVK	2140.3279	2	0.04
	3332	-.FNWYVDGVEVHNAK.-	1678.829	2	0.03
	3348	-.NQVSLTCLVK.-	1162.356	2	0.14
	3710	-.FNWYVDGVEVHNAK.-	1678.829	2	0.08
	3716	-.TTPPVLDSDGSFFLYSK.-	1875.068	2	0.35
	4122	-.TTPPVLDSDGSFFLYSK.-	1875.068	2	0.25
	4130	-.VVSVLTVLHQDWLNGK.-	1809.1023	2	0.08
		-.VVSVLTVLHQDWLNGK.-	1809.1023	2	0.04
2	gi|12082134|dbj|BAB20776.1| heat shock protein 90 beta [Equus caballus]				78.23
	2974	-.ELISNASDALDK.-	1276.3761	2	0.00
	2978	-.ELISNASDALDK.-	1276.3761	2	0.00
	3048	-.SIYYITGESK.-	1161.2866	2	0.07
	3194	-.NPDDITQEEYGEFYK.-	1848.9011	2	0.22
	3204	-.NPDDITQEEYGEFYK.-	1848.9011	2	0.20
	3366	-.HFSVEGQLEFR.-	1349.4768	2	0.10
	3372	-.ADLINNLGTIAK.-	1243.4355	2	0.09
	3386	-.ADLINNLGTIAK.-	1243.4355	2	0.12
3	gi|224053|prf||1009174A macroglobulin alpha2				60.19
	3232	-.NEDSLVFVQTDK.-	1395.4974	2	0.30
	3614	-.IAQWQSFQLEGGLK.-	1605.8193	2	0.17
	3636	-.IAQWQSFQLEGGLK.-	1605.8193	2	0.35
	3852	-.VSVQLEASPAFLAVPVEK.-	1885.1938	2	0.15
	3954	-.LLIYAVLPTGDVIGDSAK.-	1846.1576	2	0.12
	3966	-.LLIYAVLPTGDVIGDSAK.-	1846.1576	2	0.22
4	gi|6137530|pdb|1D0N|B Chain B, The Crystal Structure Of Calcium-Free Equine Plasma Gelsolin.gi|613				58.24
	2356 - 2358	-.DSQDEEKTEALTSAK.-	1652.6969	2	0.13
	2918	-.TGAQELLR.-	888.0045	2	0.00
	3338	-.AGALNSNDAFVLK.-	1320.4769	2	0.17
	3408	-.AGALNSNDAFVLK.-	1320.4769	2	0.25
	3498	-.TPSAAYLWVGAGASEAEK.-	1808.9697	2	0.19
	3506	-.TPSAAYLWVGAGASEAEK.-	1808.9697	2	0.39

Table 3.3 (continue)

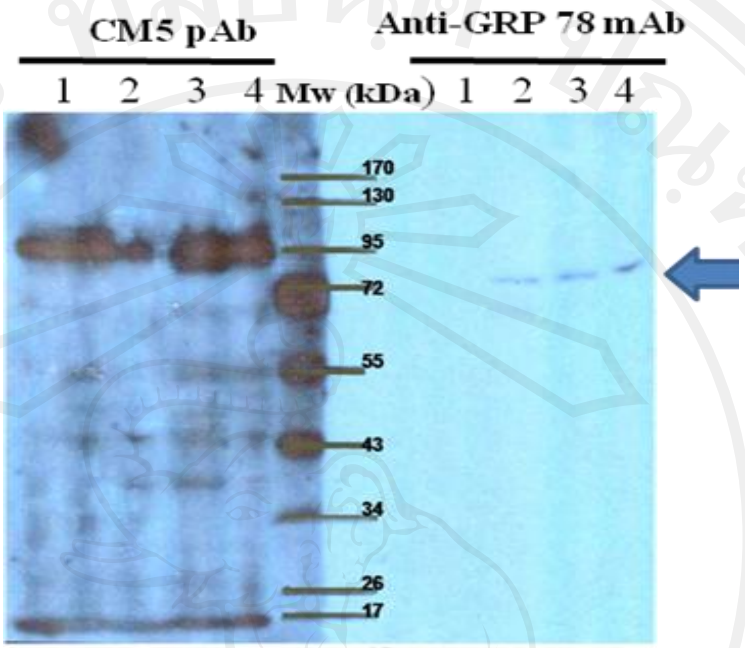
No.	Reference scan	Sequence	MH+	Charge	Delta Cn Score
5	gi|2136983|pir||I46732 Ig gamma heavy chain constant region - rabbit (fragment)gi|165110|gb|AAA312				50.19
	3114	-.LSVPTSEWQR.-	1203.33	2	0.23
	3134	-.LSVPTSEWQR.-	1203.33	2	0.19
	3516	-.TTPAVLDSDGSYFLYSK.-	1865.03	2	0.40
	3530	-.TTPAVLDSDGSYFLYSK.-	1865.03	2	0.43
	3532 - 3534	-.VVSTLPIAHQDWLR.-	1635.8918	2	0.31
6	gi|229908|pdb|1FC2|D Chain D, Immunoglobulin Fc And Fragment B Of Protein A Complexgi|229906|pdb|1				42.22
	3294 - 3296	-.TPEVTCVVVDVSHEDPQVK	2139.343	2	0.46
	3330	-.FNWYVDGVQVHNAK.-	1677.8441	2	0.43
	3348	-.FNWYVDGVQVHNAK.-	1677.8441	2	0.41
	4122	-.VVSVLTVLHQNWLDGK.-	1809.1023	2	0.01
	4130	-.VVSVLTVLHQNWLDGK.-	1809.1023	2	0.02
7	gi|227782|prf||1710352A heat shock protein 83				40.18
	2850	-.ELISNSSDALDK.-	1292.3755	2	0.00
	2868	-.ELISNSSDALDK.-	1292.3755	2	0.12
	3280	-.SLTNDWEDHLAVK.-	1528.6487	2	0.07
	3286	-.SLTNDWEDHLAVK.-	1528.6487	2	0.07
8	gi|223170|prf||0602239A fibrinogen gamma				
	2810	-.YLQEIYNSNNQK.-	1514.6219	2	38.16
	2816	-.YLQEIYNSNNQK.-	1514.6219	2	0.10
	2970	-.YEASILTHDSSIR.-	1492.6163	2	0.11
	2980	-.YEASILTHDSSIR.-	1492.6163	2	0.29
					0.45

3.1.3 Verification and confirmation of the identification result from LC-MS/MS

From the results of protein identification in section 3.1.2, two proteins showed significant delta Cn score thus represent a candidate protein of interest. In order to rule one out, western blot analysis was performed to compare their molecular weight with the CM5pAb reactive protein. Whole cell lysate from various lung tumor tissues known to overexpressed the CM5pAb-reactive protein was resolved through 10% polyacrylamide gel and separated proteins were transfered onto PVDF membrane. The blots were cut in-half and immunodetected with either CM5pAb and anti-GRP78 or anti-glucosidase II. As shown in Figure 3.2, the protein was recognized by CM5pAb has an apparent molecular weight similar to glucosidase II but not GRP-78.

Therefore, immunoprecipitation was performed using anti-glucosidase II mAb and the immunoprecipitated glucosidase II was resolved through SDS-PAGE and subjected to immunodetection with CM5pAb. As shown in Figure 3.3, the immunoprecipitated protein was recognizable by anti-glucosidase II mAb thus indicating the successful of immunoprecipitation process. Interestingly, it was found that the immunoprecipitated glucosidase II was also recognized by CM5pAb (Fig. 3.3) at the same position, thus suggesting that the CM5-reactive protein was in fact the glucosidase II.

(a)



(b)

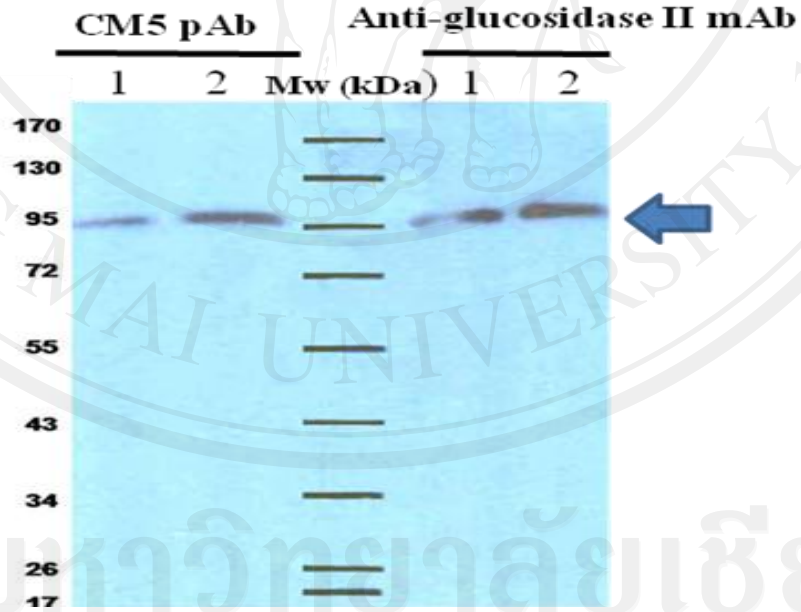


Figure 3. 2 Comparison of protein molecular weight of the CM5 reactive protein with GRP78 (A) and Glucosidase II (B); MW, protein molecular weight marker; number represents sample number

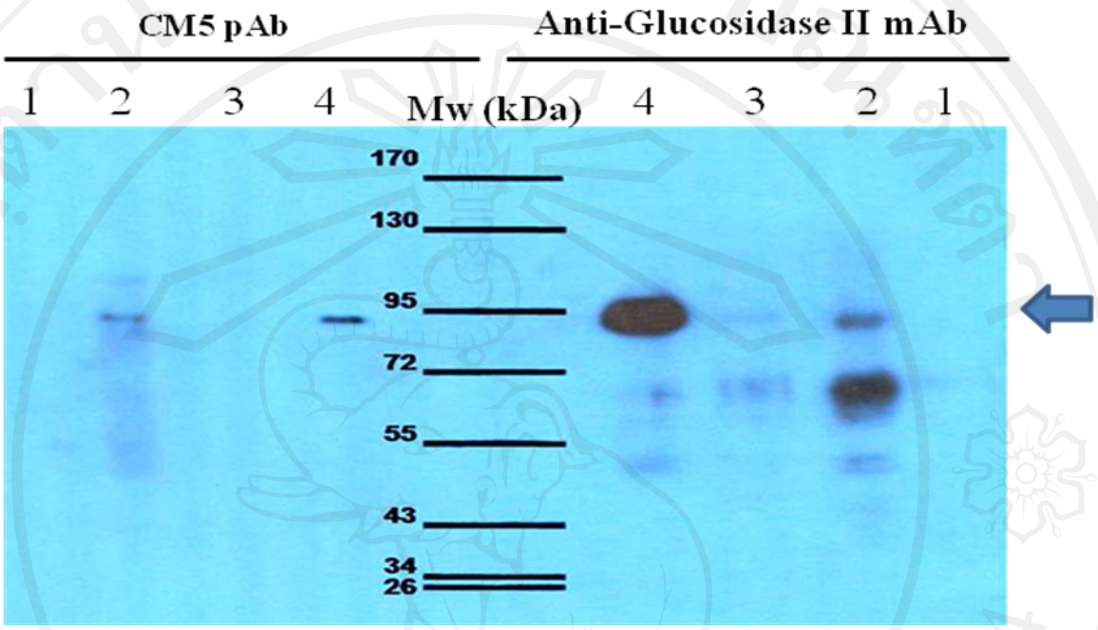


Figure 3.3 Western blot analysis showing the reactivity of the immunoprecipitated glucosidase II to CM5pAb.

Cell lysate from tumor tissues was diluted with PBS to obtain total protein concentration of 1 mg/ml and immunoprecipitated with antibody against glucosidase II. The imunoprecipitated protein was resolved through SDS-PAGE, transferred onto PVDF membrane and followed by immunodetected with CM5pAb and anti-glucosidase II mAb. Mw, molecular weight marker; lane 1, diluted tumor cell lysate before immunoprecipitation; lane 2, immunoprecipitated protein; lane 3, diluted cell lysate after immunoprecipitation; lane 4, crude cell lysate from tumor tissue (30µg of total protein)

3.2 Expression pattern of CM5pAb reactive protein and glucosidaseII in human lung tumor tissues

Paired sample of lung tumor tissues and accompanying normal tissues were obtained from each surgical specimen of the same patient. Cell lysate was prepared and separated according to their molecular weight through a 7% SDS-PAGE and subjected to western blot analysis. The representatives of Western blot results are shown in Figure 3.4. It was found that the result of Western blot analysis showed the exact same expression pattern of proteins recognized by CM5pAb and anti-glucosidase II mAb in each lung tumor tissues tested.

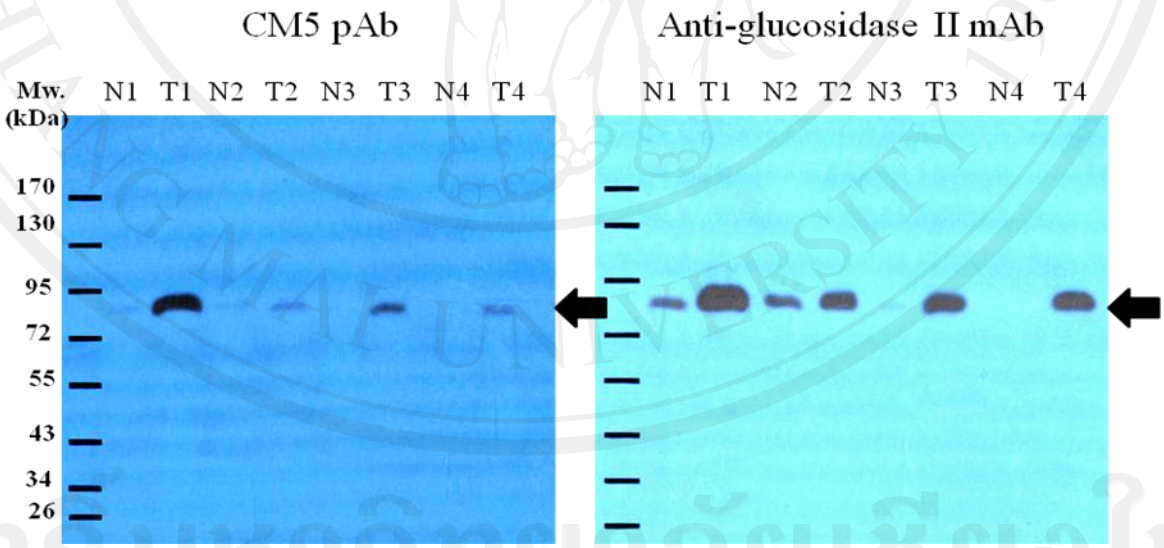


Figure 3.4 Western blotting results showing expression similarity of CM5 reactive protein and glucosidase II in lung tumor and normal corresponding tissues. T, tumor tissue; N, normal tissue

Expression of glucosidaes II in relation to clinicopathologic feathers of lung tumor tissues are summarized in Table 3.4. Of 37 lung tumor tissues, 35 showed an increase expression level of glucosidase II in tumor tissues in comparison to the normal corresponding tissues. As almost all of the tumor tissues showed overexpression of glucosidase II, no significant relationship between overexpression of glucosidase II and clinicopathologic features was seen.

Table 3.4 Expression of glucosidaseII in relation to clinicopathologic feathers of tumor tissues. ($p < 0.05$ was considered significant)

Pathological features (cases)	**GlucosidaseII overexpression (no.cases)**		**p value***
	Yes (%)	**NO (%)**	
Total (37 cases)	35 (94.6%)	2 (5.4%)	
Type of NSCLC			
Adenocarcinoma (25 cases)	24 (96%)	1 (4%)	1.00
Squamous cell carcinoma (12 cases)	11 (91.6%)	1 (8.3%)	
Age (years)			
< 60 (19 cases)	17 (89.5%)	2 (11.8%)	0.486
≥ 60 (18 cases)	18 (100%)	0 (0%)	
Gender			
Female (14 cases)	14 (100%)	0 (0%)	0.517
Male (23 cases)	21 (91.3%)	2 (8.7%)	
Tumor stage			
Early (25 cases)	25 (100%)	0 (0%)	0.099
Late (12 cases)	10 (83.3%)	2 (16.7%)	
Smoking			
Yes (28 cases)	26 (92.9%)	2 (7.1%)	1.00
No (9 cases)	9 (100%)	0 (0%)	

*Chi-square test

3.3 Characterization the CM5pAb reactive protein (glucosidase II) in response to stress signals compared with p53Protein expression when response to stress in cell culture model

3.3.1. Expression and cellular localization of p53 and glucosidase II in response to UV irradiation

Characterization of p53 and glucosidase II in response to stress signals was carried out utilizing a human lung adenocarcinoma epithelial cell line (A549) as a cell model. In order to induce DNA damage and genotoxic stress, cultured cells were irradiated with Ultraviolet (UV) 287 nm ray. To achieve optimal dose of UV irradiation, A549 cells were irradiated with various doses of UV (0, 8, 16, 32, 48, 64 J/m^2) and whole cell lysate was prepared at 3 hours after irradiation to determine protein expression levels of glucosidase II and p53. Morphologies of the irradiated cells and non-treated control cells before harvesting are shown in Figure 3.5. Non-irradiated A549 was in spindle-shaped morphology and it appeared that cells irradiated at dose 8 J/m^2 showed no morphology change in comparison to non-treated cells. However, cells irradiated with higher dose of UV became rounded and smaller in size. Expression levels of glucosidase II and p53 determined by western blot analysis are shown in Figure 3.6. The result showed that there was an increase in protein level of glucosidase II and p53 in response to UV irradiation. From this optimization result, UV irradiation at dose 8 J/m^2 was chosen for further experiment because it induces the expected response of p53 as previously described[33, 35] and with minimal cell morphology alteration.

Cell lysate was prepared from irradiated cells at different time points (3, 6, 24, 36 hours) and subjected to Western blot analysis in order to determine protein expression levels of glucisidase II, p53 and GRP (glucose regulated protein)-78, GRP-94, key regulators of the ER stress response. Blots were also probed for GAPDH to check for equal loading of protein. As shown in Figure 3.7, protein levels of both p53 and glucosidase II were increased in UV-irradiated cells. The induction appeared as quickly as 3 hours after treatment and continued to increase until after 24 hours. The induction was observed at 24 hours for both p53 and glucosidase II. The results showed that UV irradiation did not affect expression level of GRP-78 and GRP-94. An investigation of sub-cellular localization of these two proteins showed that p53 was translocalized to the nucleus in response to UV irradiation, whereas glucosidase II remained in the cytoplasm (Figure 3.8). Interestingly, it appeared that there were smaller bands of protein recognized by anti-glucosidase II mAb whose nuclear level was increased in response to UV irradiation.

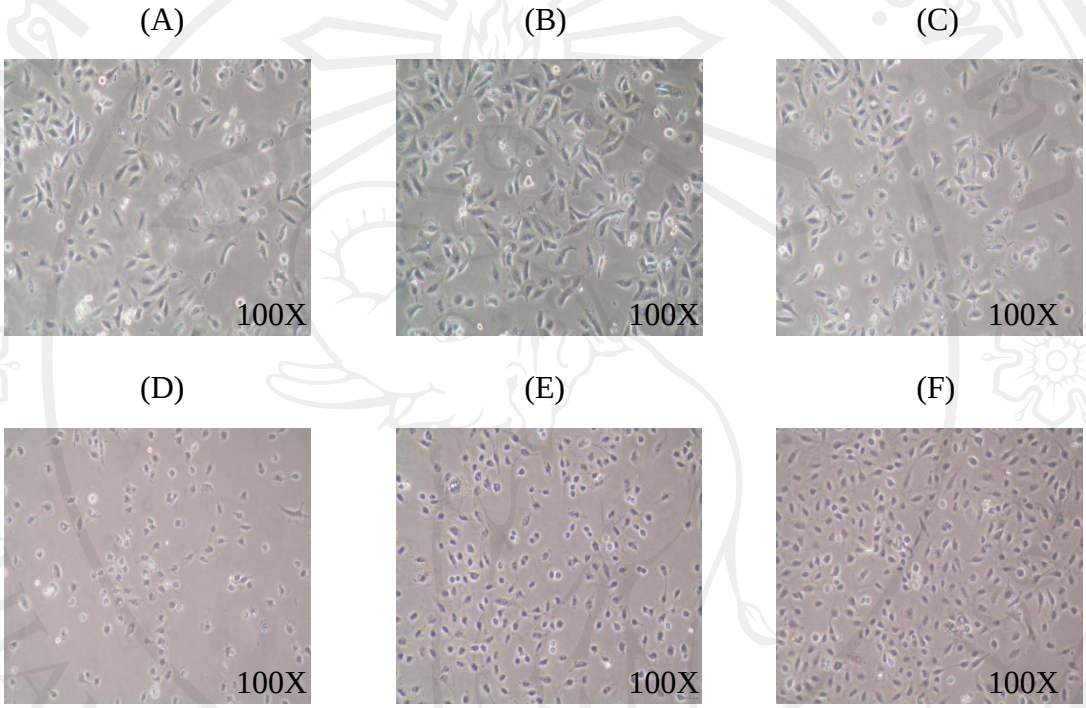
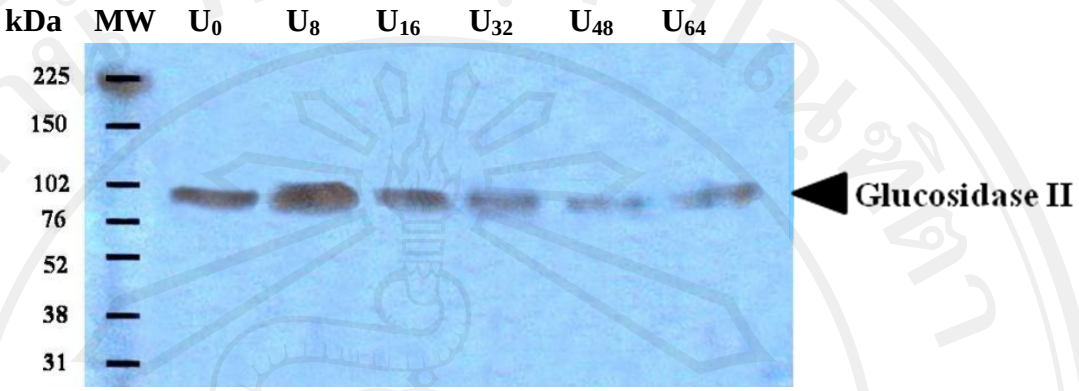


Figure 3.5 A549 cell morphology after UV treatment. A549 cells were observed after UV treatment, to optimize the exposure time by UV irradiation with various UV dose at (A) non-exposed, (B) 8 J/m^2, (C) 16 J/m^2, (D) 32 J/m^2, (E) 48 J/m^2 and (F) 64 J/m^2.

(a)



(b)

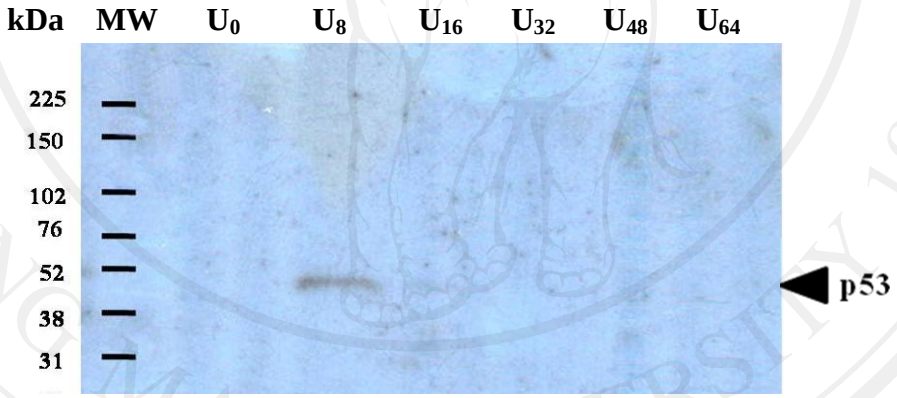
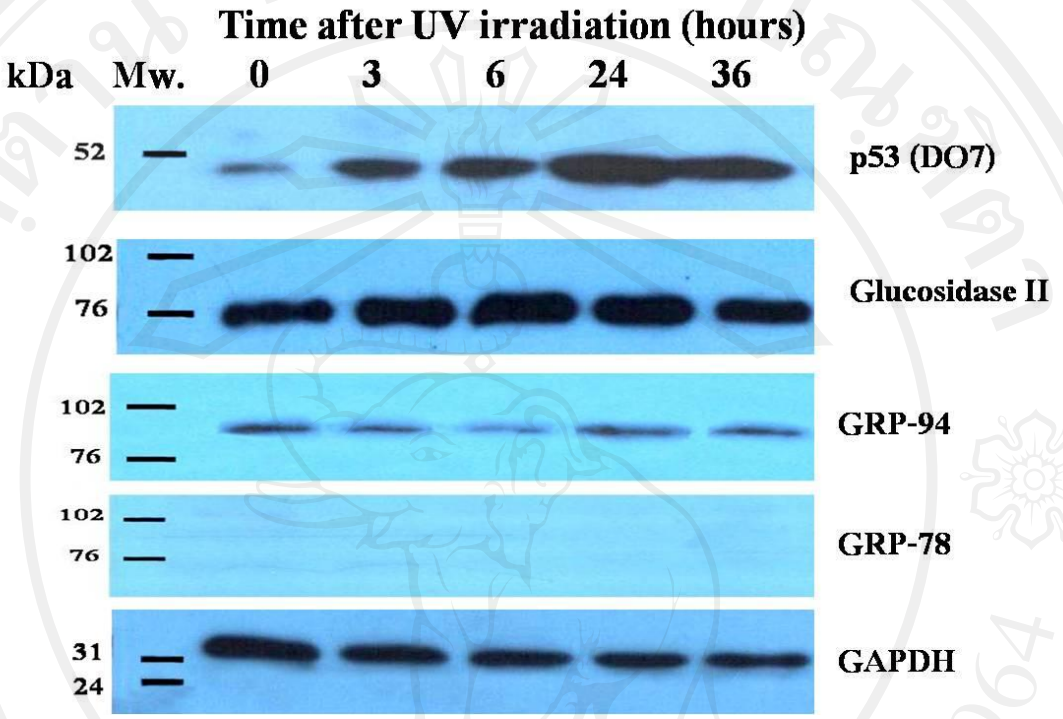


Figure 3.6 Western blot showing protein levels of p53 and glucosidase II after irradiated with various dose of UV. Glucosidase II (a) and p53 (b) protein levels were determined at 3 hours after UV treatment, (U_0) non-exposed, (U_8) 8 J/m^2, (U_{16}) 16 J/ m^2, (U_{32}) 32 J/m^2, (U_{48}) 48 J/m^2 and (U_{64}) 64 J/m^2.

(a)



(b)

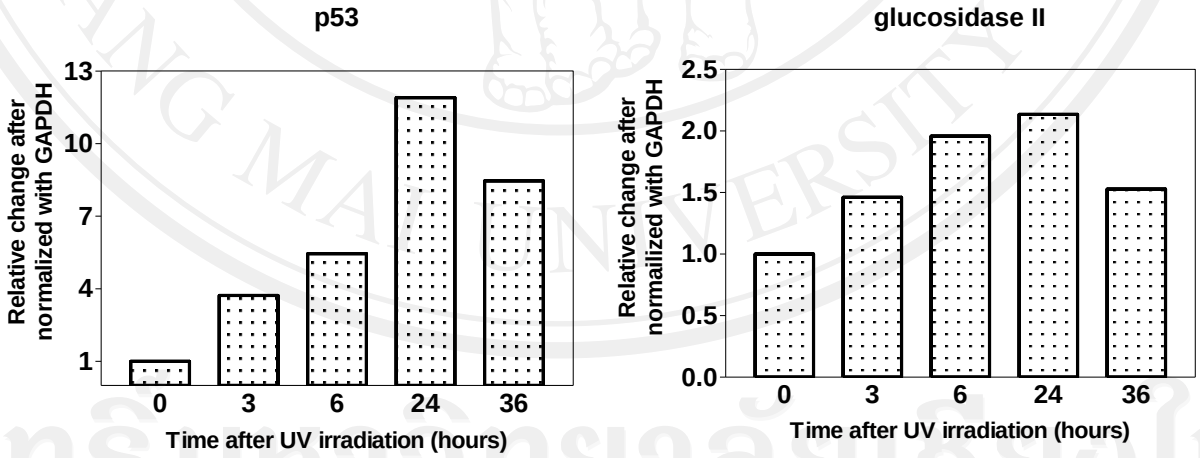


Figure 3.7 Western blot analysis (a) and quantification value (b) showing changes of various protein levels in A549 lung adenocarcinoma cells at various time points following UV irradiation. Western blot bands were quantified using Quantity One (Bio-Rad Laboratories). The quantification values of each band were normalized to corresponding values of GAPDH. The normalized values for each time points were then compared to the corresponding band of the untreated sample (0 hours).

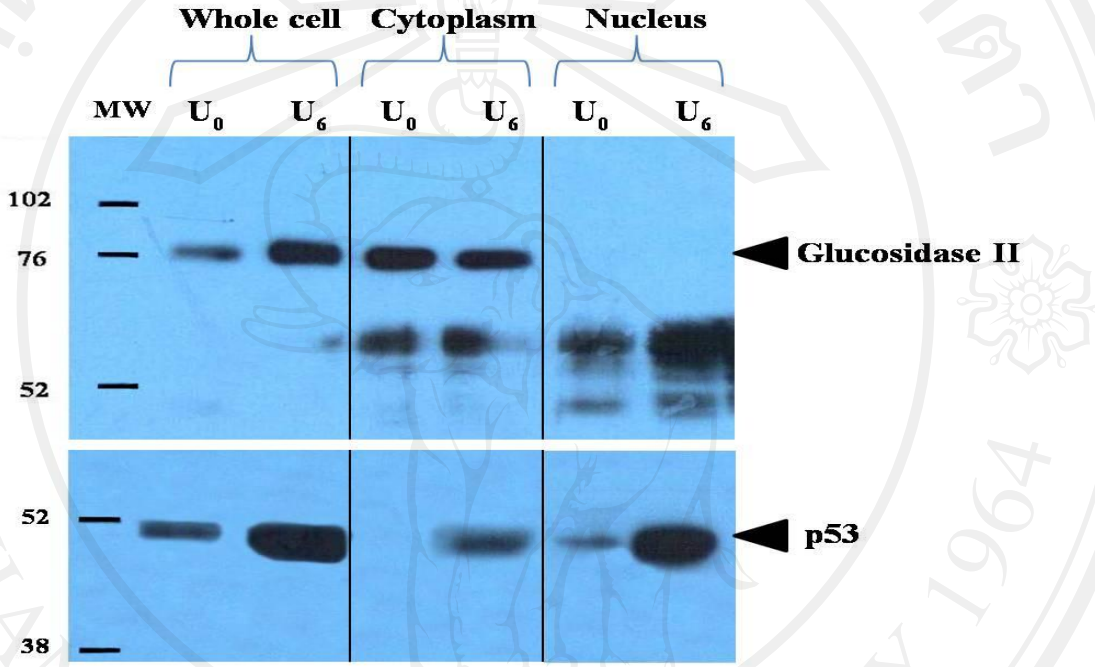


Figure 3.8 Western blot analysis showing sub-cellular localization of p53 and glucosidase II in response to UV irradiation

Cell lysate from cytosolic and nuclear fraction was prepared from UV irradiated cells at 0 (Uo) and 6 hours (U6) after UV treatment

3.3.2 Expression and cellular localization of p53 and glucosidase II in response to tunicamycin-induced ER stress

In order to induce ER stress, cultured cells were treated with tunicamycin. To get optimal dose, A549 cells were cultured with various doses of tunicamycin (0, 1, 3, 5 µg/ml) and whole cell lysate was prepared at 3 hours after treatment to determine protein expression levels of glucosidase II and p53. Morphologies of the tunicamycin-treated cells and vehicle control cells before harvesting are shown in Figure 3.9. No significant morphology alteration was observed in all tested doses. Expression levels of glucosidase II and p53 were determined by western blot analysis are shown in Figure 3.10. The result showed that there was a reduction of protein level of glucosidase II and p53 in response to tunicamycin-induced ER stress. From this optimization result, tunicamycin at dose 3 µg/ml was chosen for further experiment because it induces the prominent expected response of p53 as previously described[45] with minimal toxicity. Levels of both p53 and glucosidase II was found to be suppressed in tunicamycin-treated cells with maximum reduction appeared at 6 hours after treatment for both p53 and glucosidase II (Figure 3.11). The sub-cellular localization study showed the disappearance of both p53 and glucosidase II in response to tunicamycin-induced ER stress (Figure 3.12). Interestingly, although protein level of glucosidase II was decreased and disappeared from the cytoplasm in response to tunicamycin-induced ER stress, the smaller of protein bands recognized by anti-glycosidase II mAb was increased in the nucleus.

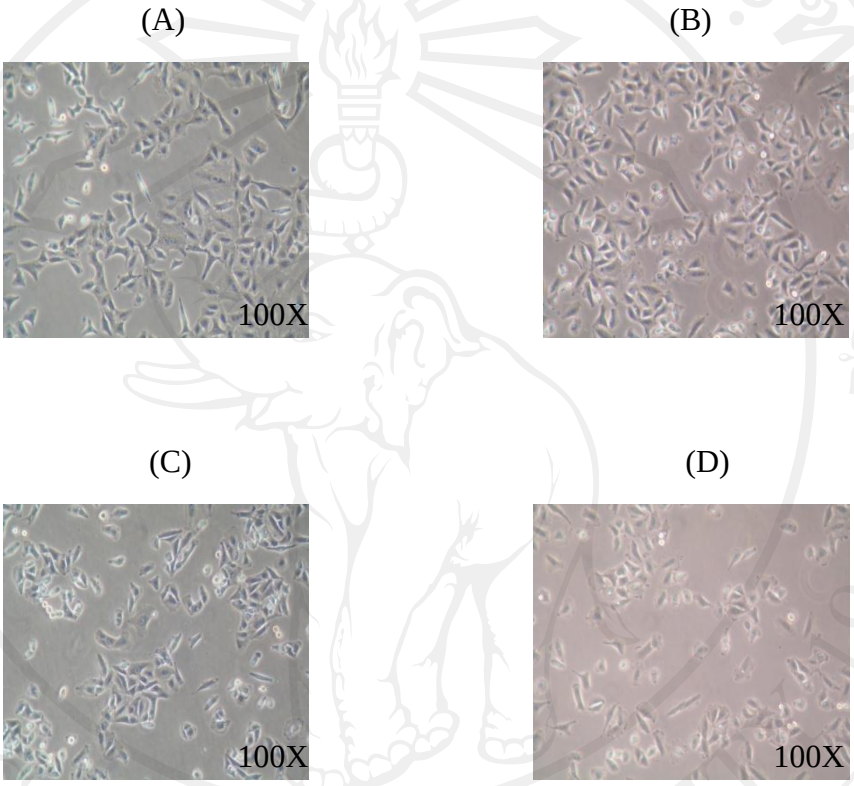


Figure 3.9 A549 cell morphology after tunicamycin treatment. A549 cells were observed after tunicamycin treatment for 3 hours with (A) 0.1% DMSO, (B) 1 µg/ml tunicamycin, (C) 3 µg/ml tunicamycin, and (D) 5 µg/ml tunicamycin.

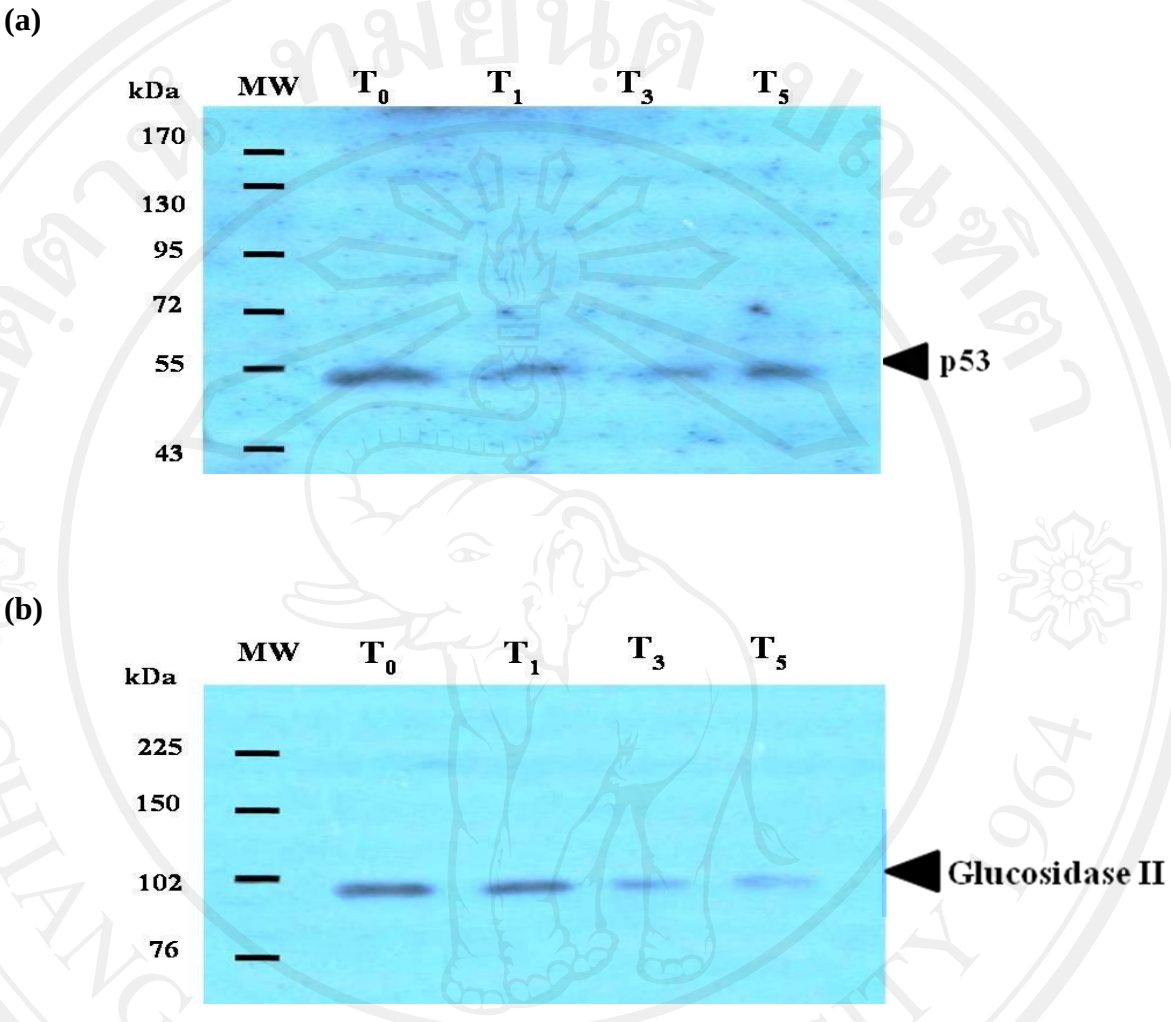
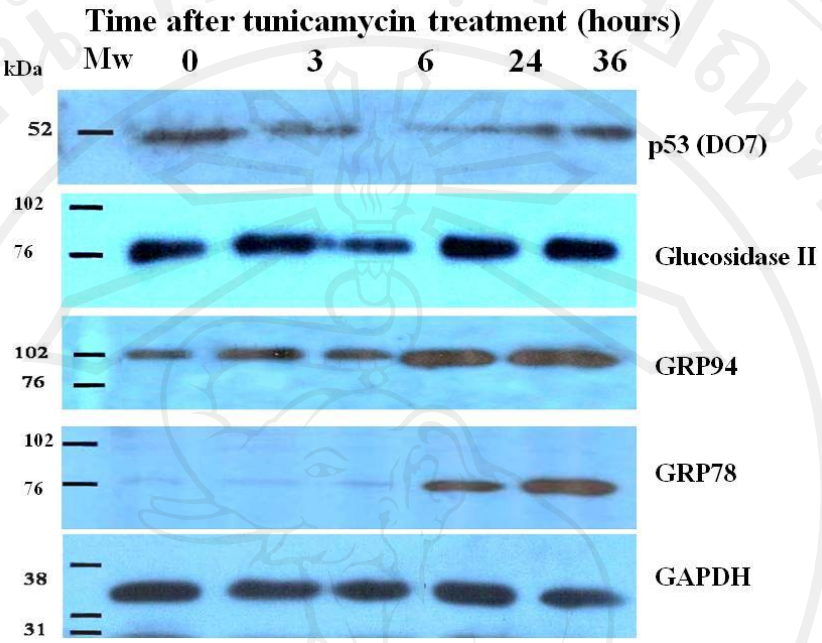


Figure 3.10 Western blot analysis showing protein levels of p53 and glucosidase II after Tunicamycin treatment. p53 (a) and glucosidase II (b) protein expression were determined at 3 hours after tunicamycin treatment with (T_0), vehicle control (0.1% DMSO); (T_1), 1 µg/ml tunicamycin; (T_3), 3 µg/ml tunicamycin; (T_5) 5 µg/ml tunicamycin.

(a)



(b)

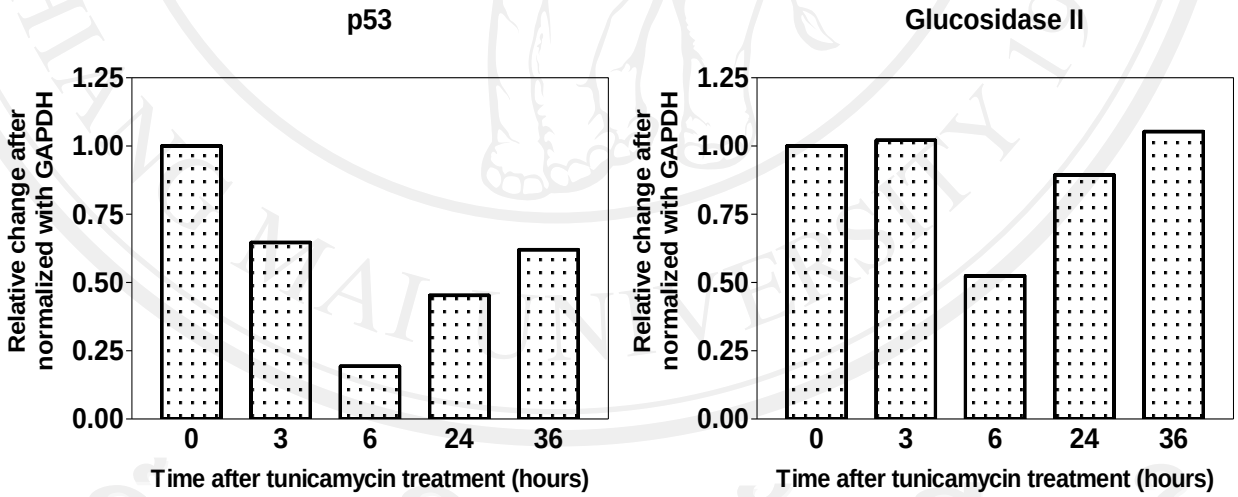


Figure 3.11 Western blot analysis (a) and quantification value (b) showing changes of various protein levels in A549 lung adenocarcinoma cells at various time points following tunicamycin treatment.

Western blot bands were quantified using Quantity One (Bio-Rad Laboratories). The quantification values for each band were normalized to the corresponding values of GAPDH. The normalized values for each time point were then compared to the corresponding band of the untreated sample (0 hours).

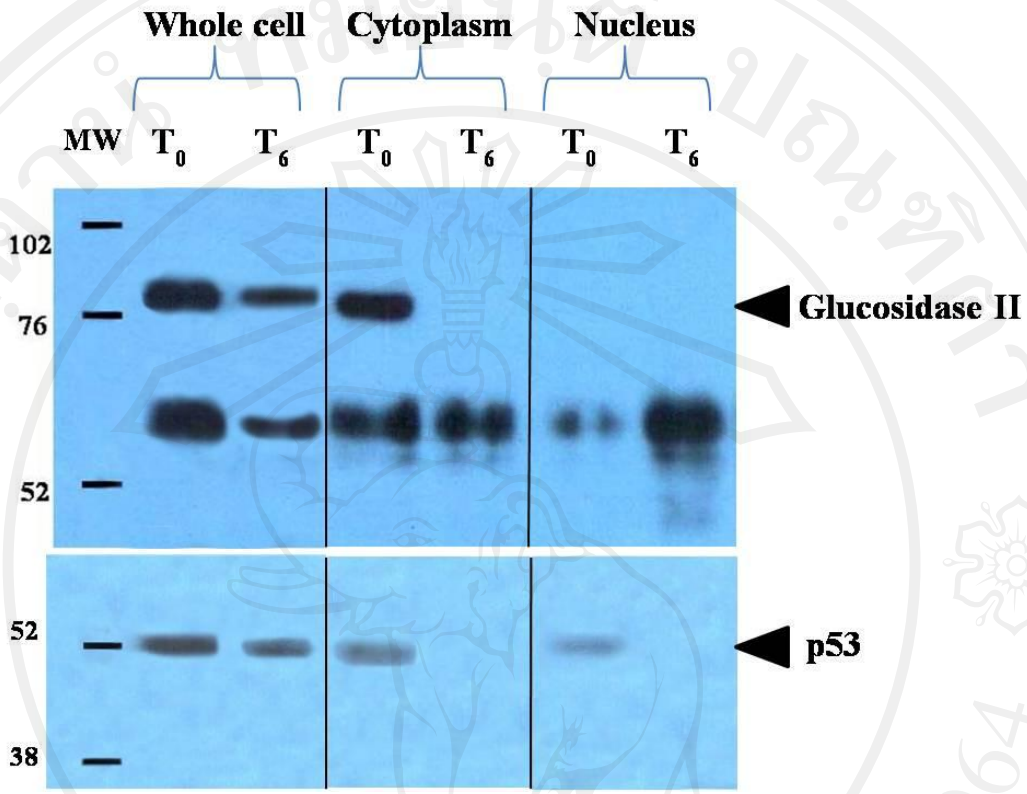


Figure 3.12 Western blot analysis showing sub-cellular localization of p53 and glucosidase II in response to Tunicamycin-induced ER stress

Cell lysate from cytosolic and nuclear fraction was prepared from Tunicamycin treated cells at 0 (To) and 6 hours (T6) after tunicamycin treatment.