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評估週邊血液淋巴球中抑癌基因 p53 活性被誘導之程度作為肺癌感受性及預後指標之可行性

Evaluation of the p53 activity induced by carcinogens on the PBMC as the susceptibility and prognostic markers for lung cancer

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主持人：柯俊良　　中山醫學大學毒理所

一、中文摘要

已知 p53 是非常重要的抑癌基因之一，在腫瘤組織中經常發現 p53 有異常情形發生，基因座缺失(LOH)、缺失突變、點突變、病毒感染及腫瘤蛋白分解 p53 蛋白質等情形而往往造成 p53 功能喪失，最後導致腫瘤發生。p53 在癌症腫瘤中的功能表現不能只以西方吸漬法(Western blot)或免疫組織染色分析法(Immuno-histochemistry)作判斷，雖然一般而言在正常細胞中 p53 的半衰期非常短，所以不易偵測到，但與 p53 是因 LOH 或病毒、腫瘤蛋白分解而造成 p53 的消失不易區分。而細胞受到紫外線或致癌物傷害時與突變的 p53 其蛋白質表現皆較穩定，所以非常容易易偵測到，但目前也有許多報告指出 p53 蛋白表現不見得是都具有突變而來，或突變的 p53 不見得就完全不具功能。在本研究中發現正常人周邊血受 BPDE 刺激後 p53 蛋白表現量增加但其下游 p21 及 MDM2 反而降低，具有功能的 p53 也相對的對化學療法較敏感，且本研究直接以 EMSA 方法分析抽煙者與非抽煙者血液周邊淋巴球中的 p53 蛋白質生化功能，抽煙者受 BPDE 刺激誘發 p53 與 mdm2 啟動子上 p53 的反應元結合能力上升，而非抽煙者反而減少，有助於了解否因人的感受性不同及活化誘導物不同，導致 p53 活性誘發不同。。

關鍵詞：p53 抑癌基因、雙微染色體、西方吸漬法

Abstract

P53 is one important of tumor suppressor genes. p53 is always abnormal in the tumors. Inactivation of functional p53 included loss of heterozygosity, deletion mutations, point mutations, viral infection and oncoproteins degrading p53. In general, it is always undetectabled in normal cells as a result of the half-life of wide type p53 is very short. We could not differentiate between p53 degraded by oncoprotein ubiqutination or LOH and wt p53. Western blotting or immunohistochemistry is not enough represented a mutational p53 that result in an increased the half-life of the p53 protein. p53 abnormalities examined by immuno-histochemistry are not always consistent with those examined by analysis of its gene. p53 is a critical tumor suppressor gene, which can respond to multiple signals of cellular gatekeeper for growth and division. MDM2 gene is one of the downstream target genes for transcriptional activation by the product of p53 tumor suppressor gene. Transactivation of MDM2 gene and p21 expression is represented by the presence of a functional p53 protein. In our work, the p21 and mdm2 were decreased in PBMC for BPDE treatment. Functional p53 is more sensitive for chemotherapy than wild type p53. It is showed the division into two opposing extremes by BPDE induced the PBMC between smoker and non-smoker. We have found that increased the inducibility of p53 and DNA binding activity in smoker population than decrease the binding activity in non-smoker. Therefore, it is beneficial for the suscetibility of an individual by carcinogen induction from this study. We will analyze induction of p53 biochemical activity by carcinogen using EMSA assay pair with p53 induction on peripheral blood mononuclear cells for lung

cancer patients, smokers or nonsmoker.

Keywords: p53 , EMSA, Western blot

二、緣由與目的

p53 在細胞中是抑制腫瘤 (tumor suppressor)的蛋白 (Chen *et al.*, 1990)，一般認為 p53 具有抑制細胞生長 (Finldy *et al*;.,1989；Vogelstein *et al.*,1992)以及維持遺傳物質完整性的功能 (Livingstone *et al.*,1992；Yin *et al.*,1992)。在臨床組織的分析中，超過半數的腫瘤細胞都可以發現 p53 有缺失 (deletion)或是突變 (mutation)的情形發生(Hollstein *et al.*,1994)，而且在人類腫瘤細胞中，是最常發生突變的基因 (Hollstein *et al.*,1996)，可見 p53 在細胞的生長與控制上，扮演著相當重要的角色 (Reznikov *et al.*,1989)。在 non transformed 的正常細胞中，p53 蛋白的半衰期 (half-life)約只有 5~10 分鐘，表現量少，極不穩定 (Rogel *et al.*,1985)。但是當正常細胞受到紫外線照射後，會因 post-translation 的改變，造成 p53 蛋白的穩定，進而促使細胞內 p53 蛋白大量增加 (Maltzaman and Czyzk，1984)。而腫瘤細胞在化學物質的刺激下，也能發現 p53 具有高表現量 (Deleo *et al.*,1979)；而在許多轉形 (transformed)的細胞株，以及許多癌症患者的初級腫瘤細胞中也都能發現大量 p53 蛋白的累積 (Oren *et al.*,1982)，因此可知當細胞面臨 oxidative stress 時，可能會提升 p53 的穩定性 (Midgley and Lane，1997)。

三、材料與方法

樣本製備：

收集 2 ml 周邊淋巴球細胞以 RBC lysis buffer 溶解紅血球，處理 B[a]P 及 BPDE 不同時間後，進行核質分離並製備蛋白質樣本以利後兩者(EMSA 及 Western)實驗進行。

EMSA (Electrophroetic mobility shift assay)

本實驗是在測定 p53 核蛋白與 Biotin 標定的 Mdm2 promoter p53 responsive element（MDM2-p53RE）鍵結的能力。當淋巴球細胞生長於 3.5 公分的培養皿待處理 BaP 或 PAH 藥物，生長並以 1x PBS 清洗兩次，再以 TD 及 BL bufferr 進行核質分離，收集上清液，並以 Bio-Rad protein assay 定量蛋白量；取 10 微克的核蛋白之後，使用 Roche 出產的 DIG GEL Shift assay kit，依序加入 4 微升的 5x binding buffer、1 微升 (1 微克)的 poly d(I-C)、1 微升 (1 微克)的 poly L-lysine、再加入 2 微升的 100 pmole 已標定 Biotin 之 Mdm2 P2 p53 Response Element (MDM2-p53RE) DNA 片段，之後其餘補水至 20 微升，混合後作用於室溫 (25℃) 15 分鐘便可進行直立式電泳操作。本實驗選用 Hoefer 之直立式電泳槽，其步驟如下：首先製備 6% native gel，依序加入二次水 6 毫升、10X TBE buffer 1 毫升、40% acrylamide-bis acrylamide 1.5 毫升、Glycerol 1 毫升、1% ammonium persulfate 0.5 毫升、TEMED 15 微升混合均勻後，然後緩緩加入 1.0mm 厚度的直立式電泳槽膠台座中，然後將梳狀膠片 (comb)放入，待凝固後小心取出梳狀膠片，所留下的凹槽可做為樣品加入之用。將製備好凝固的膠，先用 0.5x TBE running buffer 填滿，再將先前已作用好的 samples 與 5 微升的 loading buffer 混合均勻，然後緩緩注入至 native gel 的凹槽中，用 80 volt 約跑 120 分鐘，直到樣品接近底部即可停止。在膠快跑完的前 10 分鐘，準備一張大小恰當的 Nylon membrane (Boehringer Mannheim Gmbh 1209299)，先用 0.25x TBE buffer 浸泡 10 分鐘秒；將膠卸下之後，同時與 nylon membrane 及兩片濾紙一起浸在 transfer 緩衝溶液中 (使 DNA/protein 由負極往正極移動)，利用半乾式轉漬法以 350 mA 120 分鐘，將膠上的 DNA 轉移到 nylon membrane 上。將 transfer 好的 nylon membrane 先經由 UV cross linker 以 120 mJ 3 分鐘後，使用 PIERCE detection kit 顯像，其步驟如下：先將 membrane 浸泡於 wash buffer 2 分鐘，再將 membrane 浸於 9 毫升的 blocking buffer 震盪 15 分鐘後，取

10 微升的 HRP 與新的 blocking buffer 5 毫升混合，然後將 membrane 放入其中，震盪 15 分鐘後，取出 membrane，置於 1x washing buffer 中震盪 5 分鐘後，倒掉再清洗一共 4 次，之後再將 membrane 放置於 Equilibration buffer 中振盪 5 分鐘，再與 substrate (Luminol 與 Peroxide 各取 1 毫升混合)作用 5 分鐘後，再以 X 光底片 (Kodak Science Imaging Film)感光 1~5 分鐘後即可顯影。

Western blot

首先製備凝膠 15%， 以每個 well loading 20 μg 蛋白樣品，先用 90 volt 跑 10 分鍾，再用 110 volt 跑 80 分鐘，直到樣品接近底部即可停止。在膠快跑完前 10 分鐘，可先準備一張大小恰當的 PVDF membrane amersham pharmacia biotech，先用甲醇洗一下（約 15 秒），再換成清水振盪 1 分鍾之後泡於 transfer buffer 中。將膠卸下後，同時與 PVDF membrane 及兩片大小相同的 3M 濾紙一起浸在 transfer 緩衝溶液中。利用濕式轉漬法以 100 V 、120 分鍾，將膠上的蛋白以負往正極的方式轉移到 PVDF membrane 上。將 transfer 好的 PVDF membrane 浸在含 2%脫脂奶粉(skin milk）的 1xTTBS 緩衝溶液（50 mMTris，0.2% Tween 20， 150mM NaC1，PH 7.5）中，振盪 60 分鍾。接著加入可辨識欲觀察蛋白的一次抗體 anti-mouse（D0-7, P53; p21 and IF-2-Zymed; mdm2），以 250 倍含 1%脫脂奶粉（skin milk）的 1xTTBS 緩衝溶液稀釋，於 4℃經過一夜振盪或室溫振盪 1.5 小時。Anti-β-actin mouse 以一萬倍稀釋，作用條件同上(抗體加入 1/100 的 2% sodium azide 可回收在利用）。將處理完一次抗體之 PVDF membrane 用含 2%脫脂奶粉（skim milk）的 lx TTBS 緩衝溶液清洗兩次（5 分鐘/次）。接著在室溫下用 anti-mouse IgG 之二次抗體反應 1 小時。將處理完二次抗體之 PVDF membrane 用含 2%脫脂奶粉（skim milk）的 lx TTBS 緩衝溶液清洗兩次（10 分鐘/次），再用不含脫脂奶粉的 1x TTBS 緩衝溶液清洗兩次。利用 E.C.L 呈色劑（Amersham RPN 2106），將經過抗體作用之蛋白影像呈現在 X-光底片（Kodak Science Imaging Film）上，約 15～30 分鍾。

四、結果與討論

本實驗先以細胞株證實其實驗可行性，所以以 H1355 細胞處理不同濃度 B[a]P 24 小時後，以 Western blot 偵測其 p53 蛋白不同磷酸化位置及 p21 蛋白表現情形，發現 ser15 位置磷酸化表現相當明顯，且 p53 蛋白表現量亦增加，但 p21 卻不增加 (圖一)，因此細胞株的 p53 為 E285 位置突變，所以即使 p53 增加也不影調控 p21。

至於周邊血細胞因我們非常擔心 p53 不穩定所以表現量可能相當低不易測得，所以先處理 PHA（10 ug/ml）24 小時後再處理 BPDE 6 小時，以 Western blot 偵測其 p53 蛋白及 p21 蛋白表現情形，發現處理 PHA 的確會增加 p53 蛋白表現 (圖二)，且 p21 表現量亦增加，但處理 BPDE，p53 如同預期的會增加，但卻十分意外發現竟然 p21 表現量是減少這以重複 5 個正常的不同男女人次皆發現此現象。處理不同濃度的 BPDE 亦發現相同現象(圖三)。

為了能證實 mtp53 是否會因為突變而影響到 p53 結合至 DNA 的能力，特地選用經 Biotin 標的之 MDM2-p53RE (P2) 雙股 DNA，與經由 mtp53 質體 DNA 轉殖後的細胞核蛋白一起作用後，利用 native gel 區分能結合至 DNA 的 p53 蛋白與無法和 DNA 結合的 mtp53 蛋白，實驗結果如圖四。pCDNA3 (empty vector) (lane 2)因為不含 p53 cDNA 片段，所以不會產生 p53 蛋白，也就沒有 DNA 和蛋白的結合(band shift)的情形發生，而至於 p53 wild type 與 MDM2-p53RE 則有非常明顯的結合反應 (lane 3)，在 mtp53 S240R (lane 6) 和 P67C/L194R 也發現顯著的結合反應 (Data not show)，mtp53 E286Q 有微弱的結合反應 (lane 8)，此外在過飽和的情況下 (壓片時間較長)，其他不同的 mtp53 皆似乎不和 DNA 結合的情形，不同的 p53 突變點對於 p53 與 DNA 的結合會造成不同的影響，結果相當值得探討。

本研究先進行抽煙者與非抽煙者的 p53 功能活性分析，分別取自 (a)抽煙者 (b)非抽煙者的周邊血細胞進行 EMSA 分析，結果發現抽煙者 p53 蛋白與 DNA 的結合隨著處理 BPDE 後 p53 與 mdm2 promoter 上的 response element 有增加情形，但在不抽煙者的周邊血液受 BPDE 處理後 p53 與 DNA 結合量反而減少(圖五 lane 9)。此情形與圖二 週邊血受 BPDE 處理後其 p21 蛋白表現量減少有相似之處。所以以 BPDE 刺激反而造成 p53 的下游基因表現量減少。p53 在細胞中是抑制腫瘤 (tumor suppressor)的蛋白 (Chen *et al.*, 1990)，一般認為 p53 具有抑制細胞生長 (Finldy *et al*;.,1989；Vogelstein *et al.*,1992)以及維持遺傳物質完整性的功能 (Livingstone *et al.*,1992; Yin *et al.*,1992)。在臨床組織的分析中，超過半數的腫瘤細胞都可以發現 p53 有缺失 (deletion)或是突變 (mutation)的情形發生 (Hollstein *et al.*,1994)，而且在人類腫瘤細胞中，是最常發生突變的基因 (Hollstein *et al.*,1996)，可見 p53 在細胞的生長與控制上，扮演著相當重要的角色 (Reznikov *et al.*,1989)。在 non transformed 的正常細胞中，p53 蛋白的半衰期 (half-life)約只有 5~10 分鐘，表現量少，極不穩定 (Rogel *et al.*,1985)。但是當正常細胞受到紫外線照射後，會因 post-translation 的改變，造成 p53 蛋白的穩定，進而促使細胞內 p53 蛋白大量增加 (Maltzaman and Czyzk，1984)。而腫瘤細胞在化學物質的刺激下，也能發現 p53 具有高表現量 (Deleo *et al.*,1979)；而在許多轉形 (transformed)的細胞株，以及許多癌症患者的初級腫瘤細胞中也都能發現大量 p53 蛋白的累積 (Oren *et al.*,1982)，因此可知當細胞面臨 oxidative stress 時，可能會提升 p53 的穩定性 (Midgley and Lane，1997)。p53 目前是一個已知的抑癌基因 (oncosuppressor gene)，若將 p53 野生型 (wild-type)基因轉殖 (transfection) 進入到缺少內生性 endogenous) p53 的人類骨癌 (Osteosarcoma)細胞中，則可抑制細胞的癌化 (neoplasticity) (Chen *et al.*,1990)；若將 p53 送入已轉形的癌細胞中，也具有抑制癌細胞增生(proliferation)的功能 (Diller *et al.*,1990；Mercer *et al.*,1990)；細胞若經由 IR (ionizing radiation)誘導而產生的 p53 蛋白會促使細胞停留在 G1 期 (G1 arrest) (Kastan *et al.*,1991)；也有研究結果指出 DNA damage 所造成的 G2/M arrest 與 p53 dependent 有關 (Agarwal *et al.*, 1995)；Papathanasiou 等人則在 1991 年，發現經由 p53 所調控的 gadd45 與 proliferating cell nuclear antigen (PCNA)結合，能影響細胞的增生 (proliferation)與 DNA 的修復 (repair)；至於 p53 在細胞程序性死亡 (programmed cell death)或稱細胞凋亡 (apoptosis)的過程中也扮演著促進子的角色，可活化下游基因 BAX，促使細胞走向 apoptosis (Attardi *et al.*, 1996)；在 COS 猿猴細胞株中則發現 p53 能抑制 SV40 DNA 的複製；而 p53 的功能失去，則往往造成基因不穩定 (genome instability)，易發生基因轉位 (translocation)，因此 p53 也具有維持遺傳物質完整性的功能 (Bryan *et al.*,1995)。近幾年來的研究顯示，在調控細胞生長的過程中，p53 抑癌基因衍然已成為一個非常重要的控制點，其中包含細胞週期進行的控制 (cell cycle control)、DNA 的修復 (DNA repair)、細胞分化 (differentiation)、基因穩定性 (genome stability)、細胞程序性死亡 (programmed cell death)和血管增生 (angiogenesis) (Vojta and Barrett 1995；Bouck，1996)。另外在 mammalian cell 中，p53 對於 DNA damage response pathway 的調控也十分重要。

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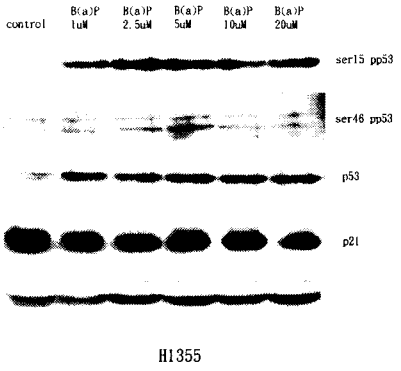
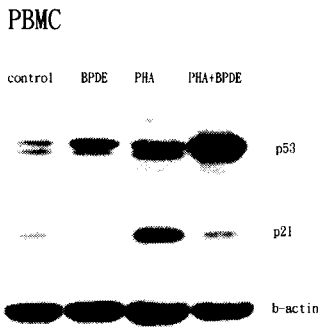
六、圖表

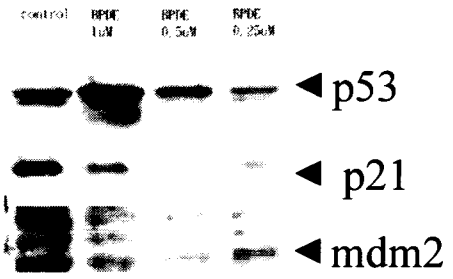
圖一 H1355 細胞處理不同濃度 B[a]P 24 小時後，以 Western blot 偵測其 p53 蛋白不同磷酸化位置及 p21 蛋白表現情形。



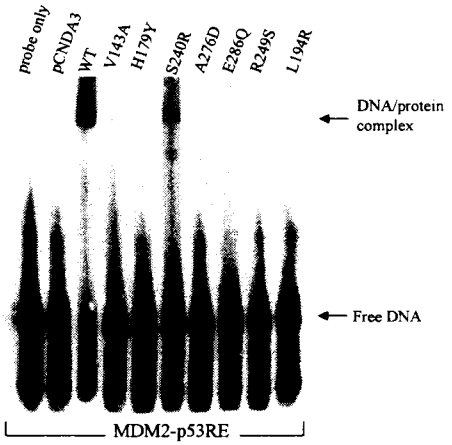
圖二 周邊血細胞(PBMC)處理 BPDE 6 小時及處理 PHA (10 ug/ml) 24 小時後再處理 BPDE 6 小時，以 Western blot 偵測其 p53 蛋白及 p21 蛋白表現情形。



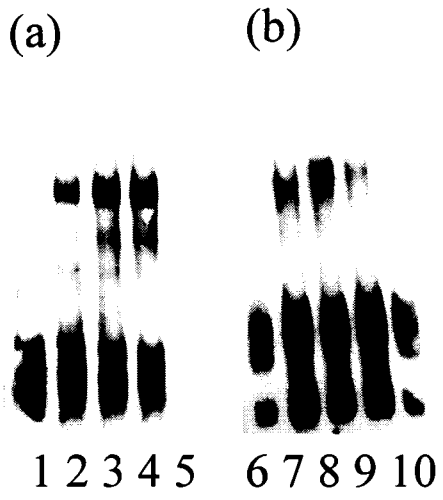
圖三 周邊血細胞(PBMC)處理不同濃度的 BPDE 6 小時以 Western blot 偵測其 p53 蛋白及 p21、mdm2 蛋白表現情形。



圖四 在H1299細胞株中構築不同p53突變株，以 EMSA 方式偵測其 p53 蛋白與 mdm2 的 probe 結合情形



圖五 分別取自 (a)抽煙者 (b)非抽煙者的周邊血細胞進行 EMSA 分析 lane1、6： free probe；lane 2、7：0.5 ml 全血；lane 3、8： 0.5 ml 全血+1 ml 培養基；lane 4、9：與 lane 3 同但有處理 1 uM BPDE；lane 5、10：與 lane 4 同，但在 binding assay 時加入 cold probe 競爭。



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A novel *p53* mutant retained functional activity in lung carcinomas

Jiunn-Liang Ko[a,*], Ming-Chiang Chiao[a], Shu-Lin Chang[a], Pinpin Lin[a], Jin-Ching Lin[b], Gwo-Tarng Sheu[a], Huei Lee[a]

[a] *Institute of Toxicology, Chung Shan Medical University, 110, Sec. 1, Chien-Kuo N. Road, Taichung 40203, Taiwan, ROC*
[b] *Department of Radiation Oncology, Veterans General Hospital-Taichung, Taichung, Taiwan, ROC*

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Abstract

The gene *p53* is a critical tumor suppressor that can respond to multiple signals of cellular gatekeepers for growth and division. The *mdm2* gene is one of the downstream target genes for transcriptional activation by the product of *p53* tumor suppressor gene. Transactivation of *mdm2* gene is represented by the presence of a functional P53 protein. To understand the biological function of mutant *p53* in tumorigenesis, we constructed a number of *p53* mutants by site-directed mutagenesis (H179Y, L194R, S240R, R249S, A276D, E286Q), followed by characterization of each P53 mutant's ability to transactivate *mdm2*, *bax and p21waf*. The transactivation properties of *p53* mutants were compared by co-transfection with pGL-3-mdm2, pGL-3-bax and pGL-3-p21waf into the P53 null cell line H1299 derived from a non-small cell lung carcinoma. Among them mt*p53* S240R and E286Q were shown to have enhanced transactivating activity of *pGL3-mdm2*, at about 43.2 and 28.2% of the wt *p53* vector, respectively, while the remaining four had nearly the same level of activity as the negative control did. Furthermore, data indicated that mt*p53* S240R had as high an ability to suppress the growth of the *p53* null cell line H1299 as wild type *p53*. Therefore, mutant *p53* alone is an insufficient indicator of poor prognosis. Instead, functional *p53* may affect lung cancer prognosis.

Keywords: Functional *p53* mutants; MDM2; Transactivation; Prognosis

1. Introduction

The *p53* tumor suppressor gene is located in the chromosomal region 17p13 and encodes 393 amino acids (aa) and a 53 kDa nuclear phosphoprotein. The protein is divided into three main structural and functional domains. The first 42 amino acids of the N-terminal compose the activation domain, the residues between amino acids 120 and 290 make up the sequence-specific DNA binding domain, and the C-terminal portion contains the oligomerization domain (aa 323–356) and the regulatory domain (aa 360–393) [1]. The P53 protein is a transcriptional transactivator inducing specific target genes by binding to short genomic sequences, called P53-responsive elements (P53 RE), which fit a consensus sequence with variable extent [2]. The transcriptional regulation dependent pathway involves induction by P53 of a growing list of P53 target genes including *mdm2* [3], *bax* [4], *waf-1/cip-1* [5], *fas/apo-1* [6], *pig3* [7], *rb* [8] and *p53* itself [9].

Among the cellular proteins that modulate P53 function, the best characterized is MDM2. The *mdm2* gene was identified as an oncogene amplified in a mouse tumor cell line [10]. MDM2 have been later found that amplified in a significant proportion of

* Corresponding author. Tel.: +886-4-24730022/1693; fax: +886-4-24720407.
E-mail address: jlko@csmu.edu.tw (J.-L. Ko).

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human sarcomas [11]. MDM2 binds to P53 both in vivo and in vitro [12,13]. This binding inhibits P53 sequence specific transcriptional activation (SST), as well as the ability of P53 to repress the activity of some promoters [12,14]. The binding of MDM2 to the transactivation domain of P53 [13] has been proposed to conceal this domain from interaction with the transcriptional machinery [15]. The binding of MDM2 to P53 has also been shown to result in in vivo complexes lacking the ability to bind to specific DNA sites [3]. Generally, there is a good correlation between the ability of P53 protein to transactivate target promoters and suppression of the growth of the *p53* null cell line [16]. Forced expression of mutant *p53* in p53 null cells confirmed pattern of association with JNK/MDM2 and prolonged half-life, as found in tumor cells [17]. Multiple lysine mutations in the putative acetylation sites of the p53 C-terminal domain interfere with ubiquitination, thereby regulating p53 degradation [18]. MDM2 was present in complexes with mutant *p53* in tumor cells, and stabilization of MDM2 required direct binding to mutant *p53* [19].

The *mdm2* expression is induced by wt P53 activity [20]. The P53 tumor suppressor protein is inactivated by mutation in a large percentage of human tumors [21]. It is not surprising that mutant *p53* is unable to bind to the DNA response element or to regulate the gene expression [22]. Recently, we reported that certain mutated *p53* with *mdm2* mRNA expression is a favorable prognostic factor in lung carcinomas [23]. However, certain mt P53 may possess a function phenotype, if the mt P53 maintains its ability to bind to and transactivate the *mdm2*-P53 response element, preserving the tumor suppressive properties of lung cancer cells. To study mutant *p53* suppression activity in tumor cells, we performed colony formation assay and compared the colony numbers. The suppression of colony formation could be achieved if mt p53 retains functional activity and may correlate with the prognosis of lung carcinoma.

2. Experimental

2.1. Cell line and plasmids

H1299 cells (a human non-small cell lung carcinoma cell line that contains a homozygous deletion of the *p53* gene) were obtained from the American Type Culture Collection (Rockville, MD, USA) and maintained in DMEM medium supplemented with 10% fetal calf serum (GIBCO/BRL, Gaithersburg, MD) in a 37 °C incubator containing 5% carbon dioxide. Reporter plasmid pGL3-MDM2-luc was constructed as described previously [3], by excising the *NheI*/*Hin*dIII fragment from the amplified *mdm2* promoter DNA and subcloning it into pGL3-Basic (Promega). Reporter plasmid pGL3-bax-luc was constructed according to accession no. U 17193 (Genebank). The representative *p53* mutants, H179Y, S240R, R249S, A276D, E286Q, were generated by site-directed mutagenesis of the parental wt P53 vector pC53SN. The pC53SN plasmids were kindly provided by Dr. W.H. Lee. The WWP-Luc plasmid encoding wild type WAF1 promoter-luciferase reporter was a kindly gift from Dr. Bert Vogelstein.

2.2. Site directed mutagenesis

Expression plasmid was the derivative of pCDNA3 (Invitrogen) constructed by ligation of a 1192 bp *Eco*RI and *Xba*I fragment derived from pC53SN by polymerase chain reaction (PCR). Primer A (G*GAATTC* ATGGAGGAGCCGCAGTCAGATCC) and primer B (AG*TCTAGA*TCAGTCTGAGTCAGGCCCTTCTG) contained the entire P53 coding sequence in frame and were used to insert the fragment into the *Eco*RI/*Xba*I sites of pCDNA3. The nucleotide sequences and amino acid substitutions of mutated primers for the construction of mutant recombinant *P53* for H179Y, L194R, S240R, R249S, A276D and E286Q are listed as follows. For H179Y: 5′-CCCAC*T*ATGAGCGCT GCTCAG-3′, for L194R: 5′-GCATC*G*TATCCGAGT GGAAGG-3′, for S240R: 5′-G TAACAG*A*TCCTGCA TGGGCG-3′, for R249S: 5′-GAACCGGAG*T*CCCA TCCTCAC, A276D: 5′-GTTTGTG*A*CTGTCCTGGG AGA- 3′, and for E286Q: 5′-CAGAG*C*AAGAGAATA TCCGCA-3′. DNA sequencing confirmed the presence of mutations in the pCDNA3 constructs. The DNA cycle sequencing was performed on GeneAmp PCR 2400 (Perkin-Elmer, Norwalk, CT) using BigDye terminators and *p53* sense and *p53* antisense primers with AmpliTaq DNA polymerase FS (Taq-FS, Perkin-Elmer/Applied Biosystems Divisions, Foster City, CA), according to the manufacturer's instructions. Briefly, purified plasmids (100 ng) were mixed

with terminator ready reaction mix (8 μl) and primer in a 20 μl reaction volume. Sequencing reactions were run under the following conditions: 25 cycles at 96 °C for 10 s, 50 °C for 5 s, then ended at 4 °C. The PCR products were resolved on the ABI Model 377 Analyzer (Perkin-Elmer/Applied Biosystems).

2.3. Transient transfections and luciferase assay

H1299 cells were seeded at 2×10^4 cells per well and incubated at 37 °C for 18 h. All of these transfections were carried out in triplicate in 24 well plates using DOTAP transfection reagent (Roche), as according to the manufacturer's instructions. An amount of 1 μg of the reporter plasmid pGL3-mdm2-luc was mixed with 1 μg of the expression vectors encoding either wt P53, mt P53 of H179Y, S240R, R249S, A276D, E286Q individually and control vector, pSV β-gal (1 μg), in DMEM medium. An aliquot (3 μg) of each mixture was diluted in DMEM without serum. DOTAP (Roche) was immediately added to the plasmid mixture. The transfectamine/plasmid mixture was then added directly to the cells followed by incubation at 37 °C and 5% CO_2 for 6 h. These cells were harvested by reporter lysis buffer (Promega) for analysis after 48 h. Equivalent amounts of cellular protein, as determined by Bio-Rad assay (BioRad), were used for luciferase assay. In luciferase assay, the transfected cells were collected, washed with PBS, and lysed in the lysis buffer provided in the luciferase kit (Promega). The efficiency of transfection was determined by co-transfecting pSV-βGal and assaying β-galactosidase activity using *o*-nitrophenyl-β-D-galactopyranoside, ONPG substrate (Promega). Transciptional activity was measured using a Luminometer (TUNEL 20/20). Successful transfection was verified by Western blotting using P53 antibody DO-7 (DAKO).

2.4. Electrophoretic mobility shift assay (EMSA)

The P53 RE of MDM2 contains two P53 binding sites (underlined regions) separated by 18 nucleotides [3]. The sequence of the region is 5-TTGAGCTGGTC AAGTTCAGACACGTTCCGAAACTGCAGTAAAA GGAGTTAAGTCCTGACTTTGTCTCCAGCTC-3′. The earlier mentioned oligonucleotides (sense, antisense) were synthesized on a Perkin-Elmer synthesizer. The 5′end was labeled with biotin. The end-labeled oligonucleotides were mixed in 0.1 M sodium chloride, heated at 95 °C for 5 min and then gradually cooled to RT for annealing.

The DNA binding reaction was carried out in DIG Gel Shift kit (Roche) binding buffer (20 μl final volume), containing 20 mM *N*-2-hydroxyethyl piperazine-*N*-2 ethanesulfonic acid (HEPES, pH 7.6), 1 mM dithiothreitol, 0.2% Tween-20, 10 mM $(NH_4)_2SO_4$, 30 mM KCl, 1 mM EDTA and poly [d(I–C)] (1 μl), for 15 min for specific DNA binding to P53 protein. Nuclear extracts (10 μl) from P53 null cells (H1299) and pCDNA 3 control vector transfected H1299 cells were added to the binding reaction as negative controls. Nuclear extracts of a stable clone of wild type P53 and mutant *p53* transfected H1299 were incubated with the above binding buffer for 15 min. The complexes were resolved in a 6% polyacrylamide gel with 0.5 × Tris–borate EDTA buffer at 100 V, then transferred to positively charged nylon membrane (Boehringer Mannheim, GmbH). Biotinylated probes were detected using the North2South TM Chemiluminescent Nucleic Acid Detection kit (PIERCE).

2.5. Growth suppression activity

H1299 cells were seeded at 1×10^5 cells per well in 35 mm dish plate for 18 h and transfected with 3 μg of P53 expression plasmid, pCDNA3 empty vector or mutated P53 plasmid with 3 μl DOTAP reagent (Roche) for 6 h. At the end of the transfection, the cells were trypsinized and replated at the density of 10^3, 10^4 cells/60 mm dish in triplicate. These cells were incubated in DMEM containing 400 μg/ml G418 medium for 14 days. The dishes were then fixed with 95% ethanol and stained with a 10% Giemsa solution.

3. Results

3.1. Clinical samples and p53 mutations

Eighty-one NSCLCs were examined for mutations in exons 5–8 of the *p53* gene by PCR direct DNA sequencing. The *p53* mutations were detected in 13 of the 81 tumors (16%) in our previously results. Eight of 13 mutations were point mutations. Six of these thirteen mutations were reported previously [24]. Seven

Table 1
The relationships between *p53* mutation, P53 protein expression, MDM2 mRNA expression and survival days

Patient no.	Age (year)	Sex	Smoking habit	Tumor type	*p53* Gene mutation			After surgery	
					Stage	Exon	Surrounding sequences	mdm2 mRNA	Day (survival (S)/death (D))
1	66	M	No	AS	IIIa	8	G<u>C</u>C→G<u>A</u>C (Ala276Asp)	–	480 (D)
2	53	M	No	AD	IIIa	6	C<u>T</u>T→C<u>G</u>T (Leu194Arg)	–	651 (D)
3	66	M	No	AD	IIIa	8	<u>G</u>AA→<u>C</u>AA (Glu286Gln)	–	336 (D)
4	52	M	No	AD	IIIa	7	AG<u>T</u>→AG<u>A</u> (Ser240Arg)	+	1228 (S)
5	60	F	No	AD	IIIa	7	AG<u>G</u>→AG<u>T</u> (Arg249Ser)	–	623 (S)[a]
6	64	M	Yes	SQ	I	5	<u>C</u>AT→<u>T</u>AT (His179Tyr)	–	689 (S)[b]

[a] Patient still surviving, but bone metastasis found.
[b] The patient had right kidney metastasis using CT scanning.

of these eight mutations were transversion mutations (H179Y, two of L194R, S240R, R249S, A276D, E286Q) and the eighth was a nonsense mutation (E298Stop). The relationships between *p53* mutation, P53 protein expression, *mdm2* mRNA expression and survival day after surgery are summarized in Table 1. All tumors with point mutation of P53 immunohistochemistry stained positive for P53 protein.

3.2. Construction of mutant p53

The earlier mentioned six mutant plasmids, as well as pC53SN production of mutant recombinants H179Y, L194R, S240R, R249S, A276D and E286Q by site-specific mutagenesis, were confirmed by nucleotide sequence analysis of mutant plasmids.

3.3. Transient transfection assays

To determine whether the transcriptional activity of *mdm2* gene is due to wt P53 or mt P53 activity, we carried out transient co-transfection into the H1299 cell line. Successful transfection was verified by Western blot analysis (Fig. 1). In each experiment, the wt P53 vector and the pCDNA3 empty vector were included as positive and negative controls, respectively. The activity of the induced luciferase gene with each mutant was tested with the wt P53 vector set at 100%. The experiments were carried out three times independently. Four of the six mutants (S240R, R249S, A276D and E286Q) altered the net charge of the P53 molecule. All the mutants were unable to transactivate the pGL-3-bax and pGL-3-p21 plasmids. Only those two mutants with S240R and E286Q mutation enhanced the luciferase activity of pGL3-mdm2 at about 43.2 and 28.2% of the wt *p53* vector, respectively (Fig. 2a). EMSA experiments revealed a retard band when wt P53 was transfected into H1299 and incubated for binding assay (Fig. 3, lane 3), and was abolished in the P53 null H1299 cells. These findings indicated the presence of P53 in the protein–DNA complex and revealed that *p53* mutant S240R still maintained a portion of the wild type P53 property to bind to MDM2-P53 response element (Fig. 3, lane 6). The *p53* mutant E286Q showed little binding activity (Fig. 3, lane 8). The other mutants, H179Y, L194R, R249S and A276D, showed *mdm2* transactivation activity at less than 10% of the wt P53. Furthermore, they did not interact with the P53 response element on *mdm2* (Fig. 3) and were accompanied by MDM2 mRNA expression.

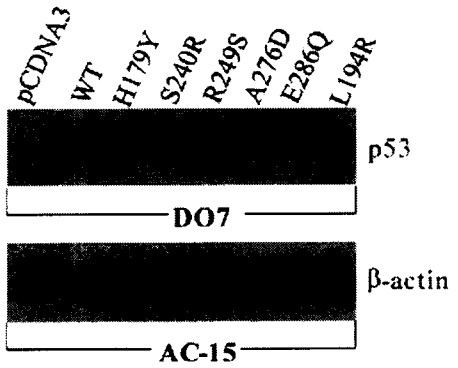

Fig. 1. Expression levels of wt P53 and mt P53 proteins. H1299 cells were transfected with 1 μg of P53 expression vectors. Cells (1×10^5) were collected 42 h after transfection and lysed in reporter lysis buffer (Promega). The wt P53 and mt P53 were detected with DO-7 anti-P53 monoclonal Ab. The β-actin as loading control was detected with AC-15 antibody.

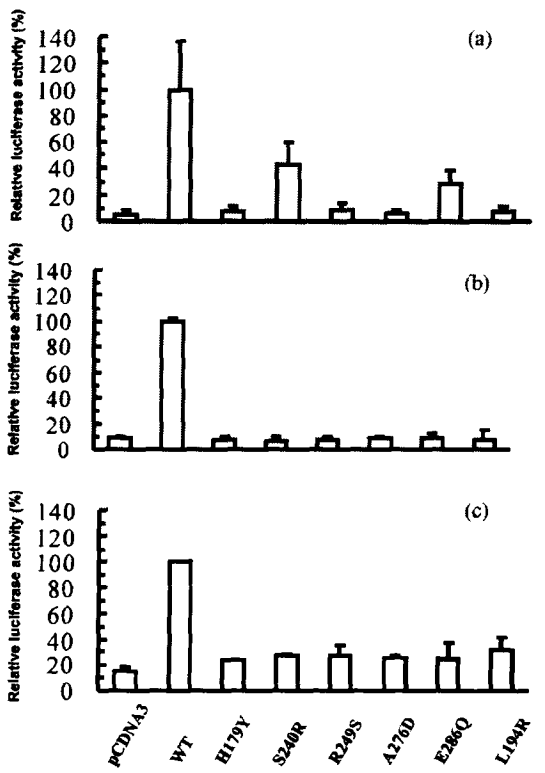

Fig. 2. (a) Effects of the *p53* mutant on the transcriptional activity of the luciferase reporter gene downstream of the MDM2-P53 RE of the reporter plasmid pGL3-MDM2-luc. (b) Effects of the *p53* mutant on the transcriptional activity of the luciferase reporter gene downstream of the BAX-P53 RE of the reporter plasmid pGL3-bax-luc. (c) Effects of the *p53* mutant on the transcriptional activity of the luciferase reporter gene downstream of the WAF-P53 RE of the reporter plasmid pGL3-WAF-luc. The total amount of DNA transfected in each experiment was 1 μg of wt or mt P53 and 1 μg of the reporter plasmid. Results shown are an average of three independent transfection experiments.

3.4. Transient expression of p53 mutants suppressed the growth in H1299 cells

There were several reports of *p53* mutants which have lost the ability to suppress the growth of P53-null human adenocarcinoma H1299 cells. The triplicate plates were examined 14 days after selection with G418-resistant colonies produced. Wild type P53 and S240R efficiently suppressed the growth of p53-null tumour cell lines such as H1299 (Fig. 4a). The transcriptionally inactive mutants H179Y, L194R, Y234N, R249S and A276D failed to suppress the growth of human adenocarcinoma H1299 cells. S240R induced

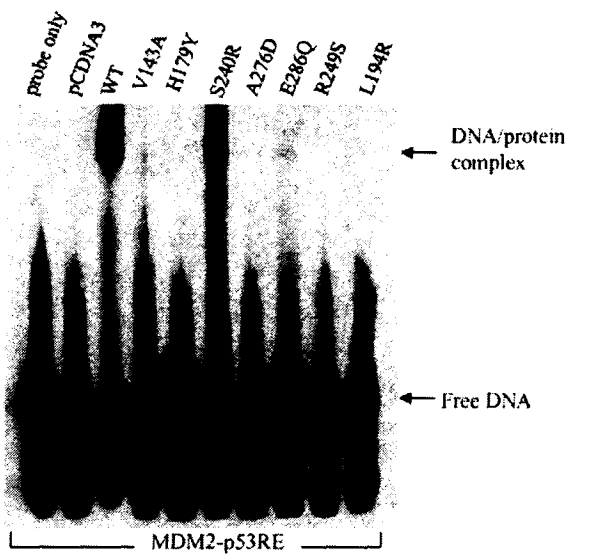

Fig. 3. Electrophoretic mobility shift assay showing sequence specific binding of P53 to the P53 RE of the *mdm2* gene. The nuclear extracts (10 μg) containing wt P53 and mt P53 from stable clone. Lane 1: the extract of H1299 cells with biotin labeled oligomers. Lane 2: the extracts of transfected pCDNA3 empty vector into H1299 as negative control. Lane 3: wt P53 transfected H1299 forms a retardation band. Lanes 4–10: the extracts from various transfected stable mt P53 clones with oligomers.

suppression of only 21.5%, comparable to wild type P53 (20.4%) (Fig. 4b).

4. Discussion

The functional activity of wt P53, which is lost following mutation, is mediated via transcriptional activation or down regulated expression of genes involved in growth arrest, survival or cell death responses through sequence-specific interactions with DNA [2]. In the present study, we examined the ability of mutant *p53* proteins derived from selected lung carcinomas to bind to MDM2-P53RE and to suppress the growth of P53 null non-small-cell carcinoma cell line H1299. Additionally, mutant *p53* loses trans-activating functions and may exert a dominant-negative effect as a result of loss of function. Studies have found that mutant *p53* may possess novel functions or a dominant-positive effect [25]. For example, multiple copies of mutant V135 *p53* allele accelerate tumor development in normal mice, but not in *p53*-deficient mice. The loss of function may lead to gain of functions when

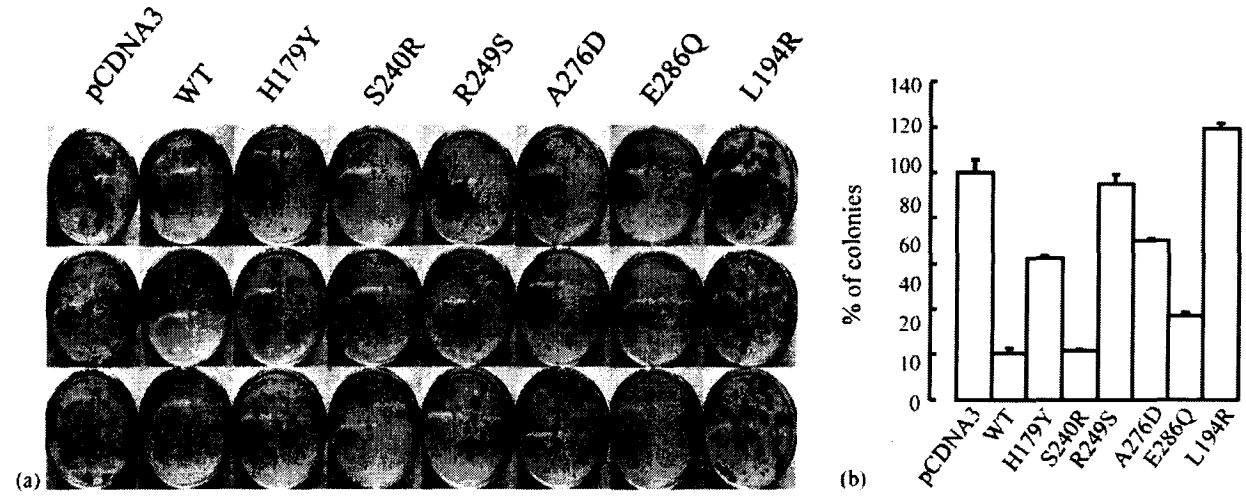

Fig. 4. Effects of the *p53* mutants on the growth suppression activity of human lung carcinoma cell line H1299. (a) Colony growth assay. Equal numbers of cells from the empty vector pCDNA3, wt P53 and mutant *p53* pools were plated and stained as described in the text. Shown are three dishes from each pool. (b) Colonogenic survival assay of wt P53 and mutant *p53*. The number of colonies was counted under a dissecting microscope. The number of cells had to be larger than 50. The percentages of colonies scored in each case were: pCDNA3 as 100%, wt P53, 20.4 ± 1.9%; H179Y, 62.4 ± 1.1%; S240R, 21.5 ± 0.7%; R249S 95.2 ± 3.7%; A276D, 69.9 ± 1.0%; E286Q, 36.8 ± 1.5%; L194R, 118.8 ± 3.0%. Each value represents the average of three separate experiments. S.E. is indicated.

p53 loses some, but not all, wt functions. The *p53* mutant 175P can lose the ability to transactivate Bax, but may retain the ability to trans-activate the *p21* promoter [26]. Mutant 181L showed a loss of ability to activate a promoter containing IGF-BP3 box B sequences [27]. Analysis of mutants with the ability to activate transcription from the p21CIP1/WAF1, BAX and MDM2 promoter in H1299 confirmed that the transcriptional activities in the mdm2 promoter of the S240R and E286Q mutants were as partially efficient as those exhibited by the wild type protein. But, both S240R and E286Q were essentially unable to activate expression from these other promoters, as expected (Fig. 2b and c). Both mutants may not be silent mutations, in that they did lose some properties than wild type p53. Levels of transactivating MDM2 in the transiently transfected cells were elevated only in cells expressing wild type p53, S240R and E286Q, confirming that the EMSA of the MDM2-p53 response element in the promoter was an accurate reflection of the activation of the promoter in the normal genomic context, further supporting the retention of transcriptional activity for these mutants. We analyzed mutation data in successive release of the database of *p53* mutations maintained at the International Agency for Research on Cancer (IARC *p53* mutation database from April 2000 update of the IARC database with 14051 entries). These six frequencies of *p53* from 1894 lung tumor mutations were H179 (1.7%), L194 (0.5%), S240 (0.1%), R249 (4.2%), A276 (0.8%) and E286 (0.6%), respectively (http://www.iarc.fr/P53). We used the crystal structure of the core domain DNA complex [28], in conjunction with biochemical studies of mutants, to provide a solid basis for understanding how mutations affect the sequence specific DNA binding activity of P53. One class of mutations involves residues that contact the DNA, and failure to bind to DNA by these mutants can be attributed to loss of critical DNA contacts. Another class of mutations involves residues that appear to be important for the stable folding of the core domain, and loss of DNA binding by these mutants can be attributed to structural defects [28]. For example, R249 affect the overall structure of the core.

The transcriptional activity of P53 is mediated in part by specific binding to P53 response elements in the promoter or distal regulatory regions, and in part by interaction with components of the transcriptional machinery. H179Y and L194R are located on the L2 loop to the ligands for zinc. A249S occurs in the L3

loop, adjacent to the minor groove contact (Arg248), and is surrounded by portions of the L2 and L3 loops and the COOH-terminal end of the S3 strand from the β-sandwich. R249 is necessary for the integrity of the structural elements at the DNA–protein interface [29]. E286Q fits within the major groove of the DNA. Although not in possession of a classical zinc finger, the tetrahedrally coordinated zinc atom is essential to the structure of the P53 core. DNA-binding experiments revealed that two of seven *p53* mutants retained their DNA binding properties. We suggest that certain P53 missense mutations, apart from stabilizing P53, generate proteins with several properties of the wt P53 proteins. These mutations were detected at codons 240 (S–R) and 286 (E–Q), respectively. One possible explanation for this phenomenon is the charge difference introduced by these mutants into the P53 amino acid sequence. Interestingly, the substitutions in *p53* mutants S240R and E286Q were conservative, and thus, did increase the net charge of molecules in the binding motif. Increasing the net charge of the P53 protein may improve the binding to the negative charge of the DNA. Furthermore, they maintained the partial ability to transactivate the MDM2 promoter (Fig. 2). In our experiments, S240R maintained apoptosis of lung carcinoma cells and transactivated the promoter of MDM2. Surprisingly, S240R mutant retained the mdm2 promoter transactivating ability, but other promoters, such as BAX and p21-WAF1, could not be activated. Therefore, the S240R mutant indeed did lose some functional *p53* activity. The contribution of the mutant S240R *p53* to the suppression of growth in the H1299 cell may be via some other mechanisms rather than p21 inducing the cell cycle inhibition or BAX inducing apoptosis. The elucidation of this mechanism required further investigation. Additionally, the same mutant *p53* gene, S240R, was found in a colon carcinoma cell line where it also expressed the MDM2 protein and possessed apoptotic activity similar to our observation in lung carcinoma [30].

It has become apparently that while the activity of *p53* mutants in transformation assays does not correlate well with transactivation. In some cases the ability of transformation positively correlated with impaired apoptotic function [26]. The wild type P53, which is central to the onset of apoptosis, is lost in many tumor cells. Many studies have shown that constitutive expression of mutant *p53* forms interferes with the ongoing process of apoptosis [31,32]. Furthermore, loss of wt P53 activity could potentially account for the increased radio- and chemo-resistance of tumors bearing *p53* mutations. Nevertheless, if one accepts the existence of function of *p53* mutations, some of the apparent increased resistance to therapy may be due to direct protective effects of mutant P53 proteins. Further studies are needed to investigate whether mt P53 S240R increases the sensitivity to radio- and chemotherapy. Site directed mutagenesis of mutant *p53* provides a framework for understanding mt P53 phenotype. The studies presented here show that retention of MDM2 transactivation is not unique to the S240R and E286 mutants. There are other tumor derived mutants (R175P, R181L and R213Q) that possess partial growth suppressive activity and are potent inducers of MDM2 expression [26,33]. In our previous investigation, median patient survival for stages II or III was 630 days [23]. One patient with the presence of *p53* mutation (S240R) at stage IIIa survived, and was still living after more than 1228 days. The two patients with R249S and H179Y mutations in *p53* are still alive but the tumors have metastasized. The patient with R249S mutation in *p53* has bone metastasis, while CT scanning showed that the patient with H179Y had right kidney metastasis. However, the sample size is entirely too small to draw any type of conclusion as to how the mutants affect clinical responsiveness. Our data indicate that functional P53 may be associated with a favorable clinical outcome. Therefore, mutant *p53* is not by itself an indicator of poor prognosis. Our evidence suggests that functional P53 may be important in the determination of lung cancer prognosis.

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