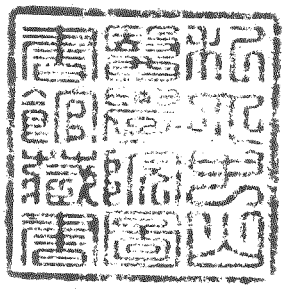
中山醫學院毒理學研究所碩士論文

Master Thesis, Institute of Toxicology, Chung Shan Medical and Dental College

指導教授： 李 輝 博士(Huei Lee, Ph. D.)

p21 蛋白和 HPV 感染對 Rb 蛋白消失在台灣地區肺癌之角色探討

THE ROLE OF P21 PROTEIN AND HPV INFECTION ON RB PROTEIN INACTIVATION IN TAIWANESE LUNG TUMORIGENESIS



研究生： 陳培亮 (Pei-Liang Chen)撰

中華民國八十八年六月八日

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(博碩士論文)

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論文名稱：p21 蛋白和 HPV 感染對 Rb 蛋白消失在台灣地區肺癌之角色探討

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指導教授姓名:李 輝

研究生簽名: 陳培亮 學號:R86503

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THE ROLE OF P21 PROTEIN AND HPV INFECTION ON RB PROTEIN INACTIVATION IN TAIWANESE LUNG TUMORIGENESIS

本論文為中山醫學院授與理學碩士學位之必備條件之一，經中山醫學院毒理學研究所碩士論文考試委員會審查合格及口試通過。

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中華民國八十八年六月八日

學生陳培亮論文題目 p21 蛋白和 HPV 感染對 Rb 蛋白消失在台灣地區肺癌之角色探討，其論文已經中山醫學院毒理學研究所碩士論文考試委員會審查合格及口試通過，並經由其指導教授審核無誤。

指導教授：李　輝博士　　簽名：李輝

中華民國八十八年六月八日

謝　　誌

隨著論文的付梓，在毒理所的日子也將進入尾聲．回首兩年來如苦行僧，辭去高薪，為實驗廢寢忘食，拋妻別子，熬夜讀 paper 算統計，雖經濟貧乏，但如今卻帶給我精神的富足，和滿滿的自信，毒理所的人、事、物，更成為我畢生難忘的回憶，心中是由衷的感激，難以言表。

文稿初成，復蒙台中榮民總醫院胸腔外科主任陳志毅醫師及中山醫學院毒理學研究所柯俊良博士之撥冗審閱，對本論文細心審查，詳加斧正，並提供諸多寶貴意見，在此致上誠摯謝意。

入研究所，執意跟隨恩師 李輝教授，以我不算年輕的年紀帶給老師極大的壓力，唯恐我的家累和體力，無法和馬拉松式的實驗競賽。但感謝 李老師不但傾心盡力帶領我進入深奧的分子生物的領域，教導我 paper 的選讀方向，於論文撰寫期間，不眠不休的指導我文章的撰寫，更以其經驗堅持我走完整個碩士訓練，不時鼓勵我繼續往學術上衝，雖辜負老師的心意，但老師的期許卻憫感五心。

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協助，使本研究得以順利完成，謝謝您們！

國豪、書林、瀅太同窗好友，和你們一起打拼的日子真好，祝福你們畢業後鵬程萬里！冠穎、玲雅兩位學妹加油，希望未來一年事事如願，要 band 有 band，要 data 有 data。

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最後，僅以此論文獻給我賢慧的老婆淑娟，感謝妳這兩年來的辛勞，不僅一人挑起家庭經濟重擔還要侍候一位老爺、一位少爺和一位千金，未見妳一絲抱怨卻還要不時給我打氣，使我心無旁騖順利完成碩士課程。謝謝妳！

陳培亮　　謹誌於

中山醫學院毒理學研究所

中華民國八十八年六月八日

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ABBREVIATIONS

Cyclin-dependent kinase	CDK
Cyclin inhibitor protein	CIP
Complementary DNA	cDNA
Deoxyribonuclease	Dnase
Diethyl pyrocarbonate	DEPC
DL-Dithiothreitol	DDT
Enhance chemiluminesence	ECL
Human papillomavirus	HPV
Horseradish peroxidase	HRP
Immunohistochemistry	IHC
Labelled streptavidin-biotin reagent	LSAB
Messenger RNA	mRNA
Non-small cell lung cancer	NSCLC
N,N,N,N,-tetramethylenediamine	TEMED
Proliferating cell replication antigen	PCNA
Polymerase chain reaction	PCR
Retinoblastoma	Rb
Reverse transcription-polymerase chain reaction	RT-PCR
Ribonuclease inhibitor	Rnasin
Sodium chloride	NaCl
Sodium Dodecyl Sulfate	SDS

中文摘要：

過去的研究顯示 p53 基因發生突變是引起肺癌的重要致病原因。但台灣地區非小細胞肺癌 (non-small cell lung cancer, NSCLC) 的 p53 突變頻率 (18%) 較過去的報告都低 (50-80%)，這顯示台灣地區肺癌發生的機轉可能有其他更重要的基因參與。本研究假設 Rb 基因可能參與台灣肺癌的腫瘤化機轉。因此本研究先利用免疫組織化學染色法分析 247 肺癌病患肺腫瘤的 Rb 表現。結果發現有 68.4% 的患者 (169/247) 無法偵測到 Rb 蛋白的表現。為了探討 Rb 蛋白消失的原因，我們推測可能是 (1)參與 Rb 蛋白的磷酸化基因，例如 p21、cyclins 和 cyclin dependent kinase 等蛋白表現不正常所致。 (2) 感染人類乳頭狀瘤病毒而將 Rb 蛋白分解。 (3) Rb 基因的轉錄層次受損。首先同樣以免疫染色法分析 p21 和 cyclin D1 的蛋白表現，結果發現有將近一半的病患 (45.7%, 113/247) 無法偵測到 p21 蛋白的表現。同時發現 p21 蛋白與 Rb 蛋白的表現呈顯著正相關 ($p=0.008$)。同樣地，cyclin D1 蛋白的表現也與 Rb 蛋白表現呈顯著相關 ($p<0.001$)。這些結果顯示 Rb 蛋白的消失可能是 p21 無法有效抑制 Rb 蛋白磷酸化所造成。其次以 nested PCR 偵測肺癌組織是否有人類乳頭狀瘤病毒的 DNA，以確定肺癌患者是否感染人類乳頭狀瘤病毒。令人訝異的是竟然發現有 55.2% 的肺癌患者感染人類乳頭狀瘤病毒，此感染率明顯高於其他國家曾經發表過的相關報告(0-36%)，因此認為人類乳頭狀瘤病毒的高感染率可能經由誘導 ubiquitin-

proteasome 路徑，將肺腫瘤組織中 p21 蛋白與 Rb 蛋白迅速分解。可能在台灣地區肺癌肺腫瘤化過程有相當程度的關係。另外利用 RT-PCR 和 genomic PCR 來分析 Rb 基因的轉錄層次是否發生改變，結果發現僅有 16-19% 的 Rb 蛋白消失是由基因轉錄機轉缺陷所致。在臨床上預後研究上，發現 Rb 蛋白表現僅在 p21 蛋白消失的患者才能作為預後指標 (P = 0.0078)，即 Rb/p21 同時消失的肺癌患者之存活時間顯著短於 Rb+/p21- 的患者。由以上的結果可知肺癌患者 Rb 蛋白消失可能和 p21 蛋白表現異常及人類乳頭狀瘤病毒的高頻率感染有關。同時 Rb 蛋白消失在台灣地區肺癌腫瘤機轉上可能扮演重要的角色。

I . Abstract

In this study, we propose that Rb protein inactivation may play a role on Taiwanese lung tumorigenesis, because relatively lower frequency of p53 gene mutation (18%) was found compared with previous data reported in other countries (50-80%). To test the hypothesis, immunohistochemical analysis was used to evaluate the expression of Rb protein in 247 primary non-small-cell lung cancer (NSCLCs) specimens. High frequency of Rb protein was undetectable by immunostaining (169/247, 68.4%). We suspect that the high frequency of Rb protein inactivation may be caused by (1) alteration of genes involved in Rb protein phosphorylation, such as p21, cyclins and CDKs, (2) inactivation by other oncoproteins from HPV infection, and (3) alteration of Rb gene transcription. Thus, we provide the following evidence to demonstrate which mechanism was important in Rb protein inactivation. Therefore, we first analyzed the protein expressions of p21 and cyclin D1 using immunohistochemistry. We found that high frequency of p21 negative immunostaining (113/247, 45.7%) and its expression was significantly associated with Rb protein expression ($P = 0.008$). Cyclin D1 protein expression was also correlated with Rb protein expression ($P < 0.001$). These results suggest that Rb protein inactivation may be caused by the increased its phosphorylation. Secondly, genomic nested PCR was used to detect whether HPV DNA was existed in lung tumor. Surprisingly, 55.2% (32/58) of HPV-18 DNA was detected in lung tumors. The HPV infection

frequency in lung cancer found in this study was significantly higher than previous reports (0-36%). We thus strongly suggest that high HPV infection may partly result in the absence of p21 and Rb proteins through the induction of ubiquitin-proteosome pathway. Finally, the defect of Rb gene transcription was examined by reverse transcription PCR (RT-PCR) with the primer which was used to hybridize exon 2 of Rb gene and we used genomic PCR to examine whether promoter site of Rb gene was defected to cause down-regulated transcription. Our results showed that Rb protein inactivation were only 16-19% of cases to be explained by the defect of Rb gene transcription. In clinical outcome, the survival rate of patients with the p21 negative/Rb negative immunostaining was significantly shorter than patients with p21 negative/Rb positive ($P = 0.0078$). This finding further support that p21 negative immunostaining plays an important role on Rb protein inactivation. From the above findings, we strongly suggest that high frequency of Rb protein inactivation caused by p21 gene alterations and HPV infection may play an important role on Taiwanese lung tumorigenesis.

Ⅱ. Introduction

Lung cancer has already become the leading cause of cancer deaths in Taiwan. Aberrations of the genes directly controlled cell-cycle progression represented the most common genetic lesions in human cancer (Cordon-Cardo et al., 1995), especially for genes participating in the regulation of cell cycle progression from G1 to S phase (Fig 1). The aberrant events in human neoplasm may be one or more these genes (Tanaka et al., 1998. Gorgoulis et al., 1998). The gene alterations in lung cancer have been demonstrated to occur mutations of 10-20 genes including the tumor suppressor genes such as p53, Rb, $p21^{WAF1}$, $p16^{INK4a}$ and oncogenes such as ras, myc, and mdm-2 (Weintraub et al., 1996; Smit et al., 1996). In this study, we hope to find out which gene alteration may be responsible for Taiwanese lung tumorigenesis. In our previous study indicated that relatively low frequency of mutation in p53 gene (~18%) was found in Taiwanese NSCLC compared to data from previous reports (50-80%). In immunohistochemistry, data was showed that high frequency of undetectable of p53 protein in lung tumor specimens. These results suggest that p53 gene alteration may be not important for Taiwanese lung tumorigenesis. Thus, in this study, we hypothesize that another important tumor suppressor gene, Rb, may play a role on the development of Taiwanese NSCLC.

1. Structure and function of Rb gene

The Rb gene was the first tumor suppressor gene to be identified, which was mapped to the region q14 of human chromosome 13 and which transcript is encoded in 27 exons dispersed over 200 kilo-bases (kb) of genomic DNA. The length of individual exon ranges from 31 to 1889 base pairs (bp). The largest intron spans >60kb and the smallest one has only 80bp (Frank et al., 1989). It has been cloned as a cDNA of 4.7kb (Friend et al., 1986), and produce a 928 amino acid 105-115kd nuclear phosphoprotein depending on its phosphorylation status (Hong et al., 1989). The Rb protein regulated cell cycle progression not only depended on the protein phosphorylation-dephosphorylation manner but also had a novel mechanism, proteolysis of Rb protein (Florence Fu et al., 1998).

The Rb protein participated in dual tumor suppressive functions, one linked to cell cycle progression and the other related to differentiation control (William et al., 1998). When wild-type Rb gene introduced into certain human cancer cell lines, exerts suppressive effects on their neoplastic phenotype (Huang et al., 1988; Bookstein et al., 1990; Takahashi et al., 1991). This suggested that Rb might have an inhibition of neoplasm development, and to prevent or suppress tumorigenesis.

Therefore, Rb gene is believed that the complete loss or inactivation may lead to tumor formation (Cavenee, et al. 1983).

2. Cell cycle control and Rb protein phosphorylation

The Rb protein is a negative regulator of cell proliferation (Wang et al., 1994; Weinberg et al., 1995; Kaelin et al., 1997). Growth inhibitory activity of Rb protein is attenuated by phosphorylation. Progressive and prolong phosphorylation of Rb protein leads to its inactivation and reduction of its growth-suppressive activity (Kato et al., 1994). Rb protein is a major substrate for the cyclin-dependent kinase (CDKs; CDK4, CDK6, CDK2, and cdc2 (Lin et al., 1995; Akiyama et al., 1992; Meyerson et al., 1994), which has been shown to phosphorylated at a particular stage of the cell cycle (Chen et al., 1989; Caprio et al., 1989; Buchknovich et al., 1989; Geng et al, 1993). Since this phosphorylation occurs in G1/S transition site, cell arrests is disrupted and cell proliferation unregulated. The D-type cyclin/CDK complex (Sherr et al., 1996) initiates this phosphorylation. The CDKs is stimulated by D-type cyclins and inhibited by the tumor suppressor protein. Recently, it has been shown that cyclin-dependent kinase (CDKs), in concert

with their regulatory cyclin subunits, can phosphorylate Rb protein (Hinds et al., 1992; Kato et al., 1993). The cyclin D1/cdk4,6 are active in G1, the time of Rb protein phosphorylation, and indeed have been shown to phosphorylate Rb protein *in vitro* and in transfection studies. The hypophosphorylated Rb protein actively suppresses the gene with promoters containing the E2F-binding motif (Paul et al., 1992).

3. Rb inactivation and human cancer

Rb protein inactivation had been found to link with the tumorigenesis of a wide range of human tumors, including hereditary and sporadic retinoblastomas (Cavenee et al., 1983; Dryja et al., 1986; Fung et al., 1987; Yandell et al., 1989), small-cell lung carcinoma (Harbour et al., 1988; Horowitz et al., 1990), breast cancer (Lee et al., 1988), bladder carcinoma (Horowitz et al., 1989), osteogenic sarcoma (Dryja et al., 1986 Weichselbaum et al., 1988), prostate carcinoma (Bookstein et al., 1990a), renal cell carcinoma (Ishikawa et al., 1991), esophageal carcinoma (Boynton et al., 1991), nasopharyngeal carcinoma (Sun et al., 1993), colon carcinoma (Kohn et al., 1997), hepatocellular carcinoma (Takuji et al., 1998) and leukemia

(Cheng et al., 1990). Mutational inactivation of Rb gene appears to initiate the development of retinoblastoma and may also contribute to the pathogenesis of osteosarcomas, soft-tissue sarcomas, small cell lung cancer and other malignancies. Rb gene structure was studied at the DNA level by Southern blot and chromosome 13 restriction fragment length polymorphism analyses. Inactivation of Rb gene investigated by the gene mutation through analysis of DNA sequence is difficult, because Rb gene is composed of 27 exons that are scattered over a region of approximately 200kb (McGee et al., 1989). Expression of the Rb gene was evaluated by Northern analysis of the transcript and immunohistochemical analysis of the protein ($p105^{RB}$). Rb protein inactivation proved by immunohistochemistry to be the most sensitive method (Reissmann et al., 1993). The first paper reported by Xu indicated that altered Rb protein expression revealed by immunohistochemistry is an independent prognostic marker for overall decreased survival in early-stage NSCLC (Xu et al., 1994). In this study, immunohistochemistry, RT-PCR, genomic PCR, and Western blot to explore the role of Rb gene alteration in Taiwanese lung tumorigenesis. Kaplan-Meier survival probability and Log-rank analysis were used to confirm Rb gene alteration whether was a prognostic factor in Taiwanese NSCLC patients.

4. The role of p21 in Rb inactivation

P21 was first discovered acting as an inhibitor of the CDKs complexes, and it is also named as CIP1 (Harper et al., 1993), SDI1 (Noda et al., 1994), and WAF1 (el-Deriy et al., 1993). The overexpression of p53 in response to DNA damage agents promoted the transcription of $p21^{WAF1}$, which encoded a potent protein inhibitor of cyclin-dependent kinase (CDK) (Harper et al., 1993). P21 was considered to be a downstream target of p53, because its promoter contains two elements recognizable by p53. Binding of wild-type p53 to these regions was found to up-regulated transcription of p21 (el-Deriy et al., 1995). The p21 protein can inhibit the kinase activity of G1 cyclin/cdk complex cdk4/cyclin D and cdk2/cyclin E, two complexes that necessary for onset of the S phase of the cell cycle. P21 inhibited cell growth and promoted differentiation (Harper et al., 1997). The C-terminus binds to proliferating-cell-nuclear antigen (PCNA) and inhibits PCNA-dependent DNA replication, but not inhibiting PCNA-dependent nucleotide-excision repair (Waga et al., 1994) (Fig 2). In fact, not only p53-dependent pathway but also p53-independent pathway was able to activate p21 transcription (Jang et al., 1994; Michieli et al., 1994). Marchetti et al. (1996) demonstrated that p21 was expressed

independently from p53 gene/protein alterations in NSCLC. P21 overexpression was more frequent in well-differentiated tumors. In addition, Marchetti et al. (1995) p21 was not mutated in a series of 73 NSCLC, 74 invasive breast carcinomas and 36 ovarian adenocarcinomas and also have not been observed pancreatic cancer (Shimizu et al., 1996), and colorectal cancer (Li et al., 1995). In this study, we thus focused on the aberration of p21 protein level using immunohistochemistry to elucidate the role of altered p21 protein in Rb protein inactivation.

5. The role of cyclin D1 in Rb inactivation

It has been shown that cyclin D1 (CCND1) may act as an oncogene in certain neoplasm, leading to inactivation of Rb protein presumably through phosphorylation and/ or the formation of a physical complex (Motokura et al., 1993). Cyclin D1 is able to bind directly to Rb protein through its LXCXE motif, occupying the pocket of Rb protein and preventing E2F binding to Rb protein, suggesting that cyclin D1 played a central role in cell cycle progression (Dowdy et al., 1993). Moderate expression of cyclin D1 in early G1 is

reported to promote cell progression through G1 phase (Jiang et al., 1993; Quelle et al., 1993; Motokura et al., 1993). However, accumulating evidences suggest that cyclin D1 may also have a role as a mediator of the G1 checkpoint response in normal cells (Pagano et al., 1994; Han et al., 1995). Pagano et al. (1994) showed that down-regulation of cyclin D1 is necessary for the start of the DNA replication. Whereas Han et al. (1995) reported that stable high expression of exogenous cyclin D1 cDNA markedly inhibits rather than enhances the growth of breast cancer cell line. Expression of cyclin D1 has been shown to be up regulated by a complicate mechanism involving Rb and p53. Thus, cyclin D1 may be an important role on tumorgenesis by either directly complex CDK4 or CDK6 and promoting the phosphorylation of Rb protein or by reducing the levels of Rb protein in the cell. However, its down-regulation can be cause by oncogenic proteins such as large T antigen of SV-40, E6 and E7 of the human papillomavirus.

6. The role of Human papillomavirus in Rb inactivation

From the above mentions, the human papillomavirus (HPV) play a role in the alteration of Rb, p21, and cyclin D1 protein expression. HPV is a double-strand non-enveloped DNA virus

and HPV may have over 60 genotypes. Some types of HPV have been demonstrated to involve in the pathogenesis of many target sites of human neoplasms, including the anogenital area, oropharynx and upper respiratory tract (Shindoh et al., 1992; Stoler et al., 1992). However, more than 90% of cervical carcinoma contain HPV DNA that was usually demonstrated to be HPV-16 and HPV-18. The inactivation and degradation of the p53 and Rb proteins via ubiquitin-proteosome pathway which was induced by HPV oncoprotein, such as E6 and E7 (Jones et al., 1997). It has been reported that ubiquitin-dependent proteolysis is involved in E7-E6 or E7 mediated degradation of Rb protein (Scheffner et al., 1992; Boyer et al., 1996). The HPV E7 oncoprotein bind to the Rb pocket domain, displace cellular Rb binding proteins, and released transcription factor E2F to progress cell cycle. Our preliminary data showed that high frequency of the absence of p53 protein expression was observed in 208 NSCLC patients (Cheng et al. Submitted). We proposed that a high risk of HPV infection in Taiwanese NSCLC patients might play a role in the degradation of p53 protein in post-translational level. Therefore we hypothesize that Rb protein inactivation may be affected by HPV-induced ubiquitin/proteosome degradation pathway.

Ⅲ. Material and methods

1. Tumor specimens

We used 247 primary NSCLC tumor sections were supplied by Department of Pathology, Taichung Veterans General Hospital from January 1987 to December 1998. The histologic diagnoses and classification of the tumor were based on the World Health Organization (WHO) criteria. The post-surgery pathological tumor-node-metastatic (TNM) stage of each malignancy was determined according to the guideline of the American Joint Committee on Cancer. Survival data were collected from hospital charts and from periodic interviews with the patients and their relatives. The follow-up period for surviving patients was 5 years. Patients dying of cause other than lung cancer during the follow-up period were exclude from this study. No patients had any adjuvant therapy. Patients' background and pathological stages are summarized in Table 1.

2. Immunohistochemistry

Surgically resected specimens containing tumor tissues and non-tumor tissues were fixed in 10% formalin and embedded in paraffin by a conventional technique. Sections (2.5 μm thick) were cut from blocks, mounted on glass , and dried overnight at 37 ℃ . All sections were de-paraffinized in xylene, and

rehydrated through a grade alcohol series, and washed in phosphate-buffers saline. This buffer was used for all subsequent washed. Section for Rb protein, p21, and cyclin-D1 staining were heated twice in microwave over 5 minutes each in 10 mM citrate buffer (pH 6) to unmask the antigen. All slides were treated with 3% H_2O_2 in 100% methanol for 30 min to quench the endogenous peroxidase activity. The primary antibody used were as follow: anti-Rb monoclonal antibody (SC-102; Santa Cruz) at a 1:50 dilution, anti-cyclin-D1 monoclonal antibody (clone G124-326 PharMingen. San Diego, CA, USA) at a 1:150 dilution, anti-p21 monoclonal antibody (EA10; Oncogene) at a 1:50 dilution. Slides were incubated with the primary antibody 2 hr at room temperature in a moist chamber. After washing in PBS three times 5 minutes each, then they were next incubated with biotinylated rabbit anti-mouse immunoglobulins (DAKO, LSAB 2 Kit peroxidase) at room temperature for 30 minutes and washed again, followed by streptavidin-peroxidase complex (DAKO) for 30 minutes. Slides were developed by the application of 0.05M Tris HCl buffer (pH 7.6) containing diaminobenzaminidine and 3% H_2O_2 for 5 minutes and counter-stained with hematoxylin.

A tumor was scored negative (-) only if all malignant cells showed no nuclear protein staining, otherwise was considered positive (+) staining. The Rb, p21, cyclin D1 positive were score as follows: + (<10%), ++ (10-50%), +++ (>50%).

3. RNA Isolation

Total RNA was isolated from 100-mg tissues by homogenizing tissue samples in 1 ml of Trizol reagent first (Life Technologies, USA). The homogenized samples were incubated at 25℃ for 15 minutes to permit the complete dissociation of nucleoprotein complexes. Add 0.2 ml of chloroform and cap sample tubes securely. Shake tubes by hand for 30 sec and incubated them at room temperature for 3 min. The samples were centrifuged at 12,000 × g for 15 min at 4℃. Following centrifugation, the mixture separates into a lower red, phenol-chloroform phase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase of the volume about 0.6ml. The aqueous phase was transfer to a fresh tube carefully. The RNA was parcipitated from the aqueous phase by mixing with 0.5ml of isopropyl alcohol. Samples was incubated at -20℃ for 20 min and centrifuge at 12,000 × g for 15 min at 4℃. The RNA was precipitated and formed a gel-like pellet on the bottom of the tube, removing the supernatant, and washing the RNA pellet with 1ml of 75% ethanol. The sample was mixed and centrifuged at 7,500 × g for 10 minutes at 4℃. The supernatant was removed and the RNA pellet was dissolved with 30 μl DEPC-H_2O. The RNA concentration was determined by measuring the A_{260}, and the quality was checked by electrophoresis in 1% agarose gel in a MOPS/formaldehyde buffer (40mM MOPS, 10mM NaOAc, 1mM EDTA and 1M formaldehyde) as well as measurement of $A_{260/280}$ ratios (which were 1.7-1.9).

4. Detection of Rb mRNA by RT-PCR

(1) Reverse transcription (RT)

Reverse transcription to cDNA was performed by using the SuperScript^{TM}II Rnase H Reverse Transcriptase (Life Technology, USA). Five μg of total RNA in 12 μl mixture containing 1 μl (500 μg /ml)oligo d(T)18 (Bio-Labs, USA) were heated to 70 ℃ for 10 minutes and quick chill on ice. Then add 4 μl 5×First Strand buffer (250mM Tris-HCl, 375mM KCl, 15mM $MgCl_2$), 2 μl DDT (0.1M) and 1 μl dNTP mixture (10mM each dATP, dGTP, dCTP and dTTP). Mix contents and incubate at 42 ℃ for 2 minutes. Add 1 μl (200units) SuperScript^{TM}II (reverse transcriptase). Reverse transcription was then allowed to proceed at 42℃ for 50 minutes. Samples were immediately heated at 70℃ for 15 minutes to inactivate any remaining reverse transcriptase. The cDNA can now be used as a template for amplification in PCR reaction.

(2) PCR for Rb cDNA

One μl of cDNA was added to 49 μl of a PCR mixture containing 1.25 units of Taq Polymerase (TaKaRa Ex Taq^{TM}, TaKaRa Shuzo CO., LTD), 4 μl dNTP mixture (2.5mM each dATP, dGTP, dCTP and dTTP), 5 μl 10× Ex Taq^{TM} PCR buffer (10 mM Tris-HCl (pH8.0), 1.5 mM $MgCl_2$, 75 mM KCl), and 1 μl (10pmol/ μl) each of the used primers (Life Technology, USA).

The primers used consisted of Rb exon 2 sense primer: (5'-GCG AAT TCG TAT GTA CTG AAT CAA TTT G-3') and antisense primer: (5'-GCG AAT TCG AAG TTG GTT TTA AAA TGA G-3') yielded a 200-300bp PCR product. A negative control containing a reaction mix without cDNA was included in each PCR run to exclude any PCR artifact. A certain tissue sample showing consistent results served as positive control for normalization in each PCR run. The PCR reaction was carried out of a 5 minute denaturation at 94℃ followed by 35 cycles, each consisting of denaturation at 94℃ for 40sec, annealing at 54℃ for 1 minute, and extension at 72℃ for 1 minute with a final extension phase of 10 minutes. The PCR reaction was performed on a programmable thermal controller instrument-thermal cycle Model 2400 (Perkin-Elmer Corp.,Norwalk,CT).

5. Extraction of DNA from paraffin-embedded tissues

(1). Extraction of DNA

Section of formalin-fixed and paraffin-embedded tissues were de-paraffinized in xylene for 15 minutes two times and clear with absolute alcohol for 5 minutes at room temperature, then scraped the section from microscope slides (10 μm thickness) place in 1.5ml eppen-drof and added 200 μl lysis buffers (100 mM Tris-HCl (pH 7.6), 0.5% Sodium Dodecyl sulfate, 1mM $CaCl_2$). Digestion is carried out for 4 days at 55℃, with daily

replacement of proteinase K (250 μg /ml working concentration). This protocol was adapted from Salvador et al published in Diagnostic Molecular Pathology 6(6): 342-346, 1997. DNA extracted using a phenol-chloroform protocol. Briefly, equal volumes of phenol-chloroform-isoamyl alcohol solution (25:24:1) are added to the sample, followed by gently vortex for 5 minutes. Spinning in a bench-top centrifuge at full speed for 10 minute's separates the Organic (bottom) and aqueous (top) phases. The aqueous phase is transferred to a new eppen-drof, leaving organic phase. A second extraction with equal volume of phenol-chloroform-isoamyl alcohol solution (25:24:1) follows. After vortexing and spinning as described previously, the aqueous phase is again transferred to a new eppen-drof. One addition chloroform extraction is normally performed to remove any residual phenol contamination. DNA is precipitated with 0.2 volumes of 5M ammonium acetate and ice-cold absolute ethanol in the cold (overnight at -20℃ or 30 minutes at -80℃) and then pellet by centrifugation in a cool room (4℃) at 12000 rpm for 15 minutes. Additional washing of DNA with 40% ice-cold ethanol (5 minutes) followed by centrifugation under the same condition will visible pellets. Finally, DNA is re-suspended in 40 μl distilled water. The quality of DNA thus procured can be checked by PCR amplification of the human β-globin gene from human genomic DNA.

(2). Genomic PCR

Rb promoter DNA fragments were amplified by mixing primers Rbpro-1 (5`-TGC ACT AGC CAG ATA TTC CCT GC-3`) and Rbpro-2 (5`-TCC CGA CTC CCG TTA CAA-3`), 1 μ g of genomic DNA, and 0.2ul TaKaRa Taq enzyme with 5 μ l PCR buffer and 10 μ M d-NTP in total volume of 50 μ l. Mixture were processed through 35 cycles of denaturation at 94℃ for 30 sec, annealing at 53℃ for 40 sec, and extension at 72℃ for 1 min, follow by a single 10 min extension step in a programmable heat block. This amplification was yielded a 423 bp PCR products.

(3). Nested PCR

To efficiently determine variants of HPV 18 in the low copy number of HPV DNA, PCR-directed sequence were used with nested PCR. PCR was performed using a thermal cycler. A master mix was used, containing 5 μ l reaction buffer (TaKaRa PCR kit), 1 μ l genomic DNA, 4 μ l d-NTP, 0.2 μ l Tag Polymerase for the first PCR. The differences of the reaction mixture for second PCR, using inner primer type specific primer pairs, were 10 pmol of each primer and 2.5 μ l of the first PCR products as a template DNA. The final volume of the reaction mixture was 50 μ l for both first and second PCR. The nested PCR reaction was subjected to 35 cycle (denaturation for 5 min at 94℃, followed by 35 amplification cycles including a

denaturation step at 94℃ for 30 sec, an annealing step at 54℃ for 40 sec, and a chain elongation step at 72℃ for 1 min) for the first PCR and 25 cycles (denaturation for 5 min at 94℃, followed by 25 amplification cycles including a denaturation step at 94℃ for 30 sec, an annealing step at 53℃ for 50 sec, and a chain elongation step at 72℃ for 1 min) for the second PCR. To complete an elongation, each step was prolonged for another 10 min. Sample without DNA and with DNA containing no HPV 18 DNA was simultaneously used as negative controls in each reaction, none of which showed an amplifier bands. A total of 10 μ l of each of the PCR mixtures was finally analyzed by 2% agarose gel.

First PCR primers:(A06324, 603-920, 317bp)

Sense 5`-ATGCATGGACCTAAGGCAACATTG-3`

Anti-sense 5`-TTACTGCTGGGATGCACACCACGG-3`

Second PCR primers:(A06324, 636-886, 250bp)

Sense 5`-GTATTGCATTTAGAGCCCC-3`

Anti-sense 5`-GACAGGGTGTTCAGAAACAGC-3`

6. Western Blot Analysis

The 55 tumors used in Western blot assay were part of 247 NSCLCs in previously investigated in IHC assay. In each case, samples from tumor area and non-tumor lung tissue were store in -80℃ until study. Tissue were minced with surgical scissors in 200-400 μ l ice cold homogenized buffer (PBS, 50ug/ml

Aprotinin, 1mM Sod. Vanadate, 25mM Sod. fluoride) in micro Polytron homogenizer , then add equal volume 2X cold lysis buffer (200 mM Nacl, 0.4% NP-40, 0.4% Sod. deoxycholate, 0.2% SDS, 100mM Tris-HCl pH:8.0), and quickly homogenized.

50 μ g Protein samples were boiled in 3x sample buffer (volume ratio 1:2.5) at 94 ℃ 10 min denatured, and electrophoresis by 7.5% gel and transferred to PVDF paper. Transfer was at 25mV overnight in transfer buffer (25mmole/L Tris, 190mmole/L glycine, and 10% methanol). Blots were then incubated with blocking buffer (TBS plus 5% no-fat milk and 0.1% tween 60) for 2 hours at room temperature. Western blot analysis was performed with mouse monoclonal anti-Rb (1:200), followed by incubation with goat anti-mouse (1:500) coupled to horseradish peroxidase. Western blotting was developed using an enhanced chemiluminescence detection kit.

7. Statistical analysis

Statistical analysis was performed using an SPSS statistical software program (SPSS Inc. Chicago, IL). The analyses of the associations between Rb, p21, cyclin D1 protein expression and clinical characteristics for lung cancer (age, sex, T, N, M, tumor stage, tumor type, tumor grade, smoking habit) were performed by the Pearson Chi-Square test and logistic regression. Survival curves were estimated using Kaplan-Meier method, and

survival rates were compared using the log-rank test.

Ⅳ. Results

1. Rb Immunohistochemistry

The clinico-pathological parameters of 247 NSCLC patients were shown in Table 1. The study population had a high proportion of patients with adenocarcinoma (57.9%), grade 2 (61.5%), T2 (64.4%), N0 (49.4%), M0 (95.5%), and tumor stage I (36.8%) and IIIa (40.5%). To determine the frequency of Rb inactivation in Taiwanese NSCLC, 247 tumor specimens were used to evaluate the Rb protein expression by immunohistochemical analysis. Surprisingly, our data indicated that high frequency of Rb inactivation was found (169/247, 68.4%). In this study, not any immunostaining response of Rb found in nucleus of tumor cells was considered the Rb inactivation (Fig 3A), and the positive expression of Rb was shown in Fig 3B. Among the clinico-pathological parameters, Rb protein expression was significantly associated with histological type ($p=0.000$) and smoking status ($p=0.005$), respectively (Table 2). Inactivation of Rb protein was frequently found in NSCLC patients with adenocarcinoma (81.1%) and non-smokers. This result suggests that Rb inactivation is more important in the tumorigenesis of

adenocarcinoma lung cancer compared to low frequency of Rb inactivation found in squamous cell lung cancer.

2. P21 Immunohistochemistry

P21 acts as an inhibitor to inactivate Rb protein mediating by inhibition of CDK and cyclin protein to join an active complex, which was involved in the phosphorylation of Rb protein. Therefore, we hypothesized that the Rb protein inactivation may be partly mediated by the inactivation of p21 protein. We immunohistochemically to evaluate p21 protein expression in tumor specimens. Our immunohistochemical data showed that p21 protein was focally expressed in normal bronchial epithelium cell or stromal cell. P21 protein was frequently detected in well-differentiated cells, and the expressions were considered as an internal control. Our results indicated that 54.3% of patients (134/247) p21 had positive immunostaining (Table 3, Fig 3C, D). However, well-differentiated tumor cells had a trend showing the positive p21 immunostaining. Moreover, we found that 77% of p21-negative expression was Rb-negative tumor, and 66.7% of Rb-positive tumor was p21-positive expression. A significant correlation ($p=0.008$) was

seen in the p21 and Rb protein expressions.

3. Cyclin D1 Immunohistochemistry

Our data showed that 85 (34.4%) of the 247 NSCLC had cyclin D1 positive immunostaing (Fig 3E, F). The relationships between the cyclin D1 protein expression and clinico-pathological parameters, was only grade status was observed ($p=0.008$). Except grade status, no statistical association was found between cyclin D1 expression and clinico-pathological characteristics. In addition, we also observed that patient without cyclin D1 protein expression was frequently found patients with Rb-negative tumor (77.2%). There was a significant association between the expression of cyclin D1 and Rb protein ($p=0.000$). Therefore, we suggest that cyclin D1 protein expression may partly involve the Rb protein inactivation, but the role of cyclin D1 seem to play less important than p21 in Rb inactivation.

4. Detection of mRNA expression and promoter site of Rb gene in lung tumor tissues determined by RT-PCR and genomic PCR

To elucidate Rb inactivation in NSCLC whether was reduced in transcription level, we used RT-PCR and genomic PCR to examine whether m-RNA and promoter was detected. Unexpectedly, only 16% of patients (13/81) did not be detected Rb m-RNA by RT-PCR. The results from Rb mRNA showed that no difference was observed between patients with and without Rb inactivation by immunohistochemistry analysis (Table 4). This result suggests that transcription level in Rb gene appears to be not associated with inactivation of Rb protein. Bookstein et al. (1990) proposed that Rb gene inactivation is due to Rb gene promoter deletion. We thus used genomic PCR to detect whether deletion of Rb gene promoter caused the inactivation of Rb protein. Our data indicated that 18.9% of patients (11/58) was occurred the deletion of Rb promoter (Table 5). The patient with Rb protein inactivation appeared to have relative higher deletion of Rb promoter than patients with Rb protein expression. However, this was not a statistical significance because the patients number with deletion of promoter was remarkably lower than patient with the presence of

Rb promoter. From the above results, we concluded that the deletion of Rb promoter seem to play minor important role in the inactivation of Rb protein.

5. The Rb protein expression in lung tumor tissues determined by Western blot analysis

According to immunohistochemistry data, Rb protein was not detected in 68.4% of tumor specimens. To confirm the high frequency of Rb protein inactivation in tumor specimens, we further analyzed the Rb protein levels in fresh lung tumor tissues by Western blot analysis, and revealing Rb protein levels in tumor tissues from patients with Rb expression were higher than those of non-tumor tissues. In addition, Rb protein levels in normal lung tissues from two healthy donors by accident were significantly higher than non-tumor tissues from NSCLC patients. There were 75.5% and 100% of Rb protein in non-tumor and normal tissues from lung cancer and non-cancer controls detected, respectively. However, Rb protein in tumor tissues were mostly decreased (Fig 4). These results suggest that Rb protein expression be significantly decreased in the development of lung cancer, although the some patients with Rb protein

expression by Western blot can not be consistent to Rb protein expression by immunohistochemistry.

6. Human papillomavirus assay with nested PCR

The ubiquitin-proteasome pathway degrades both of cytosolic and nuclear protein (Spataro et al., 1998), and E6, E7 oncoproteins of HPV can promote the p53, Rb protein degradation by activating the ubiquitin-proteasome pathway (Boyer et al., 1996; Scheffner et al., 1992; Soini et al., 1996; Spataro et al., 1998). Previous studies also indicated that Rb protein degradation was found in tumor cell lines when they were infected with HPV (Patrick et al., 1994; Boyer et al., 1996). Thus, we hypothesize that Rb inactivation may be related with relatively higher infection of HPV in Taiwanese NSCLC patients. The quality of DNA from micro-dissection can be checked by PCR amplification of β-globin gene using specific primer to produce 536 bp products. Our result makes sure that the genomic DNA micro-dissected from tumor specimens was indeed amplified by PCR. We were also from this data to exclude a false negative. The negative control was used to amplify sterile water to test whether or not PCR formed, and we used

genomic DNA of HeLa cell as a positive control. Our results showed that HPV-18 DNA was shown in 32 of 58 NSCLC patients (Fig 5). According to the tumor types, the patients had positive HPV infection including 15 of 27 adenocarcinomas and 16 of 30 squamous cell carcinomas and one of large cell carcinoma patient. Although there was no significant association between HPV DNA detected and Rb protein inactivation, 21 (65.6%) of patients with HPV infection were frequently observed to be Rb protein inactivation compared with previous studies (Table 6).

7. The prognostic role of Rb, p21, and cyclin D1 in Taiwanese NSCLC

The overall survival for this cohort of patients was analysis by Kaplan-Meier plot and Log-rank test. A statistically significant difference in survival was seen in age ($p=0.0218$), stage ($p=0.0062$), N-factor ($p=0.0001$) (Table 7). However, Rb protein expression was not acted as a significant prognostic factor, although patients with Rb inactivation had a worse survival compared with the patients with Rb positive expression. The median survival time of Rb positive patients was 1174 days,

which was significantly longer than patients without Rb expression whose median survival time was 840 days.

The Kaplan-Meier survival curves of Rb, p21, and cyclin D1 protein expressions were shown in Fig 6, Fig 7, and Fig 8 respectively. The survival curves of patient with Rb, p21, or cyclin D1 protein expression were not statistically different from patients without those protein expressions, respectively. When we combined p21 and Rb protein expression to predict the survival by Kaplan-Meier survival analysis (Fig 9). We only observed the status of Rb expression to be act as a significant prognostic factor in NSCLC patients without p21 protein expression ($P = 0.0078$, Fig 10). The survival of patients with Rb inactivation was significantly shorter than patients with Rb expression. This result suggests that the expression of Rb, p21, and cyclin D1 were not acted as an independently prognostic factor in Taiwanese NSCLC patients.

V. Discussion

Many reports regarding the role of Rb-pathway in the development of human neoplasm were focused on investigations of gene alterations of Rb, cyclin D1 and p16, including lung tumorigenesis (Tanaka et al., 1998; Belinsky et al., 1998). Because cell cycle proliferation was demonstrated by the loss of p16 and Rb protein expression, and overexpression of cyclin D1 (Lukas et al., 1995a; Sherr et al., 1996). Previous studies were focused on the role of p16 inactivation in human tumorigenesis because methylation of promoter and deletion of p16 gene were frequently occurred in the development of human neoplasm. Because the role of p16 in Rb pathway has been well investigated. Therefore, in the present study, we investigate the role of p21 on the Rb-pathway in lung tumorigenesis. The expression of p21 was up-regulated mediating by either p53-dependent pathway or p53-independent pathway (Michieli et al., 1994; Marchetti et al., 1996). P21 protein inhibited Rb phosphorylation mediating though the inhibition of cyclin D and cyclin E/CDK complex activity. In this study, we investigate the role of Rb pathway consisting of p21, cyclin D1, and Rb in lung tumorigenesis in Taiwan.

Our data showed that high frequency of Rb inactivation (68.4%) was found in Taiwanese NSCLC when we compared with regarding previous studies in lung cancer, showing Rb inactivation ranged from 28% to 42% (Xu et al., 1991; Reissmann et al., 1993; Sakaguchi et al., 1996; Gorgoulis et al., 1998; Tanaka et al., 1998; Kawabuchi et al., 1999). The data were summarized in Table 8. We suspect that different antibodies used in different studies may cause the distinct results. We used two anti-Rb antibodies in our study. One is anti-Rb antibody (SC-102) purchased from Santa-Cruz Company; and its recognized epitope probably full length of Rb protein. The other antibody (PMG3-245 epitope: amino acid 300-380) purchased from PharMingen. San Diego, CA Company was commonly used previous studies. Similar results of Rb inactivation were observed between these two antibodies used. Thus, we have a strong confidence to confirm our finding showing that high frequency of Rb inactivation was actually found in this cohort study.

Rb protein expression was shown significant association with smoking status ($p=0.005$) and histologic type ($p=0.000$) when we statistically analyzed by Chi-Square test. The histologic type still had a significant correlation analyzed by

logistic regression (p=0.000). It is interesting to note that a high frequency of Rb protein inactivation was found in adenocarcinoma lung cancer. On the contrary, Relatively lower Rb protein inactivation in lung adenocarcinoma (9%-25%) was previously reported (Xu et al., 1991; Masahito et al., 1994; Dosaka-Akita et al., 1997; Lingfei et al., 1998). This result was contrast to other reports, indicating that Rb inactivation in squamous cell lung carcinoma was relatively higher than adenocarcinoma. Nevertheless, Rb inactivation may play different roles on the development of squamous cell and adenocarcinoma lung cancer in Taiwan.

45.7% of NSCLC were shown to have p21 negative immunostaining. This finding differ from previous reports, showing that p21 negative immunostaining ranged from 20% to 30% (Marchitti et al., 1996; Caputi et al., 1998a). Surprisingly, 65% of p21 negative in NSCLC was observed by Komiya et al. (1998). All of these studies used the same antibody EA-10 to detect the expression of p21 protein. Among the studies were just only different in sample size. The absence of p21 protein expression was not responsible for gene mutation of p21 according to previous reports (Shimizu et al., 1996; Marchetti et al., 1995; Li et al., 1995). Our another data indicated that 37 of

81 patients (46.7%) had undetectable p21 mRNA expression. This result indicated that the absence of p21 protein expression was imputed by the alteration of transcription level (unpublished data, 1999). In addition, high frequency of HPV infection in Taiwanese NSCLC may enhance the degradation of p21 mediating by the E6 and E7 oncoproteins of HPV which activated the ubiquitin-proteasome pathway (Chang et al., 1998).

Our data showed that no association was observed between p21 expression and clinico-pathological characteristics. When we examined the p21 immunostaining profiles, we found most of tumors with over 10% of tumor cells showing positive immunostaining in the tumor cells were poorly differentiated (Fig 3D). Caputi et al. (1998a) reported that p21 were detected in well-differentiated area of squamous carcinomas, and this finding was not consistent with our immunostaining results. The overexpression of p21 protein in poorly differentiated cells seem to be conflicting to the concept of cell cycle arrest regulated by p21 expression (Cunto et al., 1998). In our PCNA immunostaining study showed that a high degree of immunostaining was shown in tumor tissues from Taiwanese NSCLC. P21 protein may be inactivated by overexpression of PCNA to result cell progression (Waga et al., 1994). Another

evidence showed that HPV oncoprotein occupied the p21-binding domain of cyclin binding site and loss of inhibition of cyclin/CDK activity (Funk et al., 1997, 1998). All points supported p21 inactivation may be mediated by the interaction with other proteins.

Cyclin D1 expression was found in 34.5% of 247 NSCLCs in this study. Our finding was relatively lower than data from other reports (47-70%) (Shapiro et al., 1995; Betticher et al., 1996; Lingfei et al., 1998). The CDK protein expressions from Western blot analysis in lung tumor tissue were similar with those in normal lung tissue (data not shown). These results suggest that the expression of CDK and cyclin D1 may be not associated with Rb protein inactivation. Therefore we suggested that the absence of p21 protein is the major defect cause Rb inactivation, not by alteration of cyclin D1-CDK4,6 and cyclin E-CDK2 complex inactivation.

HPV-18 infection was found in 55.6% of adenocarcinoma and 63.3% of squmous carcinoma by nested PCR amplification method. Moreover 65.6% of HPV DNA was presented in Rb protein negative immunostaining (Table 6). We summarized previous studies in Table 9, showing that the infection frequency of HPV was ranged from 0% to 36%. Yousem et al. (1992) showed that higher frequency of HPV infection in squamous

carcinomas (30%) was compared with adenocarcinoma (9%). HPV infection could be a cofactor, interacting with cigarette smoking induces squamous carcinoma. In our study, women who don't smoke, had 8 in 9 of adenocarcinoma were HPV infection. The possible reason was HPV migrated from other sites of infection such as cervical, for women had high frequency of cervical HPV infection and this relationships was needed us to further clarify. Several investigations have shown that HPV oncoprotein induced Rb protein degradation through ubiquitin-proteasome pathway (Boyer et al., 1996; Scheffner et al., 1992; Soini et al., 1996; Spataro et al., 1998), but with normal level of Rb m-RNA (Wazer et al., 1995). Thus, we suggest that high frequency of HPV infection in NSCLC may be responsible for the alteration of Rb pathway in lung tumorigenesis.

Not only protein and protein interaction involved in regulating Rb protein expression, that inactivated transcription of Rb gene was probably contributed to Rb protein inactivation. We have detected the m-RNA level by RT-PCR method, only 16% tumors were shown the absence of m-RNA (Table 4). This result was not correspondent with our immunohistochemical data. Whereas the frequency of undetectable Rb mRNA in tumor tissues was higher than those of normal lung tissues from non-cancer controls. However, consistently detectable m-RNA was seen in 81% of tumor tissues and 84% of non-tumor tissues (data not shown). Rb m-RNA level was undetected in 16% tumors and was not consist with IHC data. This was not due to contamination of normal tissue, for in the p21 mRNA detection,

we had found 46.7% mRNA were undetected. Possible reason was HPV infection degraded Rb protein but not decrease mRNA level or abnormal phosphorylation of Rb protein. We also observed that 8.7% patients with Rb protein expression were not detectable its m-RNA. This may be due to the instability of 4.7kb m-RNA. When we compared Rb protein and RT-PCR results of tumor and non-tumor tissues, it revealed that 11% of cases were both undetectable. In addition, promoter deletion of Rb gene was also examined in this study, showing 18.9% of cases were the deletion of promoter region. Moreover promoter deletion of Rb gene was found in 25% of patients with Rb negative immunostaining. Therefore, we suggest that only small part of Rb inactivation be due to defect on promoter site of Rb gene and alteration of transcription machinery.

According to the Western blotting data, 90.6% (48/53) of tumor was shown to be the presence of Rb protein. This data was not consistent with immunohistochemical results. One possible reason is that Western blotting was detected the total Rb proteins of whole tissue, but immunohistochemical analysis was directly detected Rb protein of single tumor cell. Xu et al. (1991) postulated that low Rb protein level of a cell was unable to detect by immunohistochemical analysis. Our Western blotting showed that the Rb band intensity of tumors with Rb positive immunostaining was stronger than tumors with Rb negative immunostaining. The higher molecular weight of phosphorylated Rb was seen in tumors with Rb positive immunostaining comparable with tumors with Rb negative

immunostaining. Moreover, the Rb band intensity of non-tumor tissues was weaker than that of normal lung tissues. One study indicated that Rb gene promoter was positively autoregulated by its protein (Park et al., 1994). Thus, a relatively low level of Rb protein seem to be not able to induction of enough Rb protein for regulating cell cycle of tumor cells. We consider that dramatically decreased of Rb protein may be caused by aberrant Rb protein expression in lung tumor. In this 247 NSCLC patients study cohort, The important findings were summarized as follows:

1. High frequency of Rb protein inactivation was found in Taiwanese NSCLC, especially for adenocarcinoma lung cancer.

2. The most important cause to result Rb inactivation to be the high frequency of p21 negative expression (Fig 11), that may be caused by HPV infection and defect in transcription level.

3. The ubiquitin-proteasome pathway may be involved in the degradation of Rb and p21 protein to cause the high frequency of the absence of both proteins. E6 and E7 proteins from HPV infection can induce the ubiquitin-proteasome.

4. The alteration of Rb gene in transcription level and the expression of cyclins and CDK proteins may have minor contribution on the alteration of Rb pathway in Taiwanese NSCLC compared with aberration of p21 protein.

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Table 1. Patients and tumor characteristics.

Characteristics	No. of patients	%
Total no. of patients	247	
Age (mean±SD)	61.91±10.51	
≦55	67	27.1
>55	180	72.9
Range	31-84	
Gender		
Female	74	30.0
Male	173	70.0
Histological type		
AD	143	57.9
SQ	98	39.7
LC	6	2.4
Grade		
1	10	4.0
2	152	61.5
3	85	34.4
T status		
1	19	7.7
2	159	64.4
3	49	19.8
4	18	7.3
unknown	2	0.8
N status		
0	122	49.4
1	46	18.6
2	79	32.0

Table 1 (Continued).

M status		
0	236	95.5
1	11	4.5
Tumor stage		
Ⅰ	91	36.8
Ⅱ	29	11.7
Ⅲa	100	40.5
Ⅲb	17	6.9
Ⅳ	10	4.0
Smoking status		
Non-smoker	121	49.0
Smoker	126	51.0

AD: Adenocarcinoma lung cancer.

SQ: Squamous cell lung cancer.

LC: Large cell lung cancer.

Table 2. Relationships between Rb immunostaining and clinico-pathological parameters in 247 NSCLC patients.

Clinical characteristics	No. patients	Rb immunostaining - (%)	Rb immunostaining + (%)	*p* value
Total patients	247	169 (68.4)	78 (31.6)	
Age				
≦55	67	48 (71.6)	19 (28.4)	
> 55	180	121 (67.2)	59 (32.8)	0.506
Gender				
Male	173	113 (65.3)	60 (34.7)	
Female	74	56 (75.7)	18 (24.3)	0.109
Tumor type				
AD	143	116 (81.1)	27 (18.9)	
SQ	98	51 (52.0)	47 (48.0)	
LC	6	2 (33.3)	4 (66.7)	**0.000**
Grade				
1	10	8 (80.0)	2 (20.0)	
2	152	100 (65.8)	52 (34.2)	
3	85	61 (71.8)	24 (28.2)	0.461
T status*				
T1	19	14 (73.7)	5 (26.3)	
T2	159	108 (67.9)	51 (32.1)	
T3	49	31 (63.3)	18 (36.7)	
T4	18	14 (77.8)	4 (22.2)	0.664
N status				
N0	122	81 (66.4)	41 (33.6)	
N1,N2	125	88 (70.4)	37 (29.6)	0.498

Table 2 (Continued).

M status				
M0	236	160 (67.8)	76 (32.2)	
M1	11	9 (81.8)	2 (18.2)	0.328
Tumor stage				
I	91	60 (65.9)	31 (34.1)	
II	29	21 (72.4)	8 (27.6)	
IIIA	100	67 (67.0)	33 (33.0)	
IIIB	17	13 (76.5)	4 (23.5)	
IV	10	8 (80.0)	2 (20.0)	0.791
Smoking status				
non-smoker	121	93 (76.9)	28 (23.1)	
smoker	126	76 (60.3)	50 (39.7)	**0.005**
p21 expression				
-	113	87 (77.0)	26 (23.0)	
+	134	82 (61.2)	52 (38.8)	**0.008**
cyclin D1 expression				
-	162	125 (77.2)	37 (22.8)	
+	85	44 (51.8)	41 (48.2)	**0.000**

AD: Adenocarcinoma lung cancer.

SQ: Squamous cell lung cancer.

LC: Large cell lung cancer.

*: T value of 2 cases were missed.

Table 3. Relationships between p21 immunostaining and clinico- pathological parameters in 247 NSCLC patients.

Clinical characteristics	No. patients	p21 immunostaining - (%)	p21 immunostaining + (%)	*p* value
Total patients	247	113 (45.7)	134 (54.3)	
Age				
≦55	67	28 (41.8)	39 (58.2)	
>55	180	85 (47.2)	95 (52.8)	0.446
Gender				
Female	74	35 (47.3)	39 (52.7)	
Male	173	78 (45.1)	95 (54.9)	0.749
Tumor type				
AD	143	71 (49.7)	72 (50.3)	
SQ	98	38 (38.8)	60 (61.2)	
LC	6	4 (66.7)	2 (33.3)	0.146
Grade				
1	10	4 (40.0)	6 (60.0)	
2	152	65 (42.8)	87 (57.2)	
3	85	44 (51.8)	41 (48.2)	0.383
T status*				
T1	19	6 (31.6)	13 (68.4)	
T2	159	78 (49.1)	81 (50.9)	
T3	49	24 (49.0)	25 (51.0)	
T4	18	4 (22.2)	14 (77.8)	0.091
N status				
N0	122	57 (46.7)	65 (53.3)	
N1,N2	125	56 (44.8)	69 (55.2)	0.762

Table 3 (Continued).

M status				
M0	236	110 (46.6)	126 (53.4)	
M1	11	3 (27.3)	8 (72.7)	0.208
Tumor stage				
I	91	40 (44.0)	51 (56.0)	
II	29	17 (58.6)	12 (41.4)	
IIIa	100	50 (50.0)	50 (50.0)	
IIIb	17	4 (23.5)	13 (76.5)	
IV	10	2 (20.0)	8 (80.0)	0.065
Smoking status				
non-smoker	121	51 (42.1)	70 (57.9)	
smoker	126	62 (49.2)	64 (50.8)	0.266
cyclin D1 expression				
-	162	90 (55.6)	72 (44.4)	
+	85	23 (27.1)	62 (72.9)	**0.000**
Rb expression				
-	169	87 (51.5)	82 (48.5)	
+	78	26 (33.3)	52 (66.7)	**0.008**

AD: Adenocarcinoma lung cancer.

SQ: Squamous cell lung cancer.

LC: Large cell lung cancer.

*: T value of 2 cases were missed.

Table 4. The correlation between the Rb protein expression from immunohistochemistry and Rb mRNA detected by RT-PCR in 81 NSCLC patients.

Rb m-RNA	No. of patients (%)	Rb immunostaining	
		- (%)	+ (%)
-	13 (16.0)	9 (69.2)	4 (30.8)
+	68 (84.0)	41 (60.3)	27 (39.7)

Table 5. The association between Rb protein expression evaluated by immunohistochemistry and the detection of Rb gene promoter detected by genomic PCR in 58 NSCLC patients.

Promoter site	No. of patients (%)	Rb immunostaining	
		- (%)	+ (%)
Deletion	11 (18.9)	9 (15.5)	2 (3.4)
Present	47 (81.1)	27 (46.6)	20 (34.5)

Table 6. The correlation between the Rb protein expression from immunohistochemistry and HPV18 DNA detected by genomic PCR in 58 NSCLC patients.

HPV18 DNA	No. of patients(%)	Rb immunostaining	
		- (%)	+ (%)
-	26 (44.8)	14 (53.8)	12 (46.2)
+	32 (55.2)	21 (65.6)	11 (34.4)

Table 7. Relationships between median survival and clinico-pathological parameters by Kaplen-Meier analysis and Log-rank test.

Clinical characteristics	No. of patients	Median survival	*p* value
Age			
≦55	107	1057	
>55	109	884	**0.0218**
N value			
0	106	1426	
1	110	567	**0.0001**
Tumor stage			
Ⅰ, Ⅱ	104	1174	
Ⅲ, Ⅳ	112	675	**0.0062**
p21 status			
-	107	884	
+	109	1057	0.3287
cyclin D1 status			
-	148	840	
+	68	1545	0.2283
pRb status			
-	156	840	
+	60	1171	0.1514

Table 8. Literature review of immunohistochemical analyses of Rb protein expression in non-small cell lung cancer.

Literature	Patients No.	Rb inactivation (%)	References
1.	36	13 (36%)	Xu et al., Cancer Res.(1991)
2.	100	6 (6%)	Higashiyama et al., Oncology (1994)
3.	119	19 (16%)	Xu et al., Clinical Cancer Res.(1996)
4.	100	15 (15%)	Kratzke et al., Cancer Res.(1996)
5.	61	23 (38%)	Sakaguchi et al., Int. J. Cancer (1996)
6.	208	42 (20%)	Masayuki et al., Clinical Cancer Res.(1997)
7.	51	17 (33%)	Betticher et al., Int. J. Cancer (1997)
8.	20	2 (10%)	Tamura et al., Int. J. Cancer (1997)
9.	91	19 (21%)	Dosaka et al., Cancer (1997)
10.	42	9 (20%)	Marchetti et al., Int. J. Cancer (1998)
11.	101	42 (42%)	Tanaka et al., Int. J. Cancer (1998)
12.	51*	20 (39%)	Kawabuchi et al., Int. J. Cancer (1999)

*:Adenocarcinoma.

Table 9. Literature review of Human papillomavirus detection in lung cancer.

Literature	Patients No.	Positive (%)	Detected method	References
1.	43	16.3 %	ISH	Bejui-Thivolet et al., Human pathology. (1990)
2.	58	12.1 %	ISH	Yousem et al., Cancer. (1991)
3.	40	0 %	PCR	Szabo et al Cancer. (1994)
4.	37	8.1 %	PCR	Al-Ghamdi et al., British. J. Cancer. (1995)
5.	36	9.3 %	PCR	Kinoshita et al., British. J. Cancer. (1995)
6.	43	36 %	ISH	Soini et al., Thorax.(1996)
7.	34	6 %	PCR	Bohlmeyer et al., Am. J. Respir. Cell. Mol. Biol. (1998)
8.	58	55.2 %	PCR	Present study

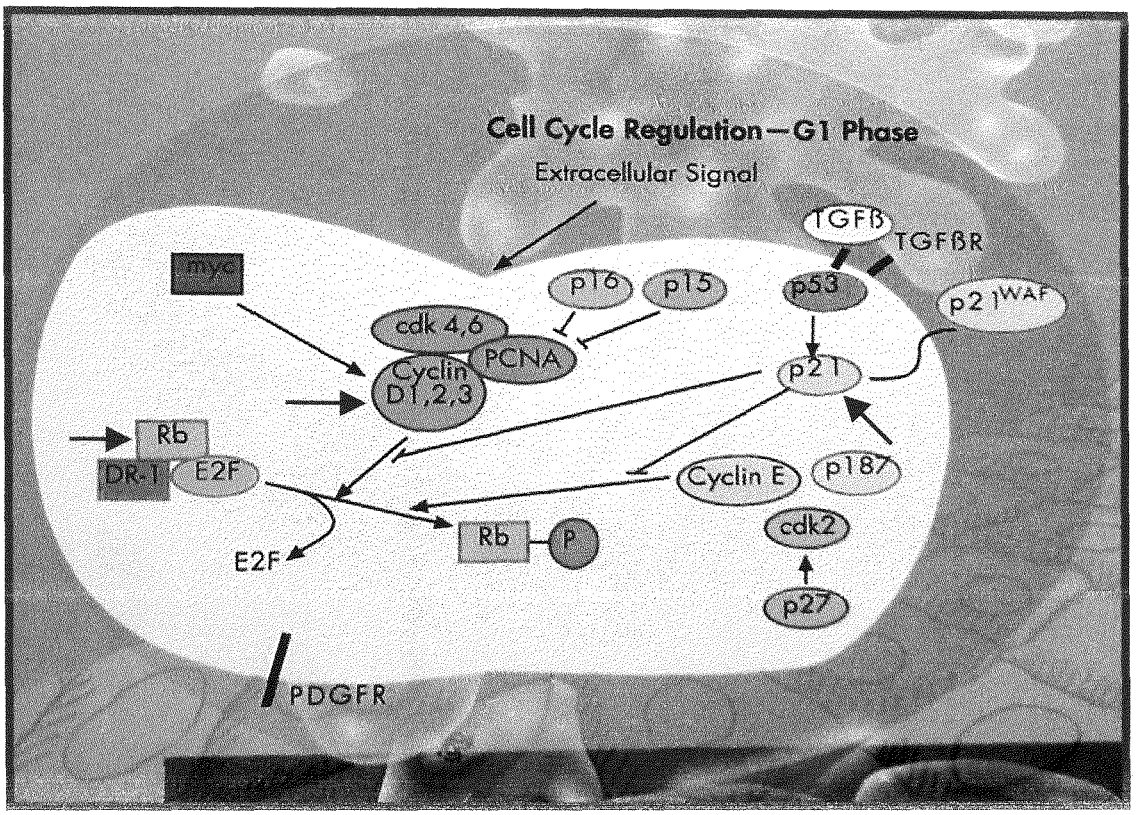


Fig 1. Genes involved in the G1 phase of cell cycle regulation, red arrows were represented the genes investigated in our study.

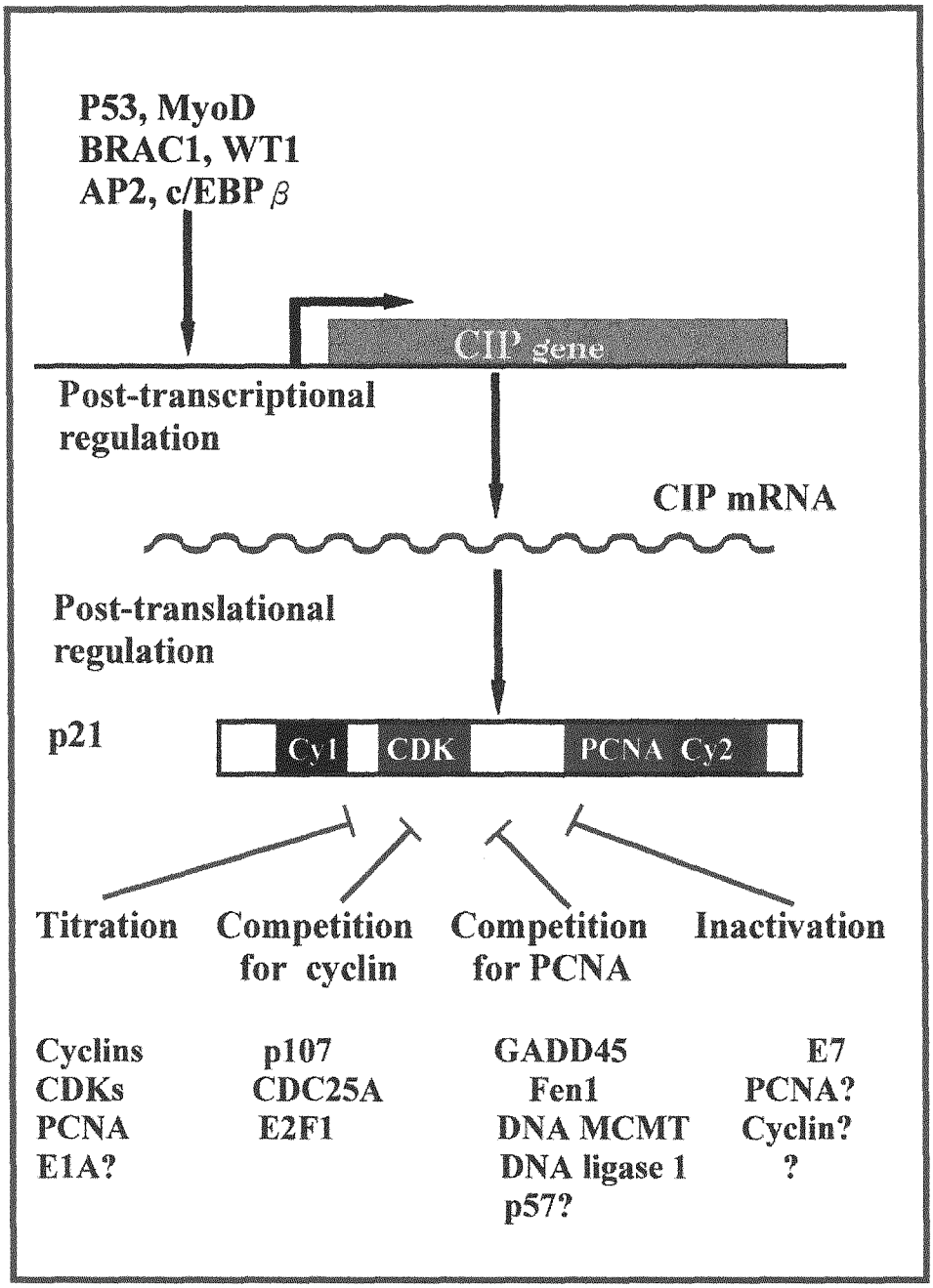


Fig 2. p21 gene expression were regulated by three different mechanisms:(1) post-transcription, (2) post-translation, (3) protein and protein interaction. Proteins interaction were included ①.titration, ②.competition, ③.inactivation.

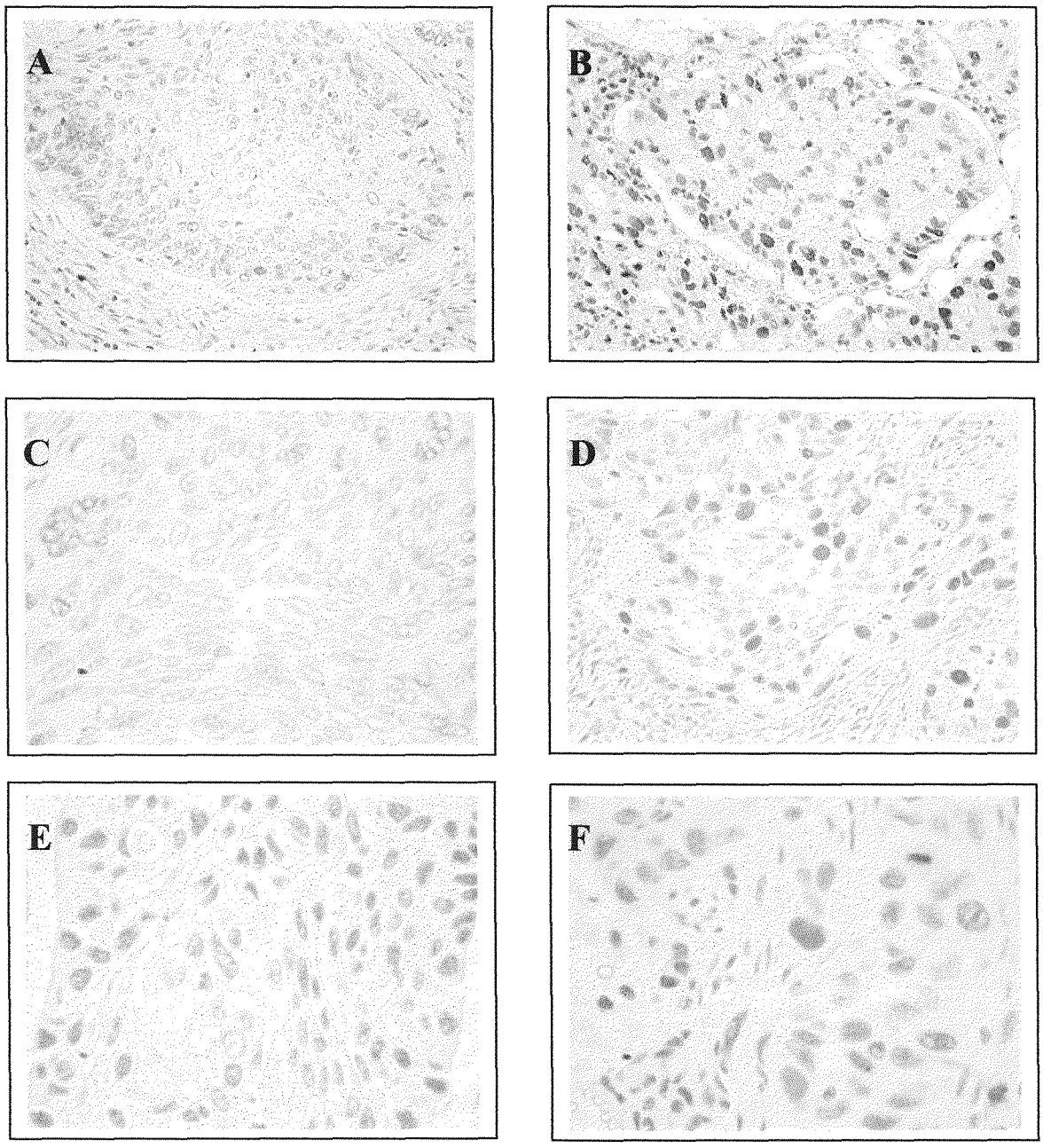
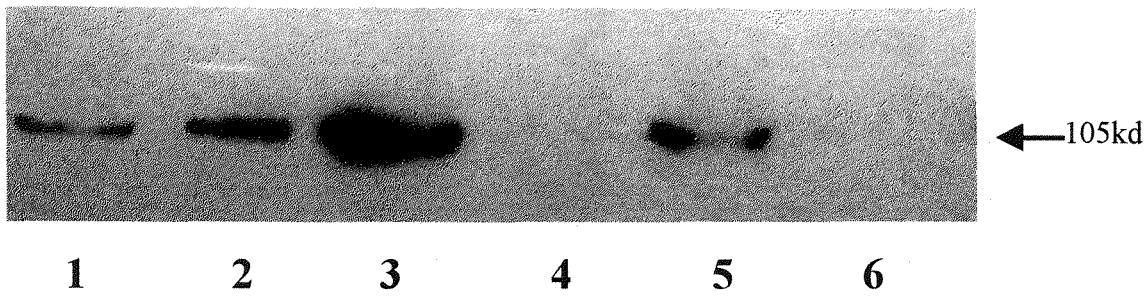


Fig 3. The immunostaining response of Rb, p21 and cyclin D1 protein in lung tumor tissue cancer from NSCLC patients. (A) Rb negative immunostaining. (B) Rb over expression (+++). (C) p21 negative immunostaining. (D) p21 over expression(+++). (E) cyclin D1 negative immunostaining. (F) cyclin D1 expression in nuclear.

A. Tumor tissue



B. Non-tumor tissue

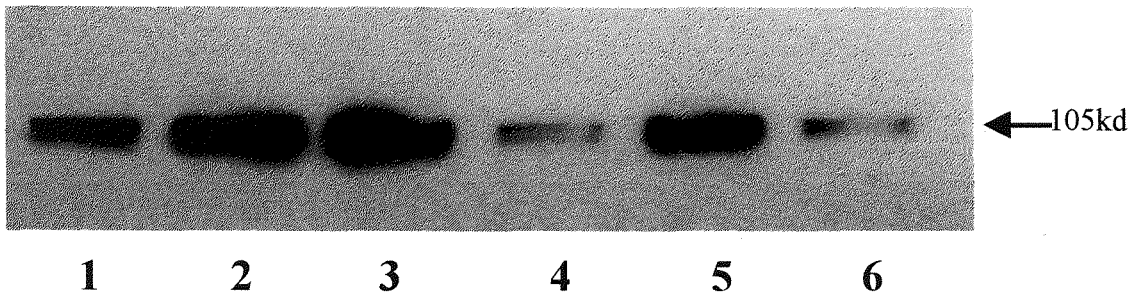


Fig 4. Representative Rb protein expression in tumor and non-tumor lung tissues evaluated by Western blot from NSCLC patients.

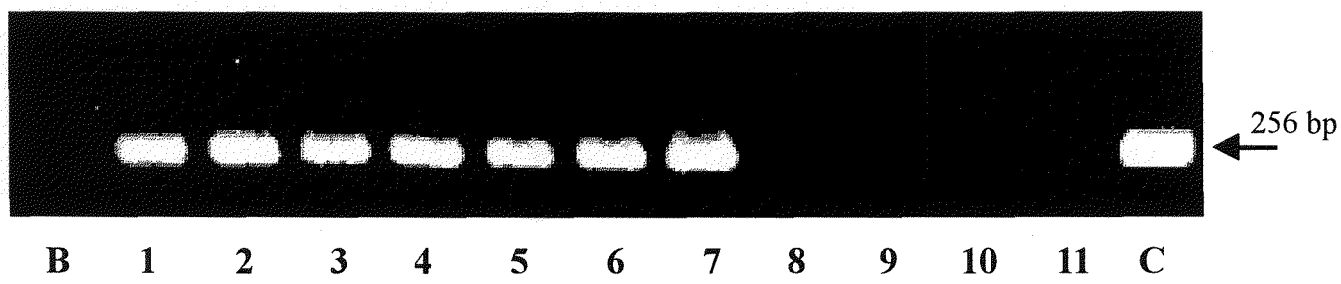


Fig 5. Representative detection of HPV-18 DNA in lung tumor specimens by genomic nested PCR. 'C': Positive control with HeLa cell, 'B': negative control with sterile water. (lane 1-7:Rb negative expression, lane 8-11:Rb positive expression).

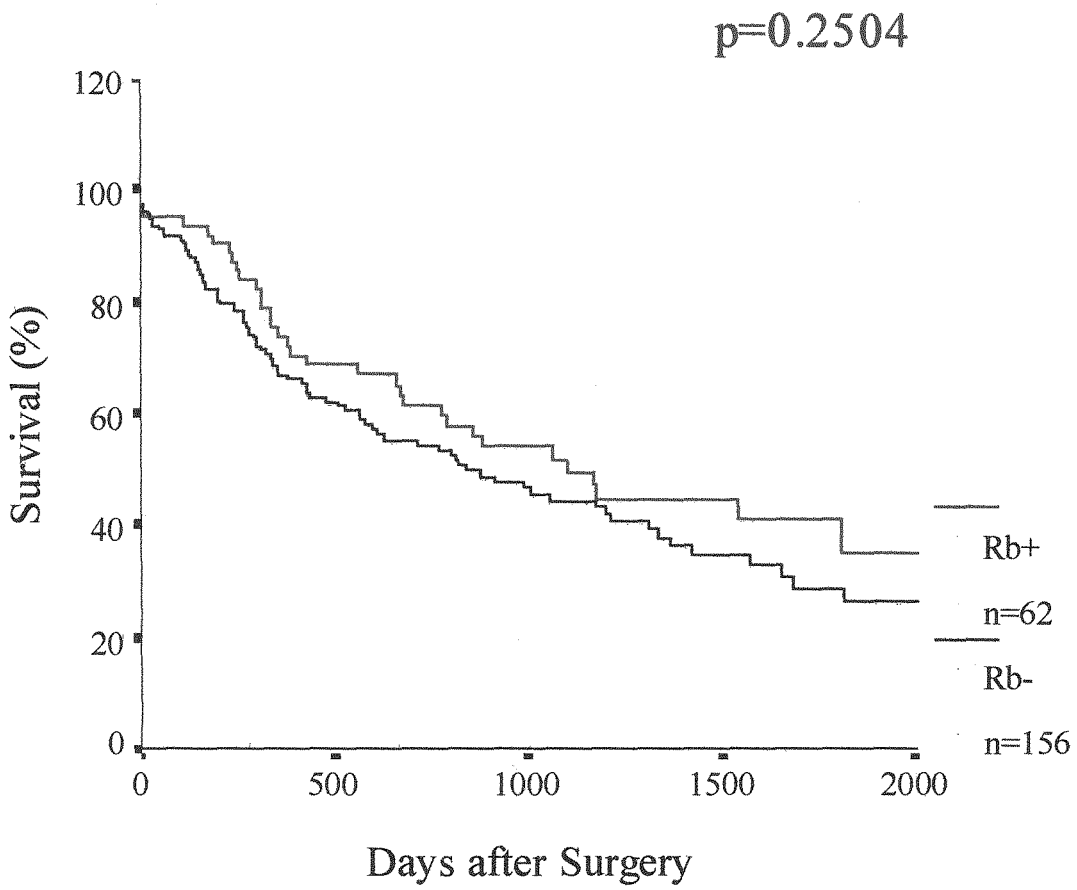


Fig 6. Survival curves of patients with Rb protein positive and negative immunostaining. The p value was shown in the right corner.

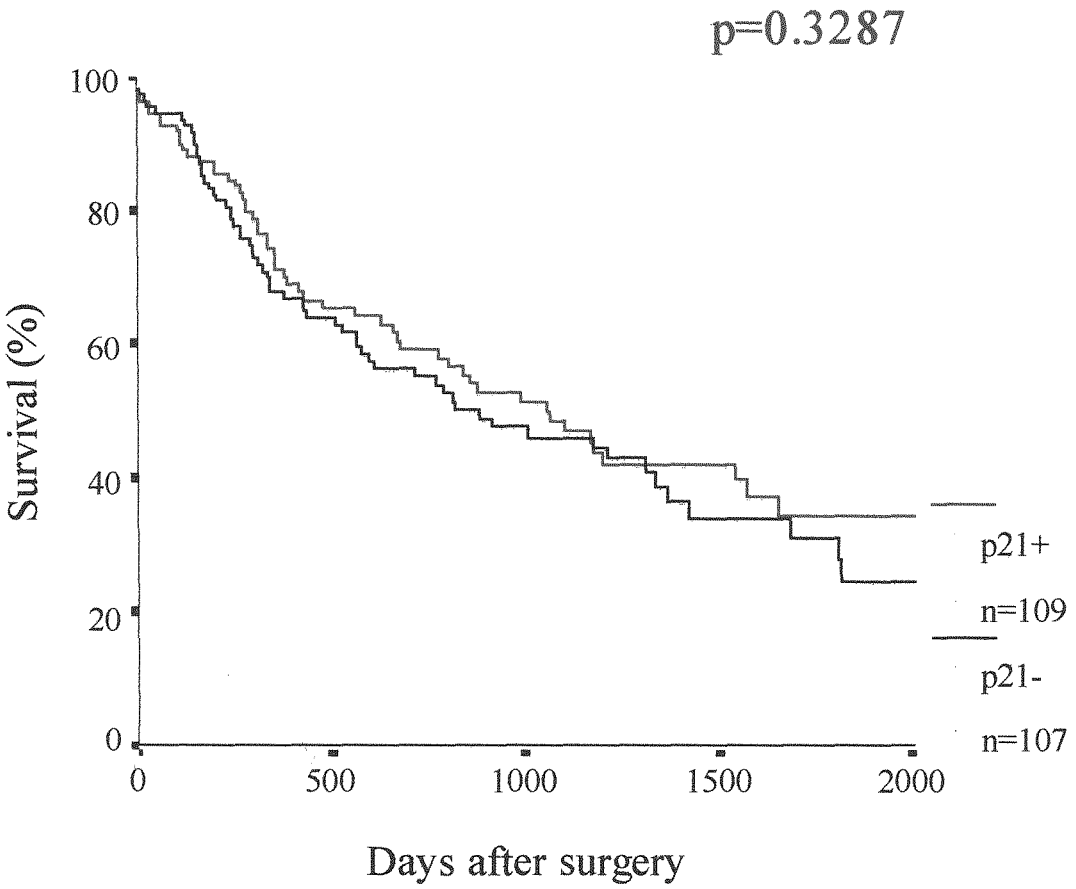


Fig 7. Survival curves of patients with p21 positive and negative immunostaining. The p value was shown in the right corner.

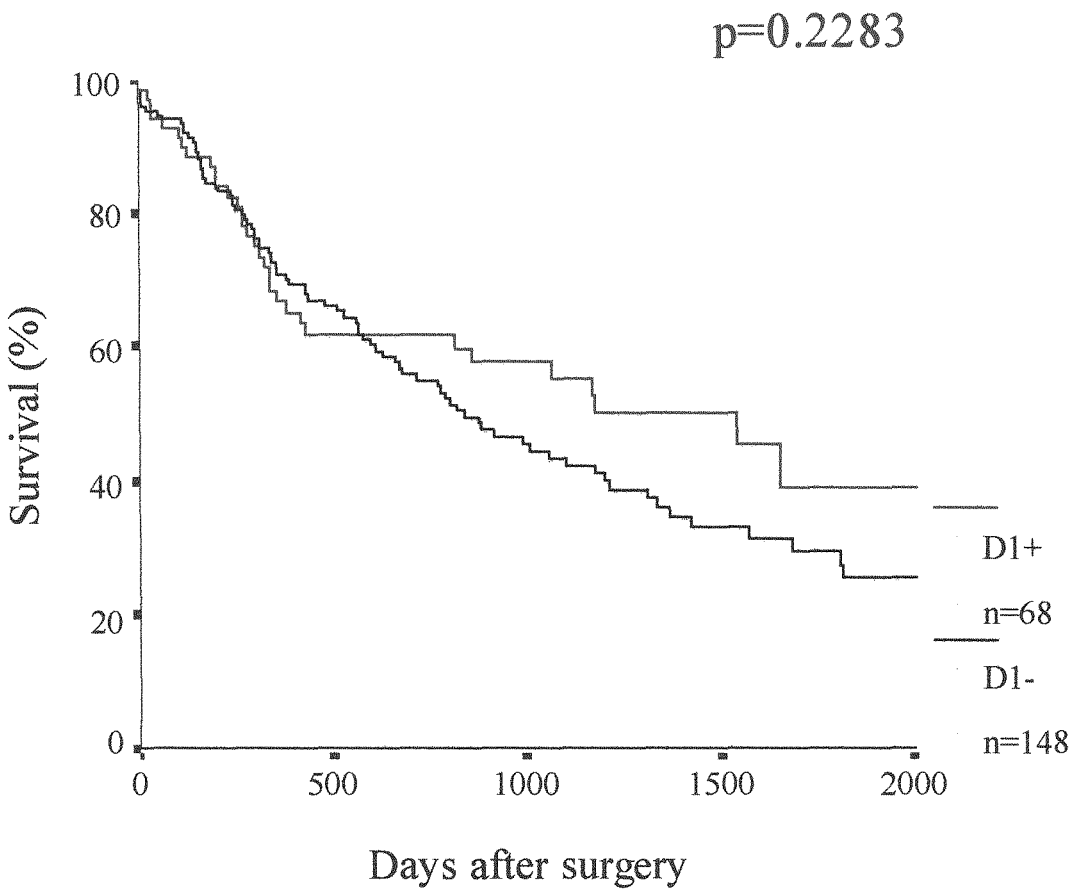


Fig 8. Survival curves of patients with cyclin D1 positive and negative immunostaining. The p value was shown in the right corner.

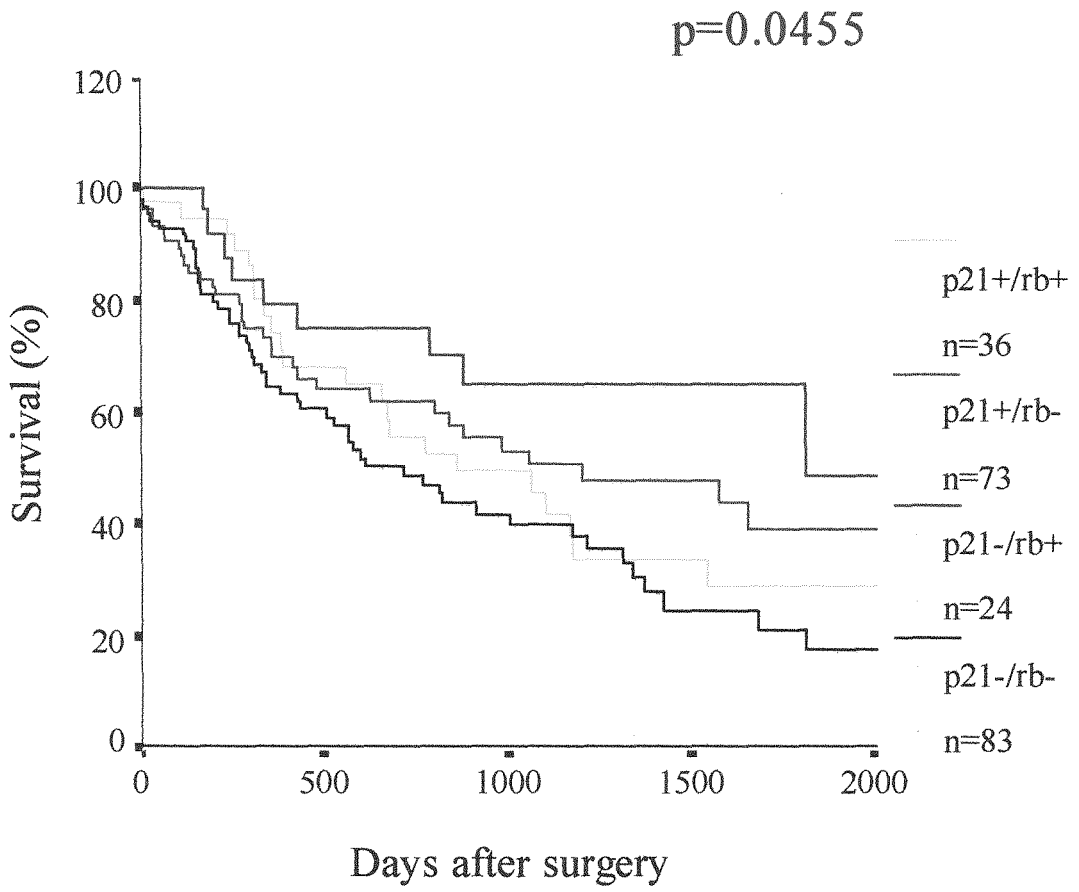


Fig 9. Survival curves for lung cancer patients, based on different p21 and pRb expression levels and constructed through Kaplan-Meier analysis. The p value was shown in right corner.

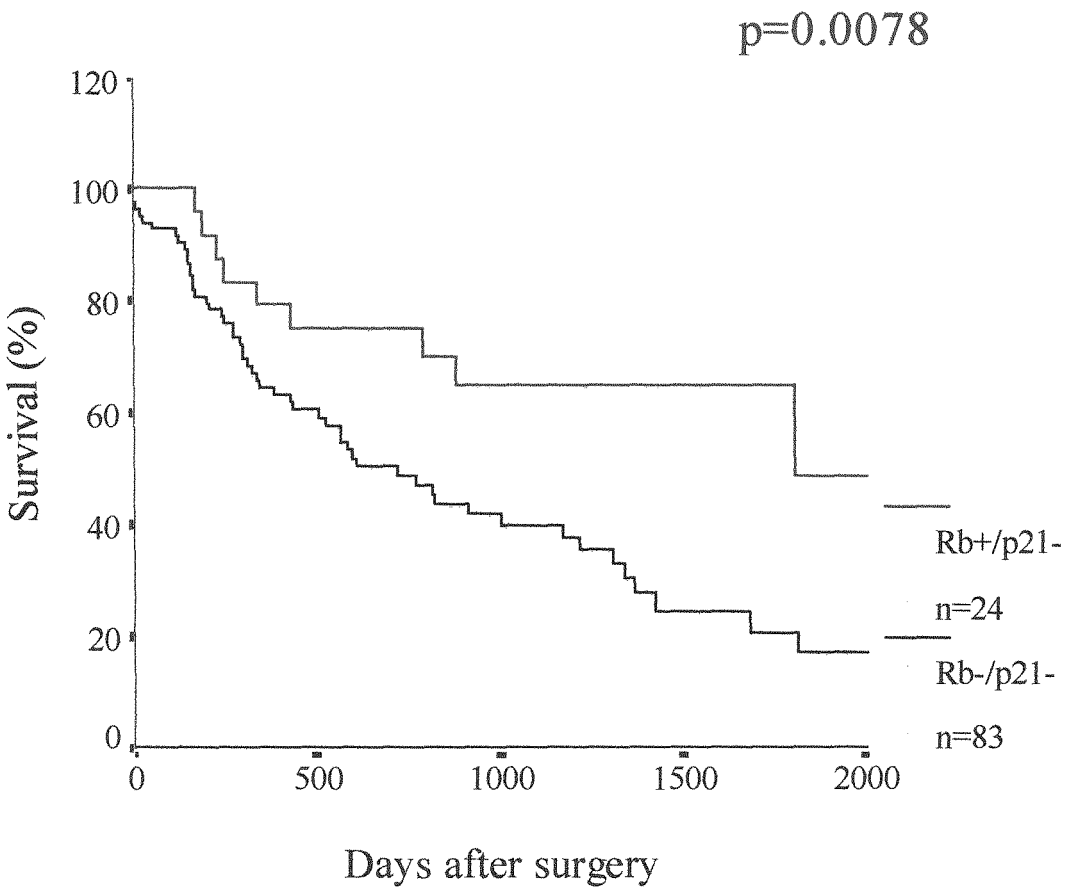


Fig 10. Survival curves of patients with Rb expression and Rb noexpression in p21 negative expression. The p value was shown in the right corner.

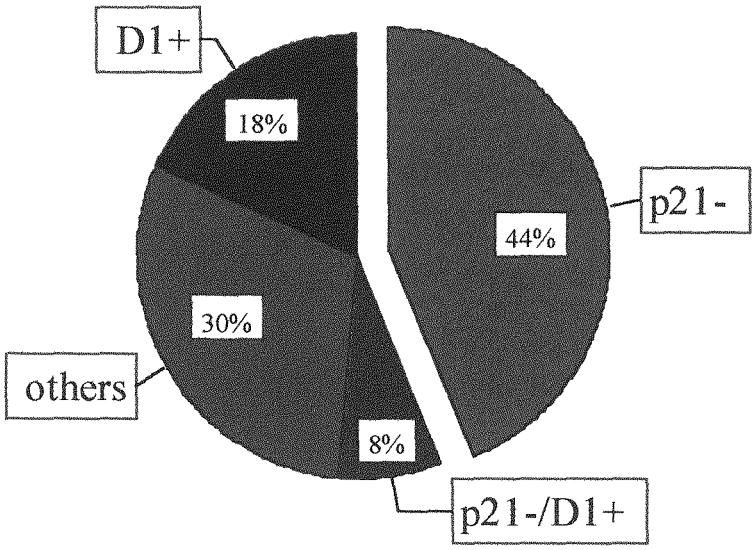


Fig 11. The contribution of alteration of p21, cyclin D1 and other factors on Rb inactivation of Taiwanese NSCLCs (n=169).