行政院國家科學委員會專題研究計畫 成果報告

細胞週期調控蛋白在泌尿上皮癌細胞株及腫瘤組織上的表現
研究成果報告(精簡版)

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簡介（Introduction）

Approximately 80% of these urothelial carcinomas (UCs) are superficial lesions. Despite of the good prognosis, these tumors have a strong propensity to recur. More than 70% of these patients have a second primary lesion within 2 years. and about 20-30% of them show disease progression and eventually die from the disease. Although clinically useful, histologic assessment of these tumors is not a sufficiently good indicator at predicting prognosis. Morphologically similar tumours may behave in different ways. Therefore, new biological markers may be useful in predicting recurrence or disease progression. . Imbalance in cell cycle control is essential in development of cancer. The mechanism of cell cycle control is primarily by the interaction of regulatory proteins. Many retrospective studies have demonstrated the importance of cell cycle regulatory proteins in the development and progression of UC.

With the progress in diagnosis and treatment, the prognosis of urothelial carcinoma (UC) improved greatly in recent years. However, we still encountered several problems in clinical practice. First, patients with superficial bladder cancer are also at risk of recurrence and progression. Several studies have indicated that the recurrence rate is 50% to 80% and the risk of progression is 10% to 25%. Determining which bladder tumors will recur and progress is a problem of major clinical interest and could be useful in designing clinical follow-up and treatment strategies. There still no biomarkers for clinical use to predict tumor behavior. Second, in the metastatic UC, the response of chemotherapy is around 40-70%. We still had no inidicators for choice of clinically responsive chemotherapy regimen.

Despite the pathogenesis of UC is still not clear, UC has been thought to result from the DNA damaging effects of carcinogens on the urothelium, or from the occurrence of spontaneous genetic events that lead to genetic instability. Two fundamental mechanisms involved in this process are the activation of protooncogenes that predispose to cell proliferation and the inactivation of tumor suppressor genes, allowing proliferation to proceed in an uncontrolled manner. All these mechanisms are associated with cell cycle regulations. Many prvious studies have proved the association between cell cycles dysregulation and UC including p16 protein, p53, Rb, Kip 1, Ki-67 et al. In Taiwan, Dr. Yu DS have studied the expression of oncoproteins in transitional cell carcinoma about the correlation with pathological behavior, cell cycle and drug resistance. Some of these oncoproteins (p21, p53, c-jun) are associated with cell cycle dysregulation

Urothelial carcinoma (UC) is a relatively chemosensitive malignancy. The current standard treatment for metastatic diseases is combination chemotherapy, such as M-VAC (methotrexate, vinblastine, doxorubicin and cisplatin) or CMV (cisplatin, methotrexate and vinblastine). Response rates of metastatic UC to combination chemotherapy range from 40% to 70%. We still had no discriminators for choice of clinically responsive chemotherapy regimen. In our hospital, TP-HDFL (Paclitaxel, cisplatin, high dose fluorouracil, leucovorin) regimen for metastatic UC have been proved to be an effective treatment with a very low toxicity profile after the phase-II clinical trial. The anti-tumor mechanisms of these agents were associated with cell cycle regulations. The mode of action of platinum complexes involves the formation of positively charged moieties that form adducts with DNA. Its major focus of activity is in the G1 and S phases of the cell cycle. Paclitaxel acts on the microtubules and stabilize the microtubule. It has been shown that a microtubule is in dynamic equilibrium. The microtubules equlibrium are associated with the

spindle fomation in M phase and are controlled by cell cycle regulatory protein. In this way, the expression of these proteins is likely to be associated with chemotherapy response.

目的(Study Goals):

The project is to investigate expression of these novel cell-cycle regulatory proteins in UC cell lines and tissue and to evaluate the correlations with clinicopathological parameters, clinical outcomes, the response to TP-HDFL (Paclitaxel, cisplatin, high dose fluorouracil and leucovorin) chemotherapy. We want to achieve the following goal.

1. To identify novel makers which is correlated with tumor behavior, clinical outcomes, and chemotherapy response.
2. To establish a model of fast and efficient analysis to find the correlations of other proteins with UCs on the basis of this study.
3. We added the characteristics of UC in Taiwan to our study (High incidence of UC in BFD area residency, High incidence of uremic UC, High incidence of UT-UC). We want to find the specific carcinogenesis of UC in Taiwan.

Results:

Part 1

We first tested 13 different antibodies were by western blot analysis for their qualities and specificities on detection of antigens presented in cells of NTUB1, T24, and TSGH-8301, respectively. Result of the western blot of NTUB1 cells showed sharp single bands for detection of Eg5, ring 3, PRC-1, PLK-1, aurora A, eIf4E, and stathmin, indicating each specific antibody is highly sensitive to its corresponding antigen. However, antibodies specific to elongin B and survivin also bound to other non-specific antigens resulting in multiple bands or signals. Phosphorylated-MCM4 (MCM4-P*), TACC3, ch-TOG, and phosphoylated-NuSAP (NuSAP-P*) antibodies showed no specific binding or response toward their antigens in NTUB1 cells (Figure 1).

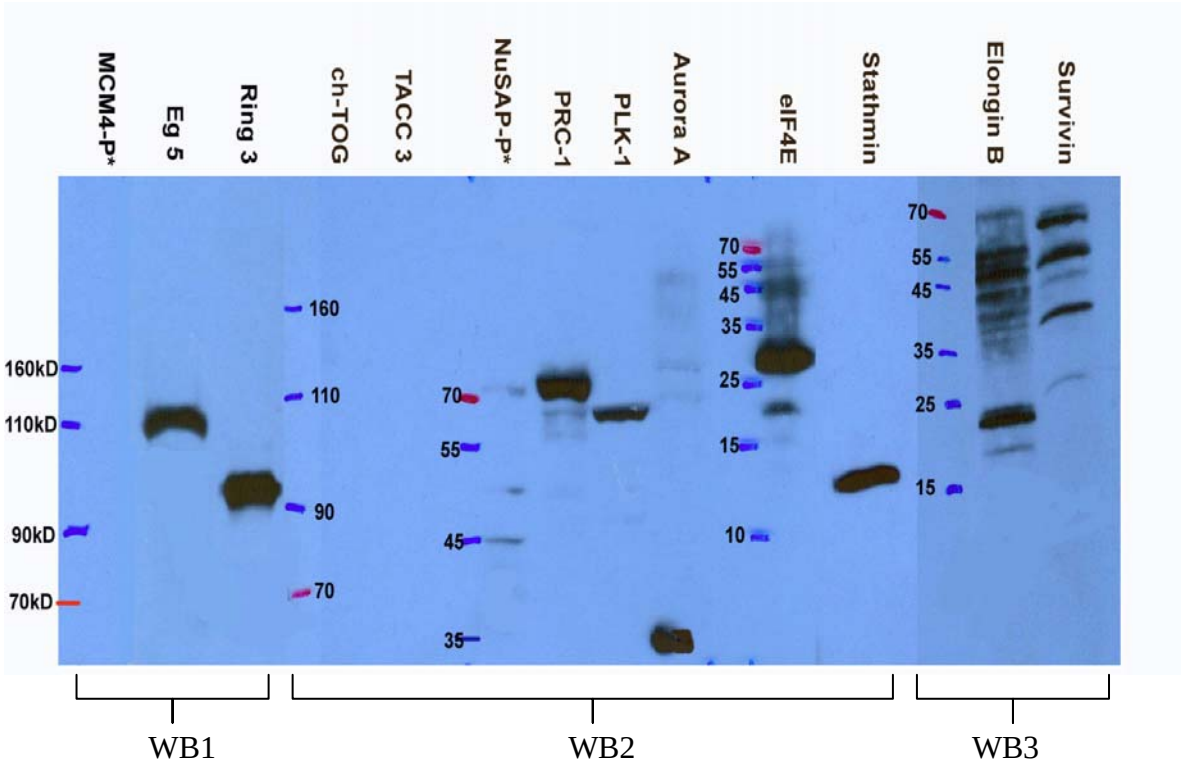


Figure 1. Western blot of high-grade urothelial carcinoma NTUB1

There are three different western blots (WB), which were joined together into one piece for display. The protein markers and their sizes are marked along on the left hand side.

Western blotting of T24, showed good sensitivity of Eg 5, ring 3, PRC-1, Eif4E, aurora A, and stathmin antibodies, except for the PLK-1, which here showed very much weaker signal compared to the NTUB1 result above (Figure 2). Elongin B antibody in this case resulted in a single band on the western blot and showed relatively low sensitivity. In addition to the MCM4-P*, ch-TOG, TACC3, and NuSAP-P*, which had no response as in accordance with the result of NTUB1, survivin antibody also had no sensitivity for antigen in T24 cells.

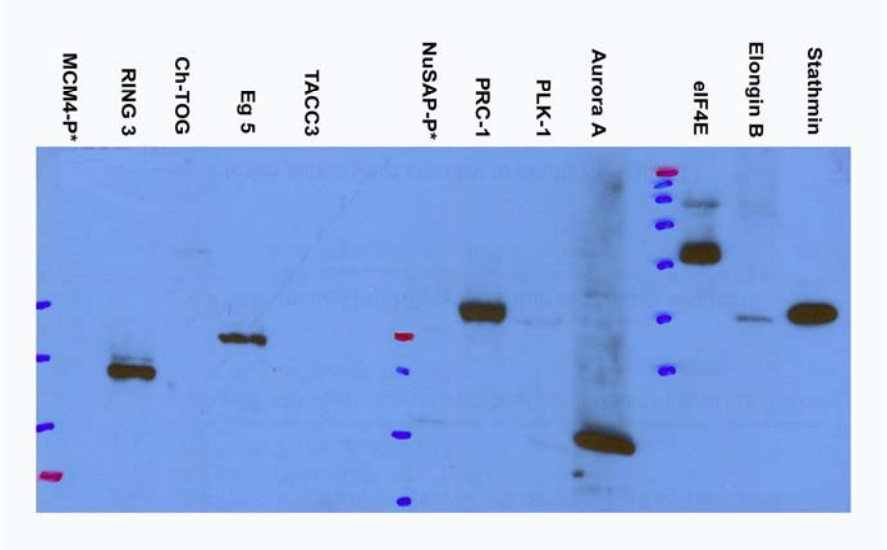


Figure 2. Western blot result of T24 cells

Result of another low grade urothelial carcinoma TSGH-8301 cells was slightly different to the ones for NTUB1 and T24 cells. Ch-TOG, NuSAP-P*, and survivn, few of the insensitive antibodies observed with the western results of NTUB1 and T24 cells were surprisingly able to detect antigens in TSGH-8301 cells and show distinctive protein bands. On the other hand, aurora A antibody gave blank result for the western of TSGH-8301 cells, which deviated from what was observed wtih NTUB1 and T24 cells.

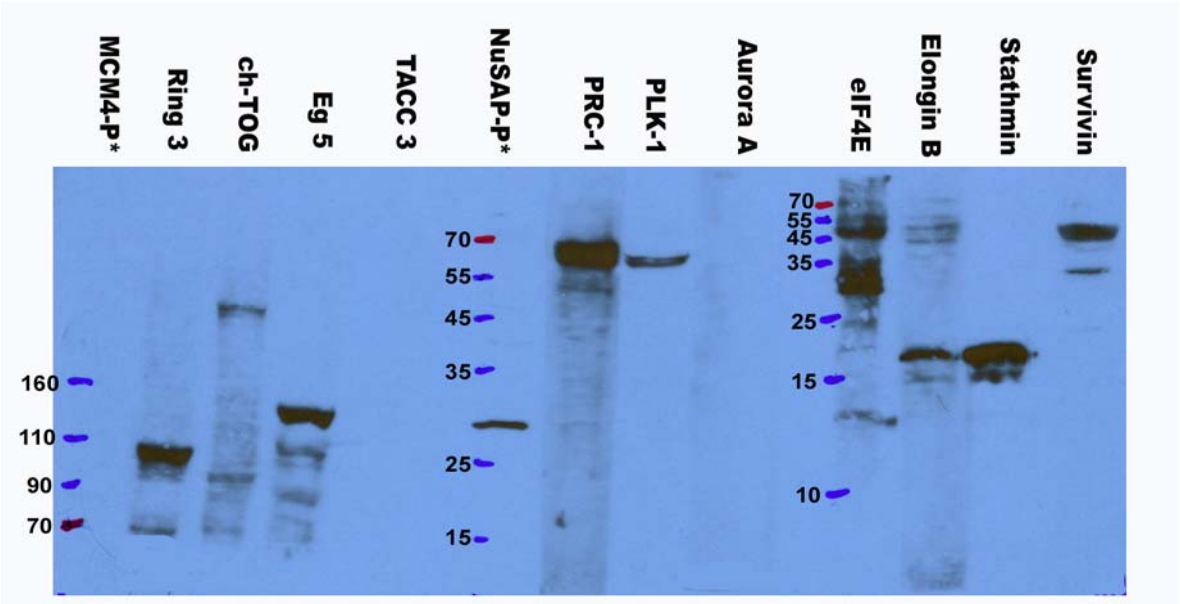
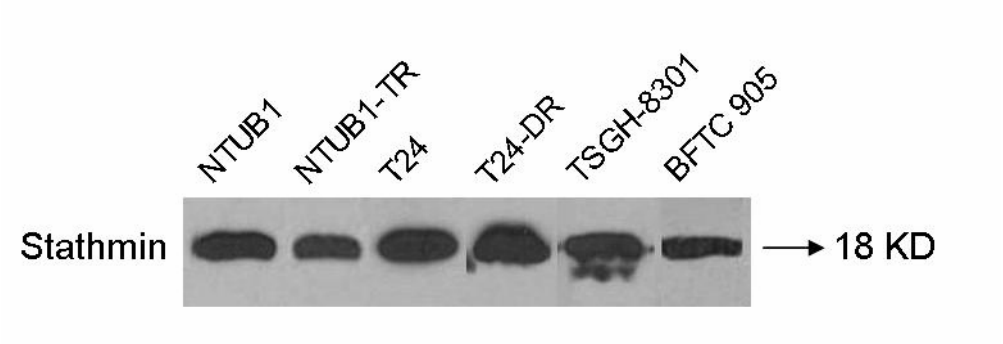


Figure 3. Western blot result of TSGH-8301

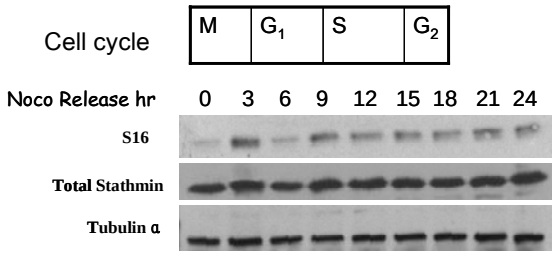
Following the quality check of the selected antibodies using western blot analysis was the immunofluorescence (IF) staining of the three cells using those antibodies in order to examine the intracellular localization of the proteins. Most of these target proteins are related to cell proliferation and therefore expressed during mitosis and cytokinesis. Eg 5, ring 3, elongin B, NuSAP-P*, and stathmin was observed to prominently localize to mitotic spindles in the nucleus during metaphase for all three cell lines. PRC-1, PLK-1, and survivn were found to concentrate at midbody during anaphase. However,

survivin was also reported to localize to mitotic spindle during metaphase, which was not observed in our study [60]. PRC-1 in our case was also found not only to localize to the midbody but also to mitotic spindle at metaphase. Moreover, ch-TOG and aurora A showed to concentrate distinctively to spindle poles in the nucleus. For MCM4-P*, eIF4E, and TACC3, intracellular localizations were not able to be clearly identified in the cells under immunofluorescence microscopy. The three cell lines shared very similar results with the identified localizations of the proteins, which were also in accordance with the findings reported from other studies.

The results showed that stathmin was the best marker with differential expression among them
Then we tested stathmin expression in different cells lines and durg resistant sublines of urothelial carcinoma(CRL-9520 (SV-40 transformed urothelium), TSGH-8301 (low grade urothelial carcinoma), J-82 (high grade urothelial carcinoma), NTU-B1 (high grade urothelial carcinoma), NTU-B1/P (resitant to cisplatin), NTU-B1/T (resitant to taxol)) to test the expression of stathmin by western blot and immunofluorescence. There are stable expression in these cell lines.



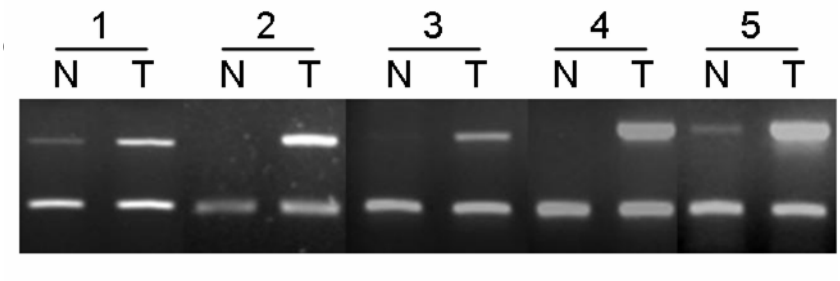
Part II Stathmin expression in different stages of cell cycle



According to what we have done above
We selected stathmin as our marker for futher study in human urothelial carcinoma samples.

RT-PCR

First we collect 7 patients speciemen including normal renal pelvic mucosa and upper tract urothelial



between normal tissue and tumor tissue.

Then we tested stathmin expression in a total of 85 patients with bladder UC

並利用臨床上收集之泌尿上皮癌之組織，以此兩種抗體進行免疫組織化學染色，與病人之年齡，性別，臨床資料（如是否合併慢性腎衰竭及末期腎病變、是否抽煙、是否居住於烏腳病盛行區、腫瘤原發部位、病理分級，臨床病理分期、預後及對化學治療的反應，以chi-square test及Fischer's exact test作雙尾檢定，$p<0.05$為具有統計上之顯著意義。

結果 (Results)

The characteristics of 85 bladder UC patients

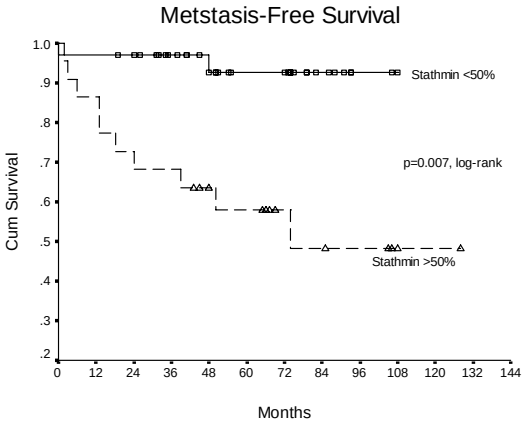
病人基本資料	總病人數 (%) N=85	表淺性 (%) N=41	侵襲性 (%) N=44
Age(years)		66.3 ± 14.4	63.3 ± 10.3
≥65	47 (55.3%)	25 (39.0%)	22 (50.0%)
<65	38 (44.7%)	16 (61%)	22 (50.0%)
Sex			
M	57 (67.1%)	28 (68.3%)	29 (65.9%)
F	28 (32.9%)	13 (31.7%)	15 (34.1%)
Site			
upper tract	20 (23.5%)	10 (24.4%)	10 (22.7%)
Bladder	65 (76.5 %)	31 (75.6%)	34 (77.3%)
pT stage			
Ta, T1	41 (48.2%)	41 (100%)	0 (0%)
T2a, T2b	22 (27.0%)	0 (0%)	22 (50.0%)
T3, T4	21 (24.7%)	0 (0%)	22 (50.0%)
Grading			
High grade	69 (81.2%)	26 (63.4%)	40 (90.9%)
Low grade	16 (18.8%)	15 (36.6%)	4 (9.1%)
ESRD (+)	6 (7.1%)	3 (7.3%)	3 (6.81%)
CRI(+)	11 (12.9%)	4 (9.8%)	7 (15.9%)
Smoking	46 (43%)	26 (63.4%)	20 (45.5%)
BFD Residency	7 (8.23%)	2 (4.1%)	5 (11.4%)

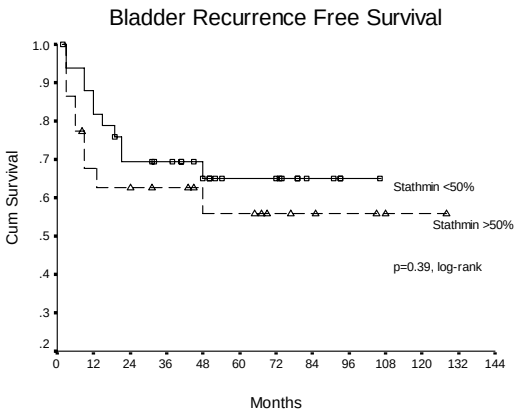
85位泌尿上皮癌病人臨床變數，病理特徵與S-16 stathmin及Total stathmin表現的關係

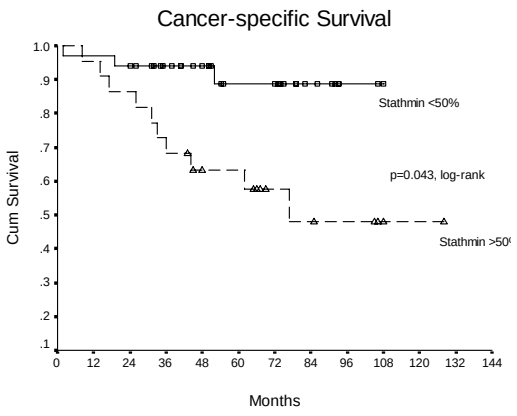
	%Pos S-16 stathmin	S-16 stathmin expression		%Pos Total stathmin	Total stathmin expression	
		OR (95% CI)	P value		OR (95% CI)	P value
All patients	79.6%			89.3%		
Age(yrs)		0.40 (0.15-1.03)	0.055		0.67 (0.28-1.60)	0.36
≥65	80.8%			85.1%		
<65	75.6%			94.6%		
Sex		2.70 (1.0-7.21)	*0.04		1.02 (0.40-2.59)	0.96
M	75.5%			86.0%		
F	85.2%			96.3%		
Site		0.56 (0.17-0.19)	0.34		0.34 (0.11-1.04)	0.052
upper tract	94.7%			94.7%		
Bladder	73.9%			87.7%		
pT stage		3.84 (1.40-8.54)	*0.006		6.86 (2.23-21.08)	*0.002
Ta, T1	56.4%			76.9%		
T2a, T2b	100%			100%		
T3, T4	95.5%			100%		
Grading		8.18 (1.02-65.78)	*0.02		1.10 (0.37-3.32)	0.9
High grade	86.7%			95.5%		
Low grade	43.8%			62.5%		
ESRD or CRI (+)	87.6%		0.6	84.3%		0.3
Smoking(+)	71.7%	0.43 (0.16-1.11)	0.2	82.7%	0.98 (0.42-2.63)	1.0
BFD Residency	83.4%	1.19 (0.10-13.7)	0.9	81.7%	2.44 (0.21-28.06)	0.5

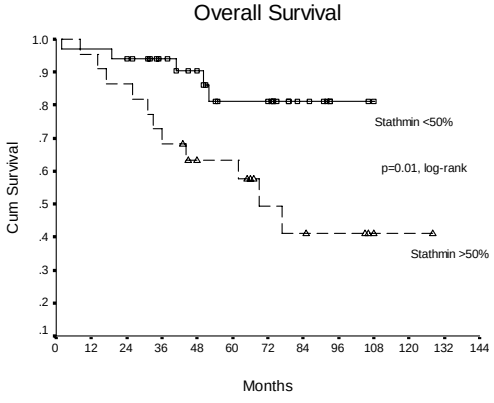
註： *p<0.05

結果可見stathmin可在泌尿上皮細胞株上表現，而在臨床上腫瘤組織的檢體表現與腫瘤之臨床分期(staging）及組織學上的級別（grading）有關 (p<0.05)，也能用以預測metastasis-free survival, cancer-specific survival, 和oveall survival.









討論 (Discussion)

這在臨床上是一相當有意義的結果，stathmin的表現可當成上泌尿上皮癌的預後因子。結果已於學會發表，將寫成論文發表於國際期刊。