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YWHAZ(14-3-3ζ) possibly activate Wnt/β-catenin signaling pathway in lung adenocarcinoma cell

YWHAZ(14-3-3ζ)蛋白在肺腺癌細胞中可能活化Wnt路徑之探討

得獎獎項

醫學與健康科學科大會獎一等獎

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關鍵詞：YWHAZ、β-catenin、Wnt

作者簡介

我想，專題研究肯定是我高中生涯最精采最離奇的冒險故事。

大家好，我是詹宗翰，目前就讀台中一中二年級高瞻計劃班級。癌症，是當今令人聞之色變的疾病，而傳統的化療又只帶給病人無窮的痛苦，如今，基因治療正逐步茁壯，滿懷熱忱的我於是一間一間去大學實驗室敲門，就這樣，便開始了我的研究。在漫漫的研究路程裡，我體驗了研究者那種發現新知與驗證想法的狂喜，也經歷了為時數週沒有進度的苦悶，它們豐富了我的專題，也豐富了我的人生。

站在巨人的肩膀上，讓我看得更高更遠，希望我這艘探險船，能秉持不懈，朝星空燦爛處前進。

摘要

YWHAZ(14-3-3δ)蛋白被證實在肺腺癌中會引發上皮-間葉的轉變作用(epithelial - mesenchymal transition ; EMT)導致癌轉移，然而其分子機制卻不清楚。先前實驗室利用 cDNA 微陣列技術(microarray)發現肺腺癌細胞大量表現 YWHAZ 蛋白會導致 Wnt 訊息傳遞路徑之相關基因大量表現。因此，本研究目的是探討 YWHAZ 蛋白在肺腺癌細胞中是否會活化 Wnt 途徑而引發 EMT。

本研究結果發現大量表現 YWHAZ 蛋白除了會增加細胞質中 β-catenin 的表現量，也會增加細胞核內 β-catenin 和 TCF4 的蛋白表現量，並且在細胞核中與 β-catenin 和 TCF4 形成複合體；而臨床肺腺癌病患檢體中發現，相較於正常肺葉組織，YWHAZ 蛋白與 β-catenin 在肺癌組織的檢體有較高的表現量，同時少部分會進入細胞核內。經由以上結果，我們推測 YWHAZ 蛋白可能會結合 β-catenin 與 TCF4 蛋白並活化 Wnt 途徑，因而引發 EMT 之產生，而促進肺癌轉移。說明 YHWAZ 蛋白有潛力成為日後診斷肺腺癌的指標蛋白(marker protein)，同時亦成為治療肺腺癌的標的蛋白(target protein)。

重要性

1.提出 YWHAZ 可能在肺腺癌細胞中活化 Wnt 訊息傳遞路徑(Wnt signaling pathway)而導致癌轉移。

2.再度指出 YWHAZ 在腫瘤生物學的重要性。

3.對於肺腺癌轉移提供了臨床診斷及治療的另一種選擇 －YWHAZ。

Abstract

In recent years, YWHAZ (14-3-3δ) protein has been reported to induce EMT(epithelial-mesenchymal transition) and promote metastasis in the lung adenocarcinoma; however, the underlying molecular mechanism remains unknown. Our previous cDNA microarray data indicated that Wnt/β-catenin signaling is the potential pathway to cause EMT in the cells with enforced expression of YWHAZ. Therefore, the objective of this study is to investigate the role of YWHAZ protein in the activation of Wnt/β-catenin signaling of lung adenocarcinoma cells.

First, we demonstrated that an increase of β-catenin in the cytosolic fraction as well as an up-regulation of β-catenin and TCF4 protein in the nuclear fraction occurred while cells overexpressed YWHAZ; Furthermore, we found that YWHAZ could associate with TCF-4 and β-catenin in the nucleus. Finally, strong expression of YWHAZ and β-catenin was seen in the specimen of lung adenocarcinomas. In addition, nuclear localization of YWHAZ and β-catenin were also observed in the specimens. With these findings, we proposed that YWHAZ protein could associate with β-catenin and TCF4 in the nucleus and play a crucial role in the activation of Wnt/β-catenin signaling of lung adenocarcinoma cells.

Importance

1. The first one who propose that YWHAZ possibly activates Wnt/β-catenin signaling pathway and leads to the metastasis of lung adenocarcinoma.
2. Again, we put stress on the importance of YWHAZ in cancer biology.
3. We provide another choice to the treatment and diagnosis of lung adenocarcinoma ─ YWHAZ。

壹、 研究背景

一、肺癌(lung cancer)的重要性

肺癌，自 1982 年起，即為國人十大死因之首，每年約奪走七千多條人命，忖均每天就有 19 人命喪於此。根據其組織型態，可分為鱗狀細胞癌(squamous cell carcinoma)，腺癌(adenocarcinoma)，大細胞肺癌(large cell lung cancer)，及小細胞肺癌(small cell lung cancer)。其中，肺腺癌因其生物特性多樣化，為目前罹患比率最高之肺癌(Edwards et al., 2005)。肺腺癌其死亡主因在於，僅管疾病早期以手術切除並配合化療，但療效不佳，仍難以遏制擴散，再加上初期的肺腺癌大都不易診斷，發現時常已發生侵襲或轉移，使得五年肺癌存活率仍為 12 ~ 15% (Jemal et al., 2006)。所以，唯有了解肺癌轉移機制，才能開發出針對的標靶藥物，進一步治癒肺癌。

二、癌症轉移(metastasis)、上皮細胞塑性(epithelial plasticity)、EMT(epithelial-mesenchymal transition，上皮細胞轉型間葉細胞過程)

癌細胞發生轉移，是經由侵犯(invasion)、內滲(intravasation)、外滲(extravasation)及他處器官增殖(proliferation)等步驟，從原處生長器官透過血管或淋巴管逃脫並轉移至遠處的另一器官生長，造成癌細胞擴散，預後極差(Fidler, et al., 2003)。一般而言，正常細胞也擁有轉移能力，但受到極嚴密的生長調控，而發生轉移的癌細胞則明顯不受控制。在癌症病例中，有 85%屬於上皮癌。正常的上皮細胞透過細胞-細胞間(cell-cell)與細胞-基質間(cell-matrix)的連接，形成細胞的極性而具有正常上皮細胞塑性(epithelial plasticity)，相當穩定且有順序的排列；但不正常的上皮細胞因基因失調導致不正常增生或失去其塑性(plasticity)調控時，就具有入侵

(invasion)及移動(migration)能力，此時便發生癌轉移。因此，上皮細胞塑性的調控與癌症轉移有極大的關係。

上皮細胞塑性的轉變過程稱之為 EMT。EMT 原本是胚胎形成時的重要過程，藉由降低細胞連接，提升轉移能力，使其到指定地點生長；後來研究發現在腫瘤轉移(metastasis)中也會發生類似現象，卻不受時程調控。EMT 的發生可藉由一些分子訊號的傳遞(例如：Wnt、EGF、TGF-β)導致轉錄因子的活化而起始，像是 Snail、Slug、Twist、SIP、ZEB1 等(見 Fig1.)。當 EMT 發生時，細胞間的連接會消失，細胞骨架會重組， 基質會被溶解，上皮細胞塑性的特徵 E-cadherin、α-catenin、β-catenin 表現量會降低，間葉細胞塑性特徵 N-cadherin、Fibronectin、Vimentin 表現量會增加，造成細胞轉移。在癌症中，EMT 常被辨識為癌轉移的特徵(Gavert & Ben-Ze'ev, 2008)。

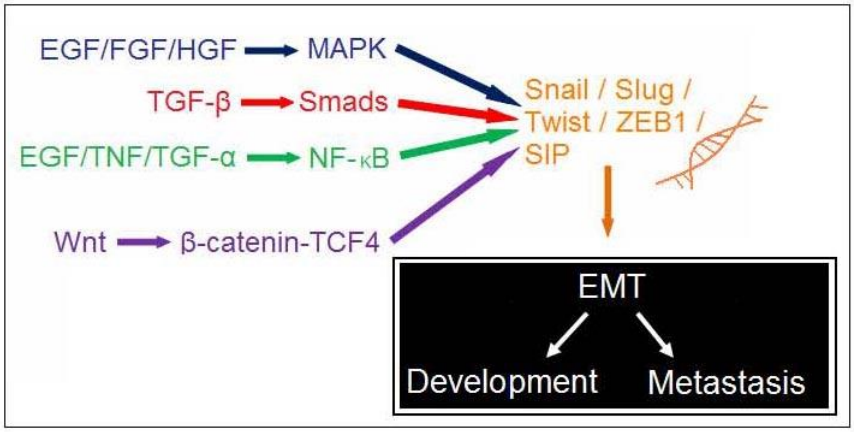


Figure1. 引發EMT的多種機制。

TGF-β、EGF、Wnt 等訊號會誘導 Snail、Slug、Twist、ZEB1、SIP 等主要轉錄因子活化 EMT 導致細胞轉移。

三、 YWHAZ (14-3-3ζ) 蛋白

YWHAZ(14-3-3δ)蛋白是 14-3-3 家族蛋白的其中一員，δ(zeta)。14-3-3 家族蛋白在哺乳動物中共有 7 個異構物(β、ε、γ、ε、σ、τ、δ)，大小約 28 ~ 33 kDa，於 1967 年首度發現在大腦中(見 Fig2.)。YWHAZ 主要調控細胞生長週期、傳遞訊號、細胞凋亡(Hermeking et al., 2005) (van et al., 2001)。近年來，YWHAZ 被發現其與肺癌有極密切的關係(Li et al., 2005) (Fan et al., 2007)，最近更發現 YWHAZ 會引發肺癌轉移，然而其相關機制仍不明瞭(Yang, 2008)。

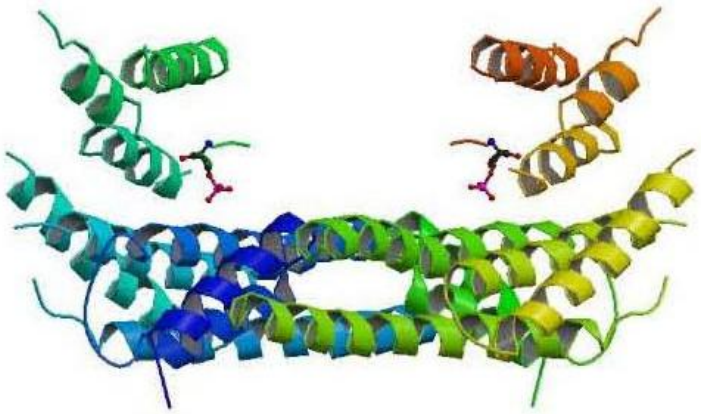


Figure2. YWHAZ 蛋白結構圖。

YWHAZ 蛋白過去被認為是細胞週期調控蛋白，近年則發現與肺癌轉移有極大關聯，然而其分子機制仍不明瞭。(http://en.wikipedia.org/wiki/YWHAZ)

四、 以基因微陣列技術(cDNA Microarray)探討在肺癌中過量表現的 YWHAZ 引發 EMT 現象之分子機制

在篩選並分析基因的表現中，基因微陣列技術(cDNA Microarray)是一個被廣泛應用的偵測技術。首先，將聚合酶連鎖反應後純化之基因片段於載玻片上製成微陣列。接著以兩種核酸探針分別標示不同波長的螢光分子與載玻片上的基因進行雜交反應。經過雷射光的激發及掃描後，放射出兩種波長的螢光同時被收集並數位化，可用以估量基因表現的程度。近年來常被用作觀察致癌基因於癌症中的表現。

先前我們實驗室以大量表現 YWHAZ 的肺癌細胞株 CL1-0 進行觀察，發現大量表現 YWHAZ 蛋白會引發 EMT，造成肺癌細胞侵襲及轉移。為探討 YWHAZ 蛋白引發肺癌轉移的分子機制，我們利用基因微陣列技術觀察大量表現 YWHAZ 的 CL1-0 肺癌細胞株中，發現數種會引發 EMT 的基因其表現情形，並進行訊息傳遞路徑之分析。結果顯示 Wnt/β-catenin signaling pathway、TGF-β signaling pathway、MAPK、EGF 四個訊息傳路徑可能參與 EMT 過程，尤其以 Wnt/β-catenin signaling pathway 的相關基因較明顯的大量表現，說明此路徑可能被活化而參與 EMT 過程。(見附錄 1)

五、 Wnt/β-catenin 訊息傳遞路徑(Wnt/β-catenin signaling pathway，以下簡稱 Wnt/β-catenin 路徑)

目前已知 Wnt/β-catenin 路徑會參與胚胎發育過程，也會誘導腫瘤生成，還會引發 EMT 導致癌症轉移。正常情況下，即路徑不進行時，β-catenin、CKI (casein kinase I)、APC (adenomatous polypsis coli)、GSK3β (glycogen synthase kinase 3β) 會組合到身為鷹架蛋白(scaffold protein)的 Axin，形成複合體。同時，GSK3β 會對 β-catenin、APC、axin 進行磷酸化作用，促使 β-catenin 被 β-transducin repeat containing protein (β-TrCP)辨認並進行降解標記(Ubiquitination)，然後被 26S proteasome 分解。此時，進入核中的 TCF4 (T-cell factor 4)受到 Groucho 的抑制，不進行轉錄表現。當 Wnt/β-catenin 路徑活化時，Wnt 蛋白會以旁泌性的方式到達並活化其受體 Frizzled (Fz)/ LRP，造成 LRP 被 GSK3β 和 CKI 磷酸化，Axin 遠離 β-catenin。因此，β-catenin 便不被降解，而在細胞質間穩定地累積，並與 TCF4 共同進入細胞核啟動其目標基因 c-myc、cyclin D1、Slug 等等，引發細胞生長或 EMT。(Clevers, 2006) (Akiyama, 2000) (見 Fig3.)。

以上可知，β-catenin 的穩定性及入核現象被視為 Wnt/β-catenin 路徑是否

活化的主要關鍵，因此了解 β-catenin 的特性便十分重要。

β-catenin 本身有三個角色：其一，是細胞外骨架 E-cadherin 與細胞內骨架 α-catenin、actin 間的黏附因子；其二，是在細胞質中的游離態，含量受控於 Wnt 訊號的表現，有未磷酸化及磷酸化兩種形式，磷酸化的 β-catenin 將被降解；最後，則是未磷酸化的 β-catenin 受到 Wnt 訊號調控而在細胞質穩定累積，並與 TCF4 共同進入細胞核轉錄生長相關基因(Gavert & Ben-Ze'ev, 2008)。

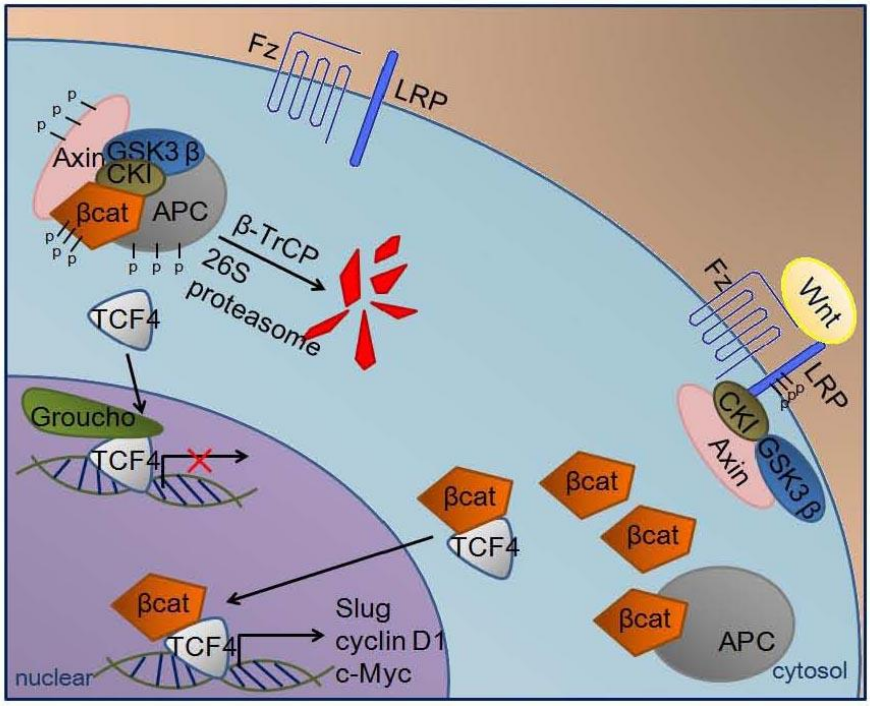


Figure3. Wnt / β-catenin signaling pathway示意圖。

正常狀況下，Wnt 訊號不傳遞，β-catenin 會與 Axin、CKI、APC、GSK3 形成複合體降解，核內的 TCF4 受到 Groucho 抑制，並不表現 Wnt 目標基因。當 Wnt 訊號傳遞時，β-catenin 不會被降解，而是與 TCF4 結合共同進核轉錄 Wnt 目標基因 Slug、c-Myc、cyclin D1 等。

貳、研究動機及目的

根據我們實驗室先前的研究結果顯示，大量表現 YWHAZ 會造成肺癌細胞 EMT 之產生。利用 cDNA 微陣列技術(microarray)發現 YWHAZ 蛋白會導致 Wnt 訊息傳遞路徑之相關基因大量表現。另外，過去文獻亦指出，在胚胎上皮細胞中，YWHAZ 會與 β-catenin 產生交互作用，促使 β-catenin 在細胞質大量累積並進入核內，造成胚胎發育(Tian et al., 2004)，而在口腔癌細胞中也發現相同的交互作用，其功能是抗凋亡(Matta et al., 2007)。因此我們進一步推測，YWHAZ 蛋白在肺癌中也可能藉由促進 β-catenin、TCF4 在細胞核內的蛋白表現量，因此活化 Wnt 目標基因，進而引發 EMT 導致肺癌轉移。故本研究目標主要可分成三部分：

一、觀察在大量表現 YWHAZ 的肺癌細胞株中，β-catenin、TCF4 的表現及分布

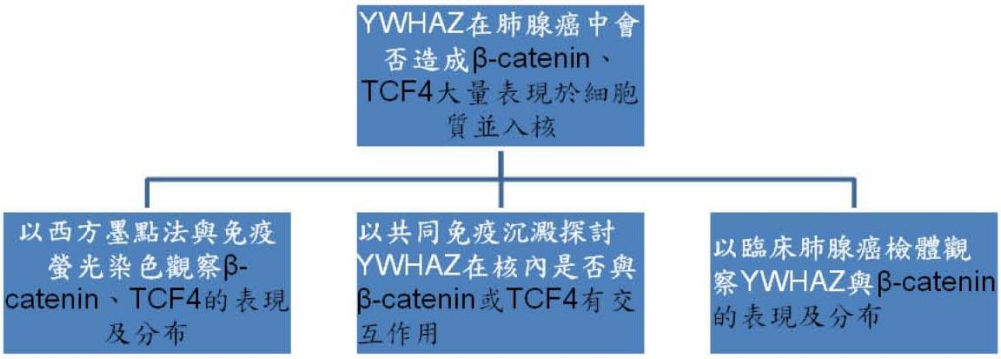
首先建立能大量表現 YWHAZ 的 CL1-0 肺癌細胞株與植入空載體的 CL1-0 肺癌細胞株，並以西方墨點技術與免疫螢光染色技術觀察 β-catenin、TCF4 蛋白在細胞內的分表現量與分佈位置，探討 YWHAZ 會否會促進 β-catenin 與 TCF4 蛋白核內外的表現量與分佈。

二、以免疫共沉澱觀察 YWHAZ 在核內是否與 β-catenin、TCF4 有交互作用

我們進一步利用網路資料庫預測 β-catenin、TCF4 蛋白是否具有 YWHAZ 結合圖域(motif)，並以共免疫沉澱(co-immunoprecipitation)法探討 YHWAZ 蛋白與 β-catenin 和 TCF4 在細胞核內的交互作用。

三、以臨床肺癌檢體鑑定 YWHAZ 與 β-catenin 的表現量及分布位置

我們以臨床檢體驗證細胞株實驗的結果，觀察 YWHAZ 與 β-catenin 在組織切片中的表現及分布。



參、實驗材料及方法

一、細胞株與細胞培養

1. 細胞株

本研究所採用的人類肺腺癌細胞 CL1-0 為台大醫學院基因體醫學研究中心楊泮池教授實驗室所提供。該細胞母株為一名男性肺腺癌患者提供，此細胞株經過 RPMI(Roswell Park Memorial Institute-1640)培養液培養約 60 代後，再依據其試管內侵入能力(in vitro invasion)建立不同轉移能力之細胞株(CL1-0 < CL1-5，CL1-0 為最低)。CL1-0 細胞株屬貼附型細胞，培養於含有 10%胎牛血清(FBS, fetal bovine serum, Invitrogen)和 1% penicillin / streptomycin (Invitrogen)的 RPMI 培養液。另外，我們採用的大腸癌細胞株 SW480、SW620、HCT116 則是從網路訂購，培養方法與 CL1-0 同。

2. 細胞培養

將細胞培養於 10 cm 培養皿(Corning)中，待細胞長至八分滿，吸掉培養液並以 1X PBS 緩衝液清洗，藉由 trypsin-EDTA 作用使細胞懸浮，吸掉 TE，加入 5 ml 培養液中和 TE，並將細胞打散，接著取 1：6 稀釋細胞至另一個培養皿，並加入適量培養液(以覆蓋過培養皿為原則)於 37℃、5 %二氧化碳培養箱中培養。(Oksvold et al., 2004)

二、質體構築與抗體

將萃取出的 YWHAZ mRNA 以反轉錄作用與 cDNA 回收技術、即時定量聚合酶連鎖反應(real-time quantitative PCR, RTQ-PCR)技術，構築於 pCMV-Tag 3 載體上。YWHAZ 外生性抗體採自 V5，材料與抗體由陳健尉教授實驗室學長姐提供。

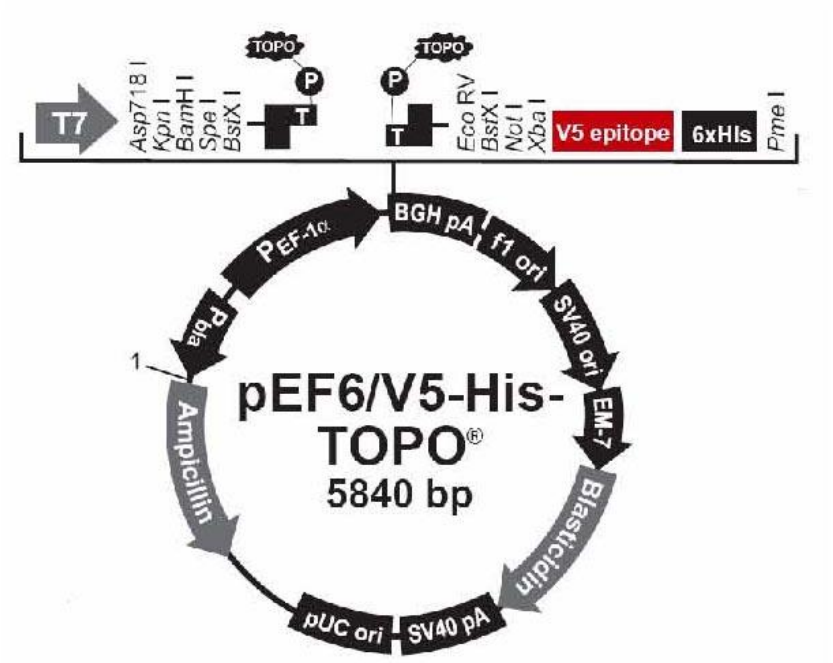


Figure4. pEF6/V5-His 的限制酶切位及圖譜。

(此圖摘自 Invitrogen vector information)

三、轉染作用(transfection)

以 24 孔細胞培養盤所培養之 CL1-0 肺癌細胞株為例，將 2 μl 的脂質體轉染試劑與 RPMI 細胞培養液混合成總體積為 50 μl，靜置 5 分鐘後，與 50 μl 有 1 μg 欲轉染質體的 RPMI 混合靜置 20 分鐘，之後將此 100 μl 混合液加入培養在 0.5 ml 含有 10% FBS/RPMI 的細胞中(不含任何抗生素)，4 到 6 小後，將培養液吸去，置換成為適當的培養液，再繼續培養 18-24 小時後，便進行穩定細胞株加藥(Blasticidin)篩選確認轉染成功，即可進行相關的細胞實驗。

四、細胞蛋白質之核質分離(Cytoplasmic and nuclear fraction protein extraction)與定量(protein amount assay)

利用 TE 將細胞從細胞培養皿打下，離心 5 分鐘後以 PBS 清洗細胞，加入 50 μl 的 Hypotonic buffer(含有 proteinase inhibitor)輕輕彈散細胞 pellet，置於冰上約 20 分鐘，其間不時輕彈管壁。於 4℃ 以 5000 rpm 離心 10 分鐘，其沉澱之 pellet 待之後備用，取上清液於 4℃ 以 13000 rpm 離心 10 分鐘後，其上清液即為含有細胞質之蛋白溶液。以 200 μl 1X PBS 清洗之前沉澱之細胞 pellet，於 4℃ 以 3500 rpm 離心，重複三次後，加入 High salt buffer(含有 proteinase inhibitor)於沉澱之細胞 pellet，置於冰上並不時 vortex 約 40 分鐘，再於 4℃ 以 13000 rpm 離心 15 分鐘後，取上清液，即為含有細胞核之蛋白溶液。

蛋白質濃度是以 Bradford 的方法來定量，利用酸性溶液 CBG (coomassie brilliant blue G-250)染劑和不同濃度的蛋白質反應，產生差異性的顏色變化。蛋白質的濃度越高，染劑和蛋白質結合而產生顏色變化的量也相對成正比。首先，製備蛋白質標準液，以 0.5 mg/ml 的 BSA(bovine serum albumin，Sigma)當作標準液，以去離子水作為空白組。第二步，取 2 μl 標準液與 1 ml CBG 染劑(0.12 mM coomaisse brilliant blue G-250、95 % ethanol、85 % orth-phospaoric acid)混合作用。第三步，以分光光譜儀(spectrophotometer DU800，Beckman Coilter)在 OD595nm 測定樣品吸光值。第四步，畫出標準曲線，以內插法計算出樣品濃度(Y=A+BX；Y：OD595nm 讀值；X：待測樣品濃度)。

五、蛋白質凝膠電泳分析(SDS - PAGE, SDS - polyacrylamide gel electrophoresis)與西方墨點法(western blot)

取出玻璃片、白色氧化鋁板和間隔條，組成三明治夾板後固定在鑄膠器上。依目標蛋白質分子量不同之需求，注入含有 SDS 之不同比例的聚丙烯醯氨的解析凝膠(resolving gel)於架設好的電泳玻璃中至八分滿，並覆上一層乙醇。待膠體凝結後，倒掉乙醇再加入 5%集焦凝膠(stacking gel)，插入齒梳，再置於室溫待膠體凝結。等待期間，取出蛋白質樣品在冰上回溶，並加入 6 倍膠體電泳染劑混合均勻，在 100℃下加熱 5 分鐘使蛋白質變性。將鑄膠完成的三明治夾板固定於裝有 1x running buffer 的電泳槽內，再把蛋白質樣品依序注入到齒模中。設定電壓 70 福特，當追蹤染料到達解析凝膠時，提高電壓至 120 福特，抵達膠體底部後即可停止。

利用 SDS–PAGE 將蛋白質進行電泳分離後，截去 stacking gel，將凝膠浸泡在轉漬緩衝液中，由負極向正極方向依序排列，3MM 濾紙、凝膠、polyvinylidene fluoride(PVDF)轉漬膜、3MM 濾紙之組合，每一層預先用轉漬緩衝液濕潤(PVDF 膜須先用甲醇潤洗)，裝置於 SEMI-PHOR TransferSystem 中，固定電流 250 mA 進行轉漬 45 分鐘。轉漬完成後將 PVDF 轉漬膜以阻斷劑於室溫下震盪浸泡 1 小時。最後以 ECL 及 X-ray film 進行壓片。(Oksvold et al., 2004)

六、免疫螢光染色(immuno-fluorescence stain)

於 24 孔盤中置入蓋玻片(cover slide)並注入細胞培養液，靜置於細胞培養箱中。24 小時後移去細胞培養液，每孔種 3 x 10 4 顆細胞(約三成)，12 小時後進行轉染；轉染 12 小時後以 4% paraformaldehyde(容於 1 x PBS，PH7.4)於室溫下固定 15 ~ 20 分鐘，以 1 x PBS (pH7.2 ~ 7.4)清洗蓋玻片二次(各 5 分鐘)。再以 0.1%Triton-X 進行細胞膜穿孔，以 5% BSA-PBS 進行阻斷，去除背景值後，加入以 PBS 稀釋之一級抗體於 4℃標記目標蛋白 16 小時，再以 1 x PBS 溶液清洗細胞。再加入螢光標記之相對二級抗體或 phalloidin，於 37℃下標記目標蛋白 1 小時，以 PBS 清洗細胞後，進行 DAPI (標記細胞核)染色，之後將蓋玻片封片，於螢光顯微鏡下觀察。

七、免疫沉澱分析(immunoprecipitation, IP)

當 10cm dish 達 100%滿度時以冰的 PBS 清洗一次，將殘留的 PBS 吸乾，加入 500 µl 細胞溶解緩衝液及 1X protease inhibitor cocktail set lll，將細胞刮下並轉置入 1.5 毫升之微量離心管中，於 4℃混合 30 分鐘後離心 20 分鐘，收取上清液並測量蛋白質濃度。再各取等量蛋白質並加入一級抗體，於 4℃充分混合 16 小時後，再加入 25 µl UltraLink Immobilized Protein G beads 於 4oC 下反應 1 小時半以結合目標蛋白。反應完後再離心，去除上清液，以細胞溶解緩衝液清洗 Protein G-protein complex 表面 6 次，最後加入 SDS sample loading buffer，於 100oC 處理樣本 5 分鐘後取上清液進行凝膠電泳及西方墨點法。轉染基因構築的細胞，則於轉染隔天才以細胞溶解緩衝溶液收下培養盤上的細胞，其餘步驟同上。(Oksvold et al., 2004)

八、免疫組織化學染色(immunochistochemistry)

1. 脫臘

首先，將組織切片按順序連續浸泡 Xylene 20 分鐘(重複二次)、不同比例酒精若干分鐘後，用 PBS 輕輕沖洗二次。接著將 300ml citrate buffer-tween 與組織切片一併放入 500ml 燒杯中，燒杯口以保鮮膜包覆並微波，再添加適量去離子水繼續微波，接著放在冰上直至室溫為止。然後將樣品清洗再以雙氧水浸砲，最後用 PBS 輕輕沖洗二次。

2. 免疫染色

將組織切片玻璃忊放，加入適量 blocking buffer 並以 parafilm 覆蓋，於室溫下避光反應，清洗後繼續加入一級抗體 IgY 於 4℃反應至隔天。隔天再以 1:100 稀釋二級抗體(Zymed)並以 parafilm 覆蓋於室溫避光反應，然後以帶有螢光三級抗體原液並以 parafilm 覆蓋於室溫避光反應。接續使用 DAB-plus substrate kit (Zymed)呈色，待組織顏色轉變為棕色時，再加入 Hematoxylin 染劑。最後加入 37mM NH_4OH 染劑，續以自來水沖洗。

3. 脫水及封片

最後，將組織切片按順序連續浸泡不同濃度的酒精各 3 分鐘、Xylene 5 分鐘(重複 3 次)後，吸乾組織切片上多餘的水分，利用滴管取適量封片膠滴在載玻片上，將蓋玻片倒蓋於載玻片上，待蓋玻片固定於載玻片上即可室溫保存備用。(Lin et al., 2009)

肆、研究結果

1. 觀察在大量表現 YWHAZ 的肺癌細胞株中，β-catenin 及 TCF4 蛋白核內外表現量及細胞分布位置

首先以西方墨點法觀察大量表現 YWHAZ(以下簡稱 YWHAZ 組)與植入空載體(以下簡稱 mock 組)的 CL1-0 肺癌細胞株中，β-catenin 及 TCF4 的蛋白表現量差異。我們發現在 mock 組中，β-catenin 在細胞核與細胞質無明顯表現，TCF4 則在核內無明顯表現；而當大量表現 YWHAZ 時，β-catenin 在細胞質與細胞核的蛋白表現量會增加；另一方面，TCF4 蛋白在細胞核的表現量也明顯增高。(見 Fig5.)

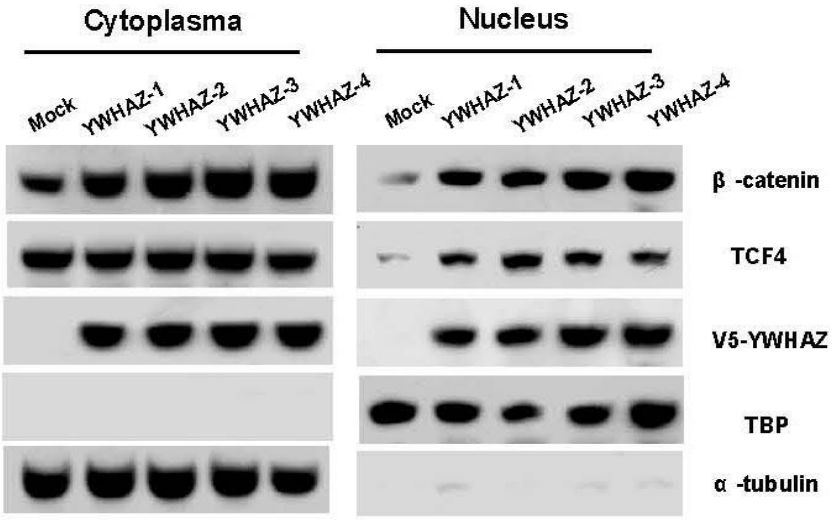


Figure5. 以西方墨點法探討 β-catenin 及 TCF4 在 mock 組與 YWHAZ 組的蛋白表現量及細胞分布位置。TBP 與 α-tubulin 各為細胞核與細胞質的定量控制。

我們再以免疫螢光染色觀察 mock 組與 YWHAZ 組中，β-catenin 及 TCF4 在細胞核與質的分布差異。我們發現在 mock 組中，β-catenin 及 TCF4 並無明顯的入核趨勢；但在 YWHAZ 組中，β-catenin 及 TCF4 均有較明顯的入核趨勢。 (見 Fig6.)

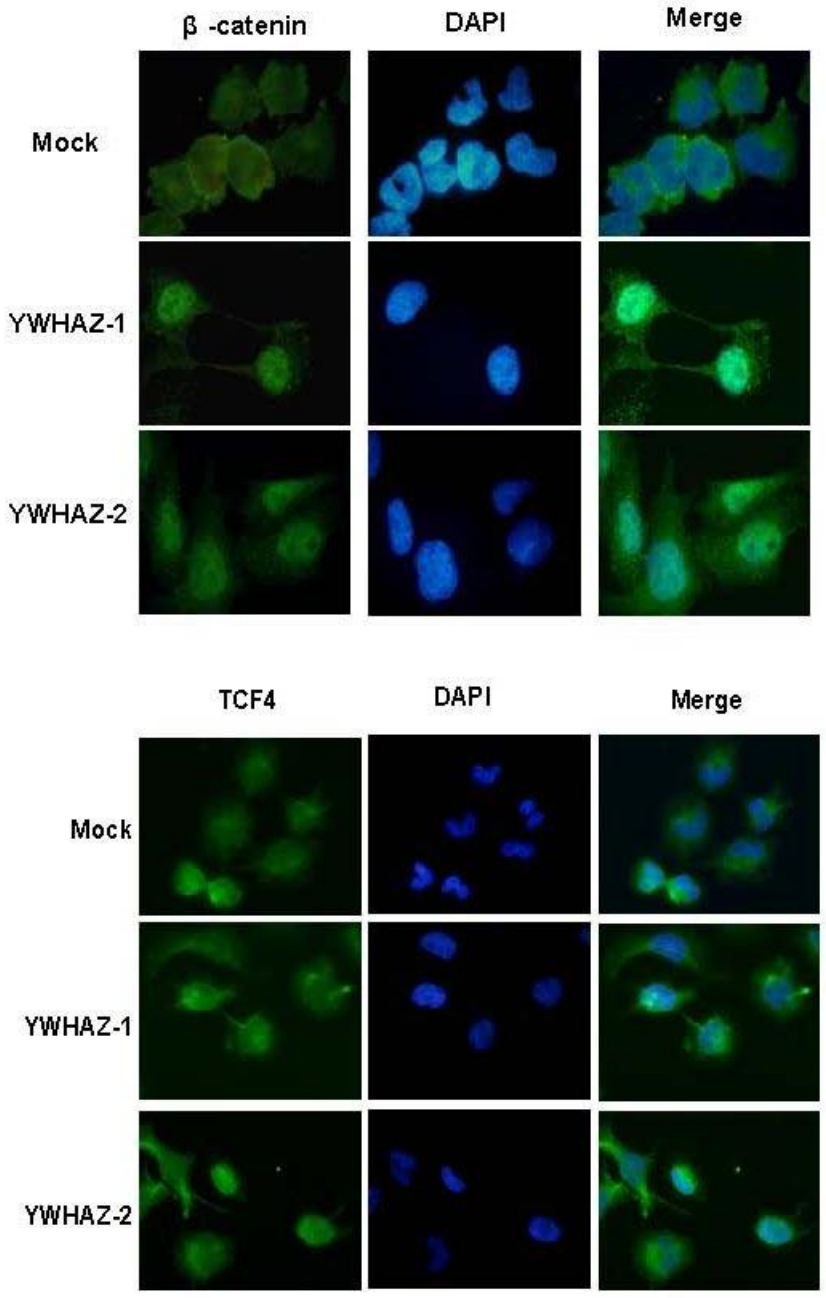


Figure6. 以免疫螢光染色觀察 β-catenin、TCF4 的核質分布。DAPI 為細胞核染色，Merge 為前二圖之重疊。

二、以免疫共沉澱法觀察 YWHAZ 在核內是否與 β-catenin、TCF4 有交互作用

由上述實驗發現 YWHAZ、β-catenin 與 TCF4 蛋白會同時在細胞核內表現，於是我們推測 YWHAZ 入核可能與 β-catenin/TCF4 的核內作用有關。首先，我們利用網路資料庫(http://scansite.mit.edu/cgi-bin/motifscan_id)預測 YWHAZ 與 β-catenin 或 TCF4 是否可能有交互作用，結果顯示 YWHAZ 與 β-catenin 和 TCF4 均可能有交互作用。(見 Fig7,8.)

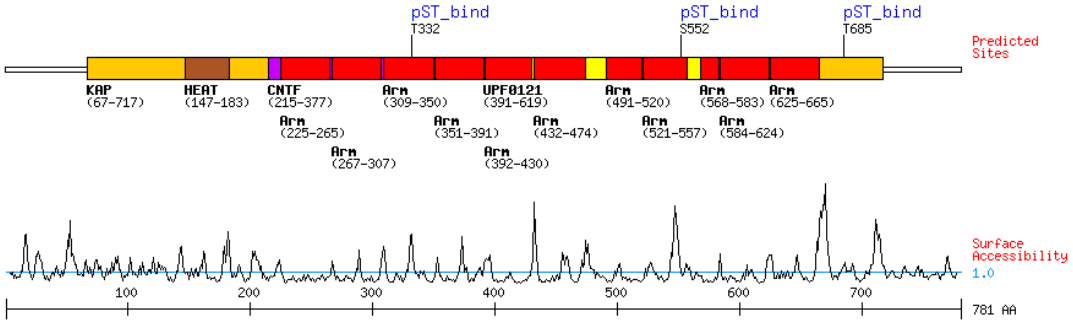


Figure7.YWHAZ 可能與 β-catenin 交互作用的位置

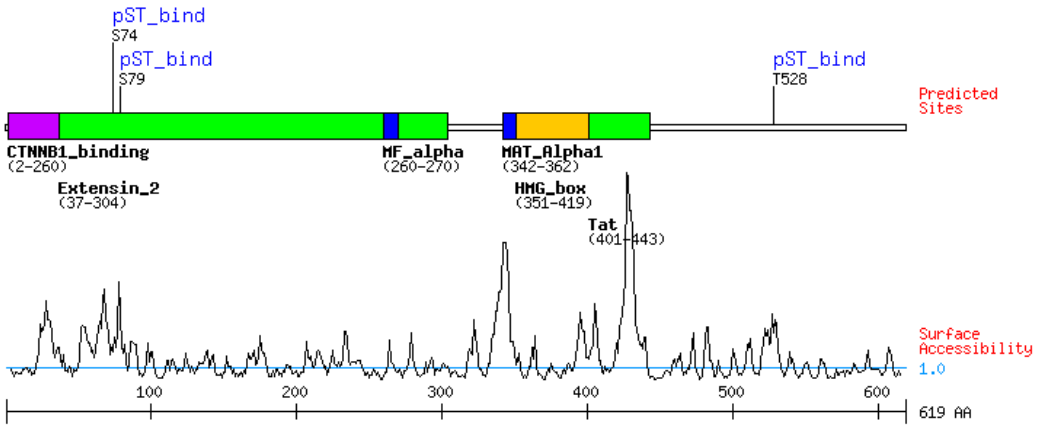


Figure8. YWHAZ 可能與 TCF4 交互作用的位置

因此我們對 mock 組與 YWHAZ 組抽取細胞核內蛋白，以 V5 的一級抗體進行 V5-YWHAZ 免疫共沉澱，再以 YWHAZ、β-catenin、TCF4 抗體辨認。結果發現當大量表現 YWHAZ 時，YWHAZ 會與 β-catenin、TCF4 在細胞核中產生交互作用。(見 Fig9.)

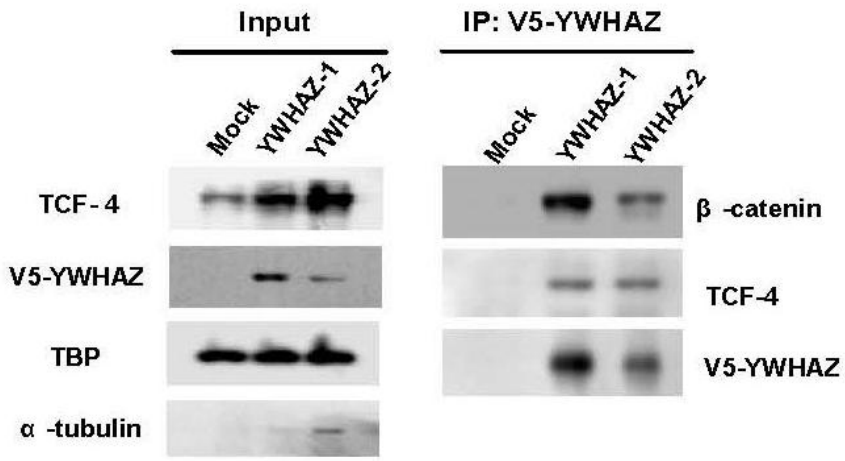


Figure9.

以 V5 抗體抓取過量表現的 YWHAZ，進行對 mock 組與 YWHAZ 組細胞核蛋白的免疫共沉澱。Input 為待測蛋白質於細胞核的總含量，對其抓取蛋白與交互作用蛋白作為輔助確定。TBP 為細胞核定量控制，α-tubulin 為細胞核定性控制，因其只存於細胞質。

由於上述實驗是以大量表現的 YWHAZ 為抓取對象，屬於外源性(exogenous)表現，我們進一步觀察內生性(endogenous)YWHAZ 蛋白是否也在核中與 β-catenin、TCF4 進行交互作用。於是，我們對 SW480、SW620、HCT116 等其內源 YWHAZ 與 β-catenin 含量較高的大腸癌細胞株(colon cancer)進行內生性的 YWHAZ 與 β-catenin 免疫共沉澱，也發現 YWHAZ、β-catenin、TCF4 三者於細胞核中有交互作用。(見 Fig10.)

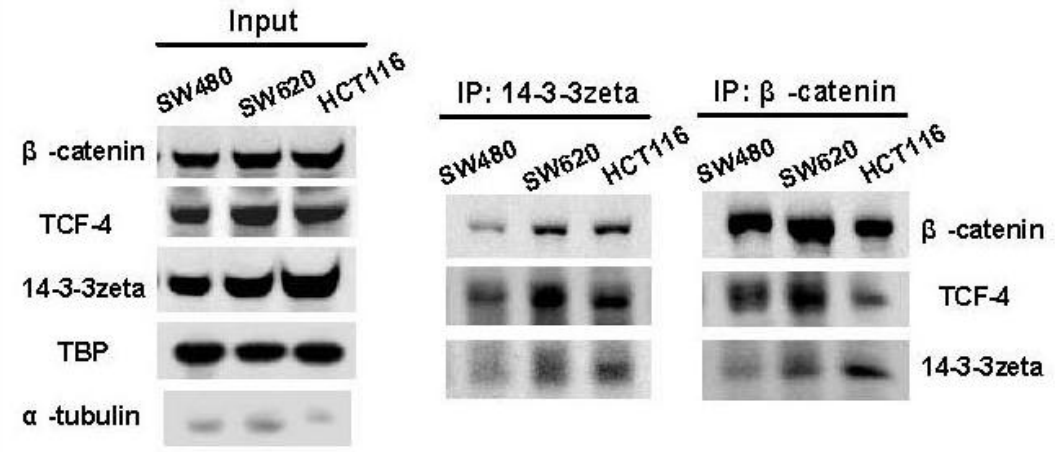


Figure10

以抗體抓取內源性的 YWHAZ 和 β-catenin，進行對 SW480、SW620、HCT116 三株大腸癌細胞其細胞核的免疫共沉澱。

三、以臨床肺癌檢體鑑定 YWHAZ 與 β-catenin 的蛋白表現量及細胞的分布位置

為進一步探討臨床上的現象，我們從台中榮民總醫院取得肺腺癌病人檢體切片(註 1)，進行免疫組織化學染色，觀察 YWHAZ 與 β-catenin 在肺腺癌組織與鄰近正常肺葉組織的表現及分布。結果顯示 YWHAZ 蛋白的表現量在鄰近正常組織的病人檢體表現量較低，而在肺癌組織的檢體有較高的表現量，同時 YWHAZ 蛋白主要分布於細胞質中，少部分會進入細胞核內。而 β-catenin 在肺癌組織的檢體也有較高的蛋白表現量，並且同樣有進入細胞核的現象。(見 Fig11,12.)

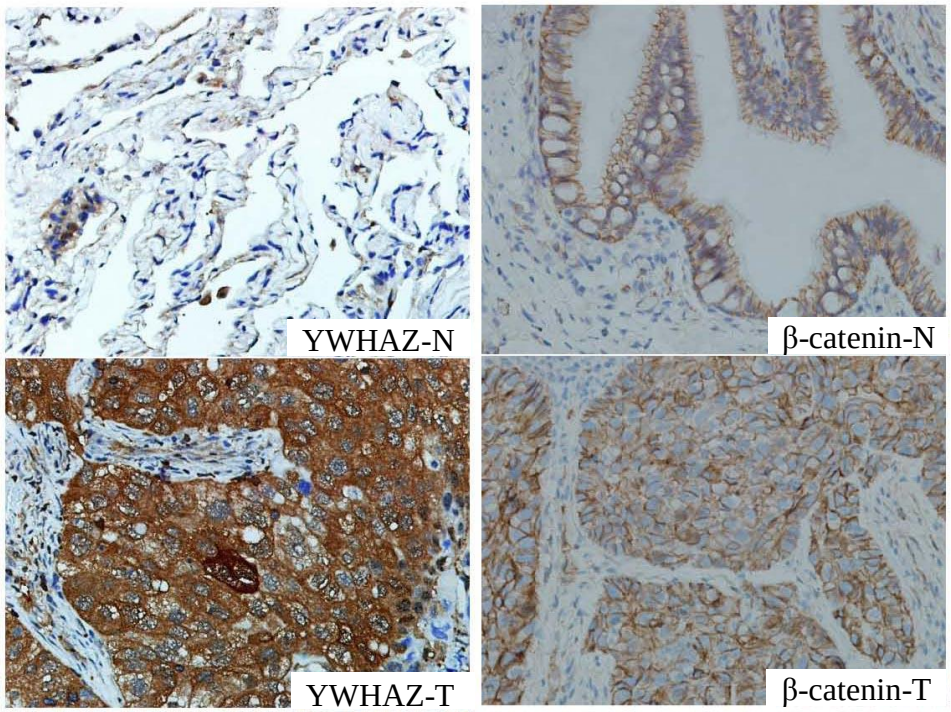


Figure11. 在正常肺部組織與肺癌病人組織中，YWHAZ與β-catenin表現及分布的差異。
上方：正常肺部組織 下方：肺癌病人組織
左方：藍色→細胞核 褐色→YWHAZ蛋白
右方：藍色→細胞核 褐色→β-catenin 蛋白

註 1：我們實驗室與台中榮民總醫院有合作關係，其肺腺癌檢體是從台中榮民總醫院接受開刀的非小細胞肺癌病人的肺癌及鄰近正常組織配對的病人檢體。

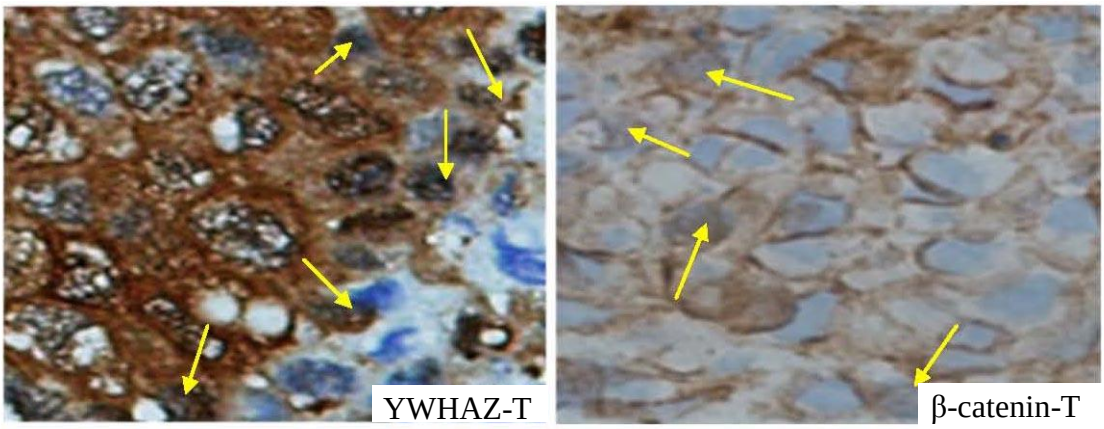


Figure12. 在肺腺癌中，當藍色(細胞核)與褐色(左為 YWHAZ，右為 β-catenin)混合時，即代表 YWHAZ 或 β-catenin 有入核的現象。

伍、討論

近年發現 YWHAZ 蛋白在其他癌症類型的重要性。2009 年 8 月，有相關文獻指出 YWHAZ 在頭頸癌中會引發 EMT 導致癌轉移(Lin et al., 2009)。2009 年 9 月，更發現 YWHAZ 在乳癌中會藉由 TGFβ 路徑造成 EMT 現象，導致乳癌轉移(Jing et al., 2009)。本實驗發現大量表現 YWHAZ 蛋白會增加細胞核內 β-catenin 和 TCF4 的蛋白表現量，且發現 YWHAZ 本身也會入核與 β-catenin、TCF4 進行交互作用，在臨床肺腺癌檢體也發現 YWHAZ 蛋白的表現量在鄰近正常組織的病人檢體表現量較低，而在肺癌組織的檢體有較高的表現量，同時 YWHAZ 蛋白主要分布於細胞質中，少部分會進入細胞核內。

在 Wnt/β-catenin pathway 中，β-catenin 與 TCF4 大量表現並共同入核即代表 Wnt/β-catenin 路徑的活化，造成細胞生長或具有轉移能力(Clevers, 2006；Gavert & Ben-Ze'ev, 2008)。我們發現 YWHAZ 在肺腺癌細胞中會增加細胞核內 β-catenin 和 TCF4 的蛋白表現量，因此 YWHAZ 蛋白可能會引發 Wnt/β-catenin 路徑之活化。

當 β-catenin 與 TCF4 結合並進入核內時，會啟動其下游基因 c-Myc、cyclin-D1、Slug 的表現(Akiyama, 2000)。我們於實驗中也發現 YWHAZ 也會進入細胞核並與 β-catenin、TCF4 交互作用，共同形成複合體，因此推測 YWHAZ 可能也共同參與其下游基因轉錄的表現。

在肺腺癌病人檢體中，觀察 YWHAZ 與 Wnt/β-catenin 路徑的主要特徵：β-catenin，我們也再度獲得證實 YWHAZ 與 β-catenin 蛋白表現量在肺腺癌中會增加，並且也會進入細胞核內。(見 Fig13.)

我們推測 YWHAZ 可能藉由結合 β-catenin 並引發 Wnt/β-catenin 路徑的活化，進而參與 EMT 的產生而促進肺腺癌轉移。我們期望，未來除了可以利用 YWHAZ 作為肺腺癌轉移的預後評估，也可以開發阻斷 YWHAZ 表現的相關藥物，就能抑

制肺腺癌轉移，進而將癌症殺傷力降至最低，肺腺癌將不再是不治之症。

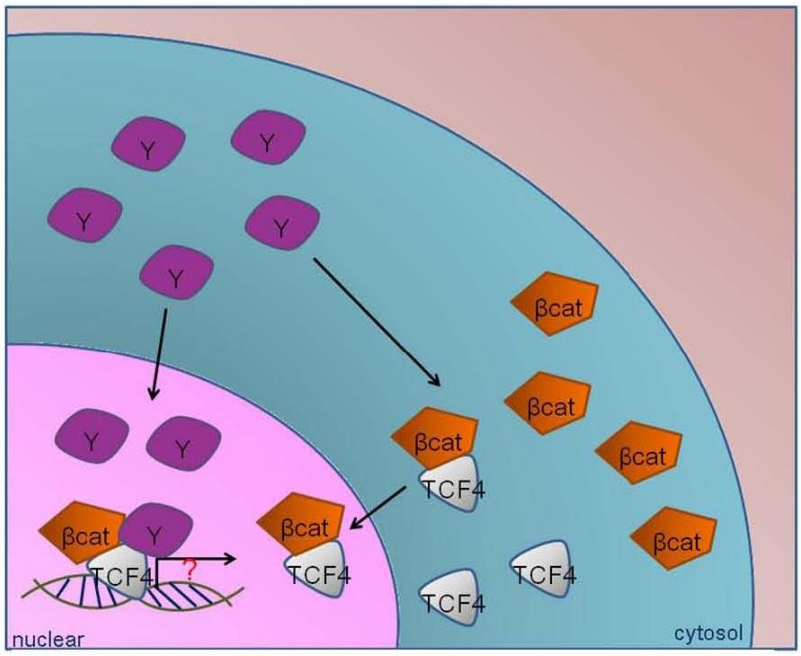


Figure13. YWHAZ 可能活化 Wnt/β-catenin 路徑想像圖

陸、感謝

在此，我要特別感謝中興大學陳健尉教授與靜嫻學姐、俊豪學長、文馨學姊的指導、高中陳孟宏老師的全力支持、親愛的家人無條件付出、一中學長的不吝建議、晚上還要幫我開大門的警衛叔叔與舍監、一路走來同學們給我的加油打氣，還有所有曾經幫助過我的人。謝謝你們，沒有你們，就沒有現在的我。

柒、參考資料

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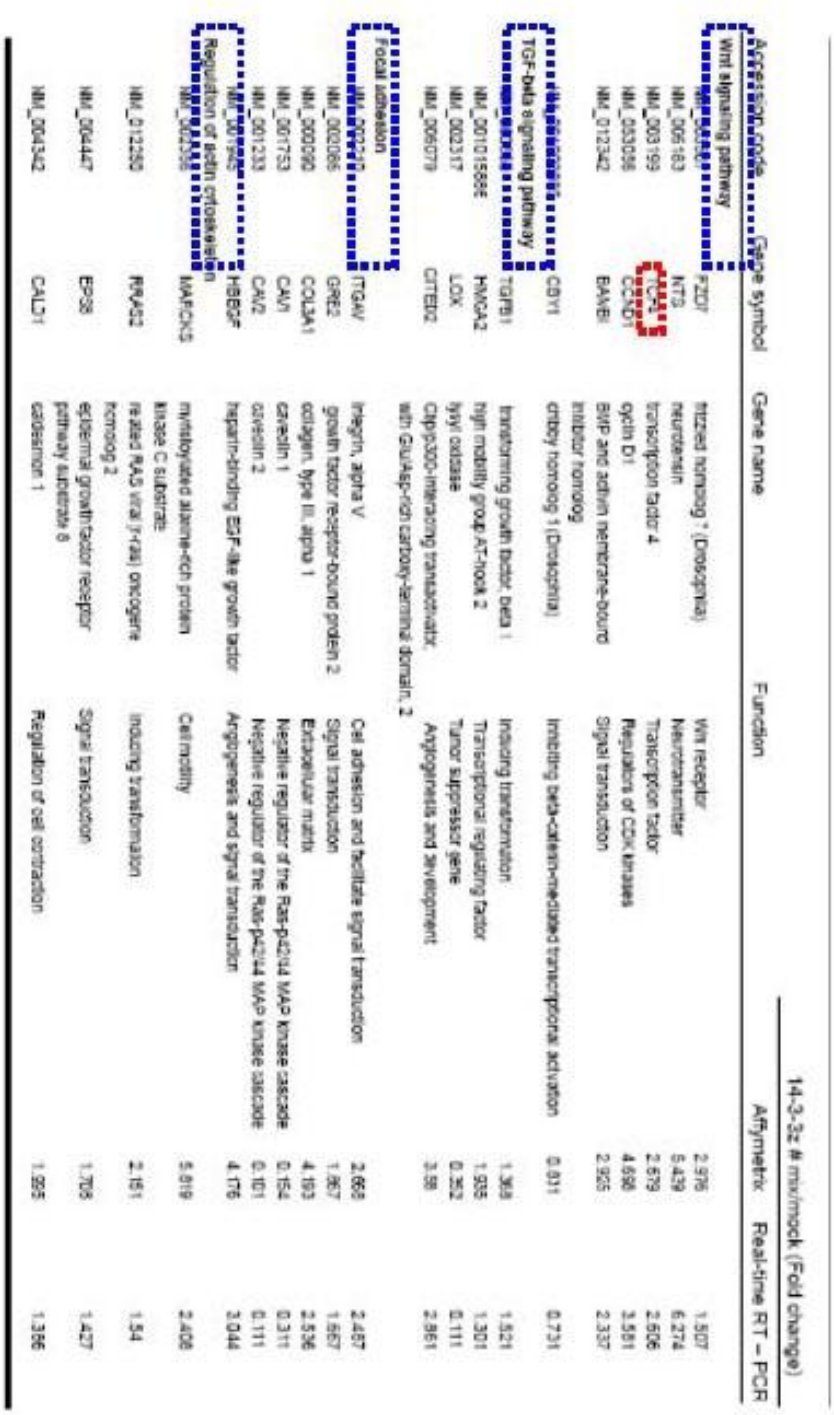
捌、附錄

一、在過量表現 YWHAZ 的 CL1-0 肺癌細胞株中，前人探討數種會導致 EMT 的相關基因其表現，發現 Wnt signaling pathway 的基因表現比較明顯。



Accession code	Gene symbol	Gene name	Function	14-3-3ε # mix/mock (Fold change)	
				Affymetrix	Real-time RT – PCR
Wnt signaling pathway					
[redacted]	FZD7	frizzled homolog 7 (Drosophila)	Wnt receptor	2.976	1.507
NM_005163	NTS	neurotensin	Neurotransmitter	5.439	6.274
NM_003199	TCF4	transcription factor 4	Transcription factor	2.679	2.606
NM_053056	CCND1	cyclin D1	Regulators of CDK kinases	4.698	3.561
NM_012342	BAIAP1	BAI1-associated protein 1 (novel inhibitor of hemo	Signal transduction	2.955	2.337
[redacted]					
TGF-beta signaling pathway					
[redacted]	CBY1	chibby homolog 1 (Drosophila)	Inhibiting beta-catenin-mediated transcriptional activation	0.931	0.731
[redacted]	TGFB1	transforming growth factor, beta 1	Inducing transformation	1.359	1.521
NM_001015885	HMGA2	high mobility group AT-hook 2	Transcriptional regulating factor	1.935	1.301
NM_002317	LOX	lysyl oxidase	Tumor suppressor gene	0.352	0.111
NM_006079	CITED2	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2	Angiogenesis and development	3.58	2.861
Focal adhesion					
[redacted]	ITGAV	integrin, alpha V	Cell adhesion and facilitate signal transduction	2.668	2.467
NM_002086	GRB2	growth factor receptor-bound protein 2	Signal transduction	1.867	1.667
NM_000090	COL3A1	collagen, type III, alpha 1	Extracellular matrix	4.193	2.536
NM_001753	CAV1	caveolin 1	Negative regulator of the Ras-p42/44 MAP kinase cascade	0.154	0.311
NM_001233	CAV2	caveolin 2	Negative regulator of the Ras-p42/44 MAP kinase cascade	0.101	0.111
NM_001945	HBEGF	heparin-binding EGF-like growth factor	Angiogenesis and signal transduction	4.176	3.044
Regulation of actin cytoskeleton					
NM_004309	MARCKS	myristoylated alanine-rich protein kinase C substrate	Cell motility	5.819	2.408
NM_012250	RRAS2	related RAS viral (r-ras) oncogene homolog 2	Inducing transformation	2.151	1.54
NM_004447	EPS8	epidermal growth factor receptor pathway substrate 8	Signal transduction	1.705	1.427
NM_004342	CALD1	caldesmon 1	Regulation of cell contraction	1.995	1.386

YWHAZ (14-3-3zeta) involves in the metastasis of lung adenocarcinoma through Wnt signaling pathway

ME025

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Abstract

Recently, YWHAZ(14-3-3ζ) protein has been reported to promote various cancer types, such as colon cancer and breast cancer. However, a deeper understanding of mechanism in lung cancer is still required. In a previous study, 14-3-3ζ was expressed strongly in high migratory lung adenocarcinoma cells than that in low migratory cells. Therefore, I hypothesized that 14-3-3ζ might play a crucial role in the metastasis of lung adenocarcinoma.

Herein, I demonstrated that when low metastasis lung cancer cells overexpressed 14-3-3ζ by transfection with 14-3-3ζ gene, an increase of β-catenin and TCF4, the key component of Wnt signaling pathway, in the nucleus was observed. Furthermore, I found that 14-3-3ζ formed a complex with β-catenin and TCF4 in the nucleus. And the metastatic activity stimulated by 14-3-3ζ was also found by cell proliferation and invasion activity.

The results suggest a model of 14-3-3ζ in the metastasis of lung adenocarcinoma through the activation of Wnt signaling pathway. Therefore, 14-3-3ζ could be a potential therapeutic and prognostic biomarker for lung cancer.

Keywords Lung adenocarcinoma . 14-3-3ζ . β-catenin . TCF4 . Wnt signaling pathway

Introduction

Lung cancer has become one of the most common causes of cancer-related mortality in the whole world. There are approximately 1.3 million deaths in the world each year, and 5000 deaths each day on average according to the report from WHO (1). Among all types of lung cancer, 80% of lung cancer cases are non-small lung cancer (NSCLC) (2). Its overall 5-year survival rate for patients is less than 15% (3-4). The staging system is not sufficient enough to the prognosis of NSCLC, even if we've already had TNM staging. Hence, a great understanding to developmental mechanism of NSCLC is necessary.

Recently, 14-3-3ζ, also named as YWHAZ, was considered as a candidate of biomarker for its differential expression in the different migration lung adenocarcinoma cell model by cDNA microarray (5); Fan *et al.* also indicated that 14-3-3ζ is a potential therapeutic and prognostic target of lung cancer in 2007 (6), but it's role in promoting metastasis of lung cancer remains unclear.

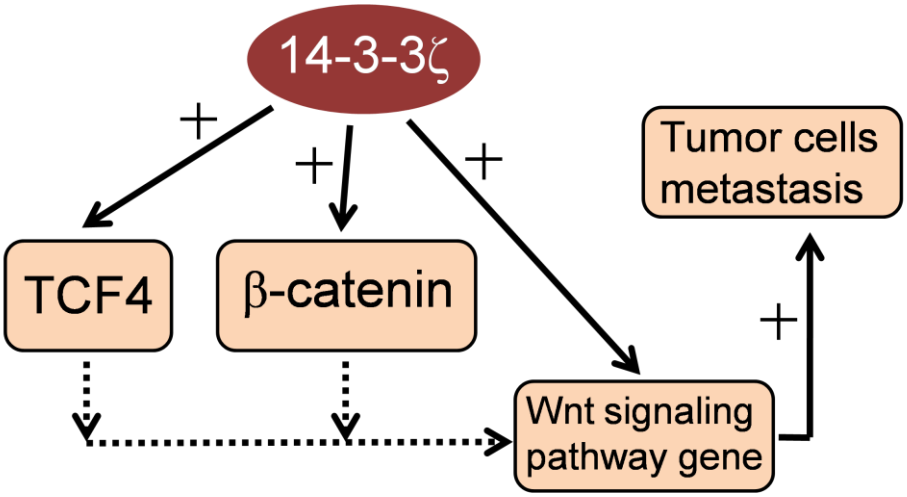
Several researches showed that 14-3-3ζ has a correlation with β-catenin in signal transduction cascade in normal cells, but not in cancer cells (7-8). β-catenin is a vital component of Wnt signaling pathway, and its expression controls the embryonic development and cancer (9-12). High accumulation of β-catenin binds to TCF frequently, causing the dissociation of cell contact and the activation of Wnt target genes, such as Myc, CCDN1 (28-29), inducing the cancer development. Also, the translocation of β-catenin into the nucleus promotes EMT and stimulates tumor cells invasion and metastasis (13-14). We wondered whether there's a correlation between 14-3-3ζ and abnormal β-catenin in the metastasis of lung cancer, which imposes a strong restoration of β-catenin in the nucleus.

In this report, we demonstrated that 14-3-3ζ enhances β-catenin transduction pathway, which leads to the metastasis of lung adenocarcinoma.

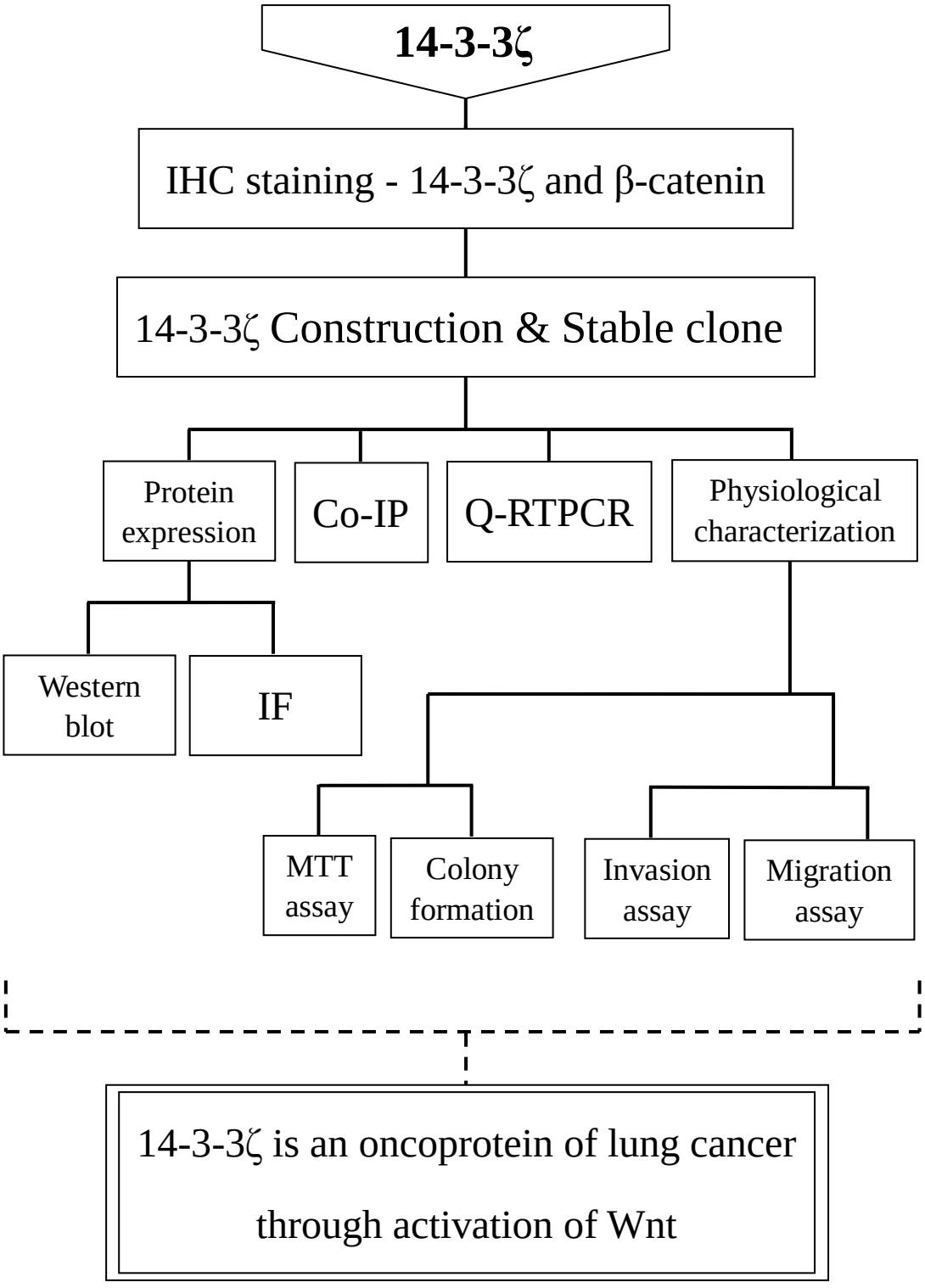
Abbreviations: TCF4, T cell factor 4; EMT, epithelial-mesenchymal transition; CCDN1, Cyclin D1; YWHAZ, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein.

Hypothesis

A. To study whether the expression of 14-3-3ζ is correlated with that of β-catenin in lung adenocarcinoma tissues
B. To investigate whether the overexpression of 14-3-3ζ induces the expression of β-catenin and TCF4
C. The role of 14-3-3ζ in the upregulation of Wnt signaling pathway's genes
D. Overexpression of 14-3-3ζ enhancing the proliferation and metastatic activity of low malignant lung cancer cells



Research Flow



Materials and Methods

Reagents & Antibodies

Protein A-Sepharose beads and glutathione Sepharose were purchased from Amersham Biosciences (Piscataway, NJ). Anti-V5 antibody was purchased from Invitrogen (Carlsbad, CA). Anti-TBP (N-12), anti-14-3-3zeta (C-16), and anti–mouse antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-β-catenin (14) was purchased from BD Biosciences (San Jose, CA). Anti-TCF4 (6H5-3) and anti-α-tubulin (DM1A) were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY).

Patients and Tissue Specimens

Lung tumor tissue and adjacent normal tissue specimens were obtained from patients with histologically confirmed 3A NSCLC who underwent surgical resection at the Taichung Veterans General Hospital (Taiwan, ROC) with the permission of IRB of the Taichung Veterans General Hospital (C07255). None of the patients had received pre-operative adjuvant chemotherapy or radiation therapy.

Immunohistochemical staining

Immunohistochemistry for 14-3-3ζ and β-catenin were performed by the avidin-biotin alkaline phopharase method, as previously reported (30). A heat-induced antigen retrieval step (deparaffinized sections were immersed in 0.01 mol/L sodium citrate buffer (PH6.0) and incubated in a pressure cooker for 3 minutes) was conducted. The mouse anti-human 14-3-3ζ and β-catenin monoclonal antibody were applied at a dilution of 1:200.

Cells and Cell Culture

The low malignant human lung adenocarcinoma cell lines CL1-0, in ascending order of invasive competence, was established in previous studies. Cells were cultured in Dulbecco's modified Eagle's medium (Life Technologies) supplemented with 10% fetal bovine serum (FBS; Life Technologies) and each of penicillin and streptomycin at 37℃ in a humidified atmosphere of 5% CO_2.

Construction of expression vector and Stable Transfection

For identification and cloning of 14-3-3ζ full-length cDNA, total RNA was isolated from CL1-5 cells using Trizol reagent (Life Technologies, Inc.). First-strand cDNA was reverse-transcribed with SuperScript II reverse transcriptase (Life Technologies, Inc.) and oligo-dT primer. The 14-3-3ζ coding region was amplified by polymerase chain reaction (PCR) using the forward primer:

5’-GGGGTACCCATGGATAAAAATGAGCTGGTT-3’, which introduced a Kpn I site, and the reverse primer: 5’-TCCCCCGGGATTTTCCCCTCCTTCTCCTGC-3’, which introduced an EcoRV site. The amplified product was cloned into pEF6/V5-His TOPO vector (Invitrogen Corp; Carlsbad, CA; pEF6/V5-HisTOPO-14-3-3zeta). The cDNA was then fully sequenced to ensure that no mutations were introduced during the PCR amplification.

Subsequently, CL1-0 cells were seeded in 6-cm dishes at 5 $\times 10^5$ cells/dish and transfected using lipofectamine reagent (Invitrogen), according to the manufacturer’s protocol. After culturing in medium containing 400 μg/mL of Blasticidin (Invitrogen) for 2-3 weeks, invidual clones were isolated. Clones that expressed the 14-3-3ζ cDNA coding region were maintained in medium containing 200 μg/mL of Blasticidin and used for further investigation.

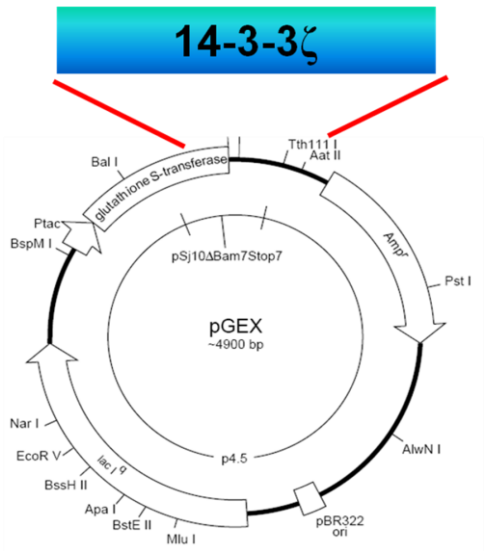


Fig1. The construction of 14-3-3ζ

Isolation of nuclear and cytosol

Nucleui, cytosol of cells were islotated using the nuclear extract kit from Active Motif North American (Carlsbad, CA) and the ProteoExtract subcellular proteome extraction kit from Calbiochem.

Western blot analysis

14-3-3ζ stable clones and CL1-0 cells were immunoblotted as described previously (30). 30 μg total protein was loaded in each lane of a 12% SDS-PAGE gel. Proteins were resolved by electrophoresis and blotted onto PVDF membranes. After blotted with PBST containing 5% skimmed milk, the membranes were incubated overnight at 4℃ with the primary antibodies diluted 1:300. Following this, the membranes were incubated with secondary antibodies diluted at 1:5000 for 1 hour at room temperature. Washed again by PBST for three times, the membranes were

detected by the enhanced chemiluminescence ECL Western blotting system.

Immunofluorescence staining assay

Cells were plated on 12-mm collagen-coated glass coverslips for 24 hours, fixed with 15 minutes in phosphate-buffered saline containing 4% paraformaldehyde. Coverslips were stained with DAPI for 30 minutes. Coverslips were mounted in anti-fading solution and viewed using a Zeiss LSM510 laser-scanning confocal microscope image system with a Zeiss 63X Plan-Apochromat objective. Wavelengths 488 nm and 359 nm were used to excite GFP and DAPI, respectively. A beam path filter (BP 505-550 nm) and a long path filter (LP 465 nm) were used to acquire images for the emission from GFP and DAPI, respectively, in a multi-track channel mode.

Coimmunoprecipitation

One milligram of whole-cell lysate in 500 μl in lysis buffer was precleared with 6 μg of IgG1 and 50 μg of protein A-Sepharose for 2 hr, then immunoprecipitated with 12 μg of specific primary antibody and rocked at 4℃ overnight. Fifty microliters of protein A-Sepharose was used to pull the pellet. After washing the pellet once in lysis buffer and twice in water, the protein complex was eluted in 100 μl of 5% acetic acid, speed-vacuum dried, and resuspended in water. The accurate procedures were described previously as (7).

Cell proliferation assay

Cells from each clonal line (CL1-0, 14-3-3ζ-1, 14-3-3ζ-2 and 14-3-3ζ-3) were seeded on 96-well plates (3×10^3 cells/well, 1 plate per cell line). After culturing for various durations, cell proliferation was evaluated by thiazolyl blue tetrazolium bromide (MTT) assay according to the manufacturer's protocol (Chemicon) In brief, 10 μL of the MTT solution (5 mg/mL) was added to each well, the cells were cultured for another 4 hour at 37℃, and 100 μL of 0.04 N HCl in isopropanol was added to each well and mixed vigorously to solubilize colored crystals produced within the cells. The absorbance at 570 nm (630 nm as the reference) was measured by using a multiwall scanning specreophotometer Victor3 (Perkin-Elmer, Boston, MA). Experiments were performed thrice.

Soft agar Colony formation assay

Cells (5×10^2) were resuspended in RPMI medium1640 (1.0 ml with 10% FBS) and plated over a layer of solidified RPMI medium 1640/10% FBS/0.35% agar (2.0 ml). Plates were incubated at 37℃, and colonies were stained with

Iodonitrotetrazolium chloride after 10 days culture.

In vitro Invasion and Migration assay

In vitro invasion assays were performed as previously described (38) using transwell chambers (8 μm pore size; Costar, Cambridge, MA) and transwell filter coated with Matrigel (Becton Dickinson, Franklin Lakes, NJ). Cells (5×10^4 each of Mock, 14-3-3ζ-1, 14-3-3ζ-2 and 14-3-3ζ-3) were seeded onto the Matrigel and incubated overnight. The next day, membranes coated with Matrigel were swabbed with cotton, fixed with methanol, and stained with 10% Giemsa solution (Sigma Chemical, St. Louis, MO). The cell that were attached to the lower surface of the polycarbonate filter were counted under a light microscope (200× magnification). The experiments were performed thrice.

Migration assay was described like invasion assay above. The only different condition is 1×10^4 seeding cells on transwell not coating with matrigel.

Results

1. 14-3-3ζ Expression in Tumor and Adjacent Normal Tissue of Patients with Adenocarcinoma

Advanced cancer specimens with histologically confirmed NSCLC were studied by immunohistochemistry assay. The data show that 14-3-3ζ and β-catenin expression in tumors was strongly higher than that in adjacent normal tissue. In addition, parts of 14-3-3ζ and β-catenin were also observed in the nucleus.

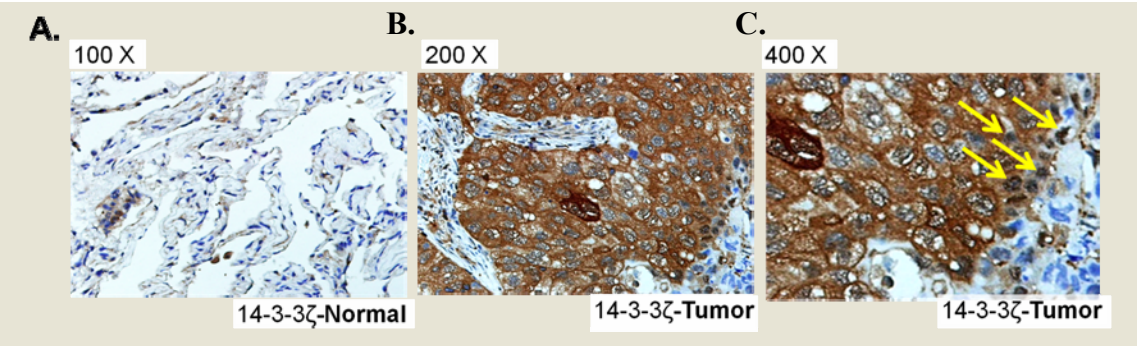


Fig.3 Immunohistochemical analysis of 14-3-3ζ in NSCLC patient's tissue. **A.** Low expression of 14-3-3ζ in adjacent normal tissue was observed **B.** Strong expression of 14-3-3ζ in tumor tissue can be detected. **C.** Expression of 14-3-3ζ in tumor tissue showing cytoplasmic and nuclear immunostaining. Arrow shows nuclear staining.

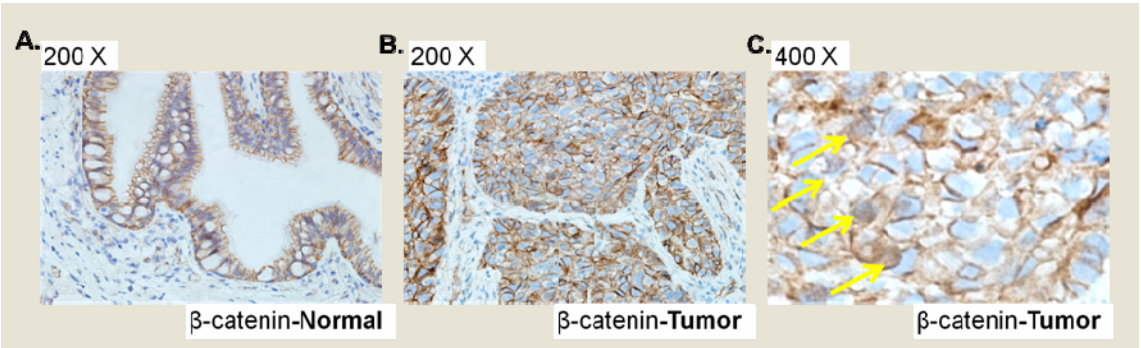


Fig.4 Immunohistochemical analysis of β-catenin in NSCLC. **A.** Low expression of β-catenin in adjacent normal tissue was observed **B.** Strong expression of β-catenin in tumor tissue was detected. **C.** Expression of β-catenin in tumor tissue was observed. Arrow shows nuclear staining.

2. Distribution of β-catenin and TCF4 in the nucleus and the cytoplasm of CL1-0 cell and 14-3-3ζ stable clones.

A. The nuclear and cytosol lysates detected by Western blot assay

There is a higher expression of β-catenin and TCF4 in the 14-3-3ζ stably expressed clones (14-3-3ζ-1, 14-3-3ζ-2, 14-3-3ζ-3) than in the Mock control (Fig.5). Compared with Mock control, both of β-catenin and TCF4 have a high tendency to translocate into the nucleus of 14-3-3ζ stably expressed clones. Moreover, we also find the obvious nuclear 14-3-3ζ expression in the 14-3-3ζ stably expressed clones than that of CL1-0 cells.

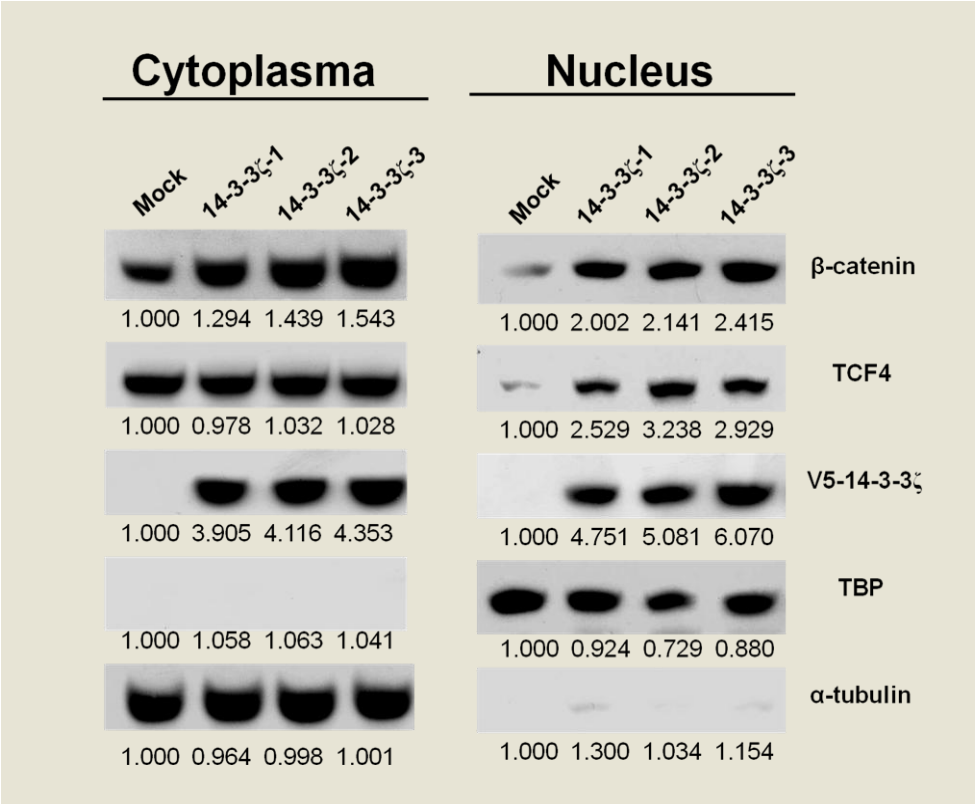


Fig. 5 Whole cell lysates extracted from the transfectants were fractionated into cytosolic and nuclear proteins and immunoblotted. TBP (TATA box binding protein) was used as nuclear internal control. α-tubulin was used as loading control.

B. The subcellular localization of proteins measured by Immunofluorescence staining

Since the strong nuclear expression of β-catenin and TCF4 in the 14-3-3ζ stable clone validated by western blot analysis, we performed Immunofluorescence stain to detect the nuclear protein translocation tendency followed by β-catenin and TCF4 antibody (Fig.6). Both β-catenin and TCF4 have a higher nuclear translocation in the 14-3-3ζ stable clone than that in mock control. These results are in concordance with the western blot assay (Fig.5).

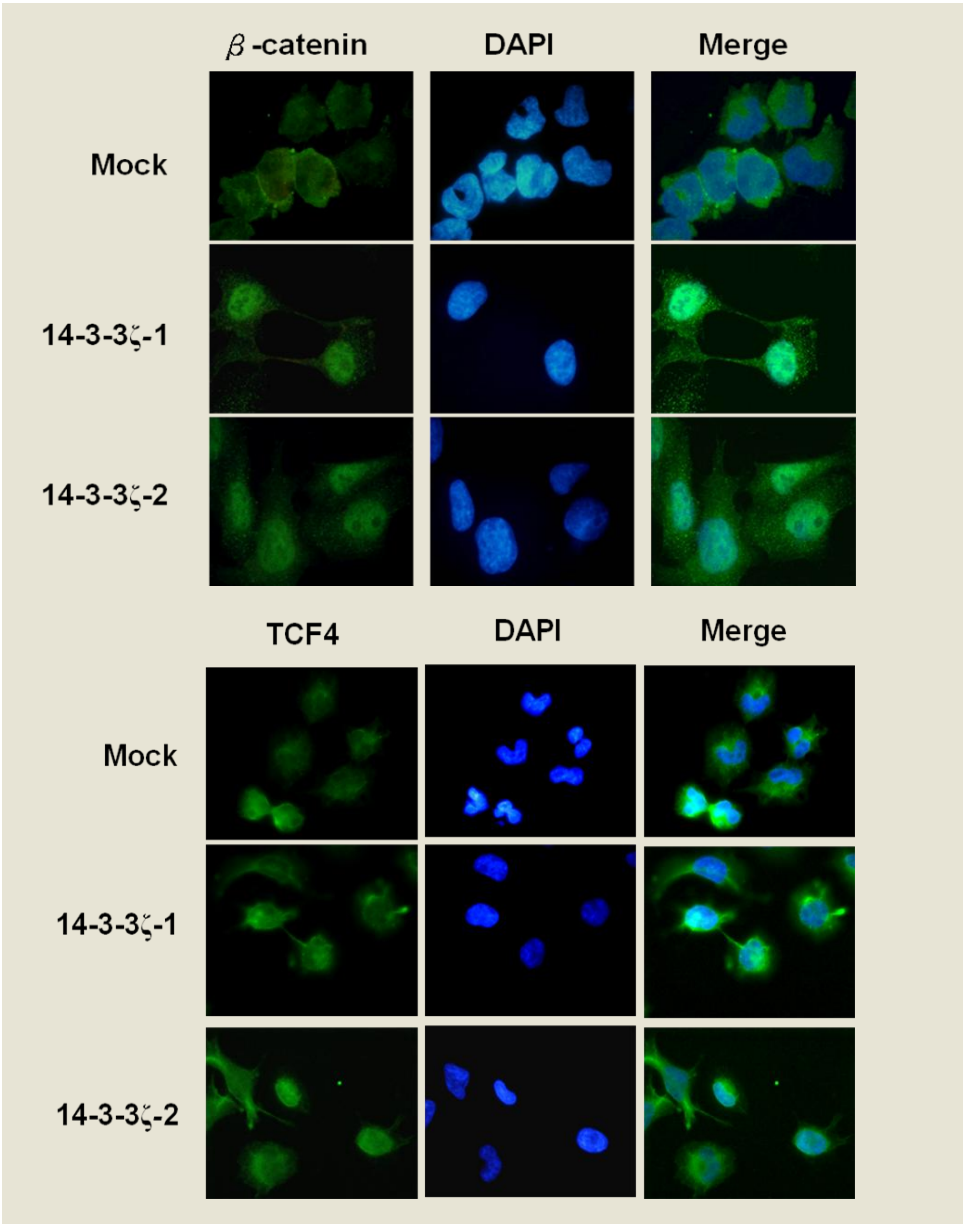


Fig.6 Confocal microscopic images of β -catenin (**A**) and TCF4 (**B**) are subcelluar localization in Mock and 14-3-3ζ-transfectant cell lines. β -catenin and TCF4 present in green color, nuclear DNA in blue color (DAPI). The coexistence regions presented in Merge images.

3. The interaction between 14-3-3ζ andβ -catenin, TCF4 in 14-3-3ζ stably expressed cell lines by Co-immunoprecipitation.

Although the nuclear expression of 14-3-3ζ was observed obviously, its function and gene regulation is mostly unknown. The Co-immunoprecipitation was carried out on 14-3-3ζ stably expressed clones nucleus by using antiV5 antibody followed by immunoblotling with anti-β-catenin or anti-TCF antibodies. The results demonstrate that 14-3-3ζ can form a complex with β-catenin and TCF4 in the nucleus (Fig.7).

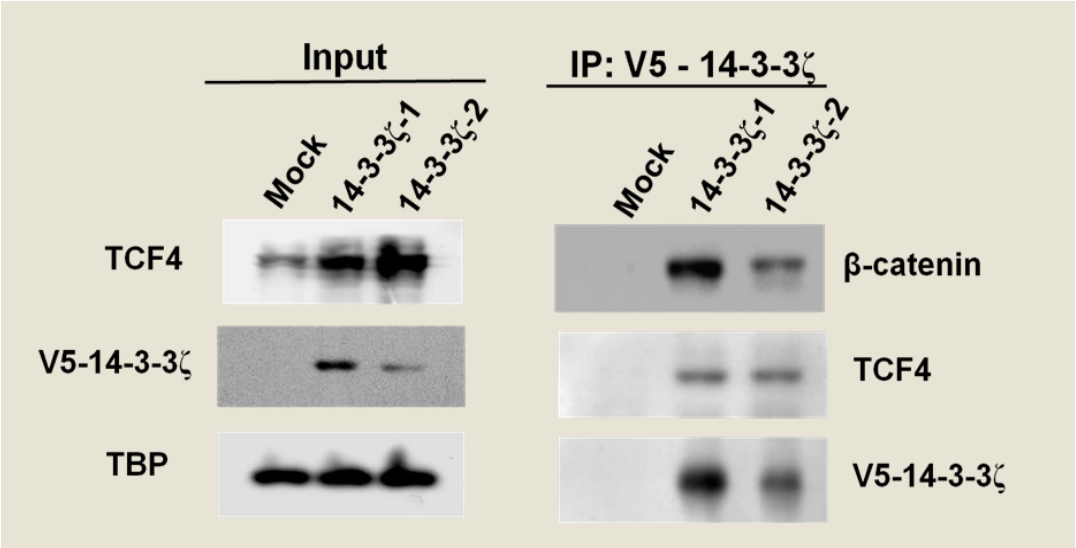


Fig.7 Co-immunoprecipitation in the nucleus was performed with V5-14-3-3ζ antibody followed by immunoblotting with β-catenin and TCF4 antibodies. 10 μg of nuclear lysate was used as an input control. TBP (TATA box binding protein) served as nuclear internal control. α-tubulin was used as loading control.

4. Identification of downstream Wnt signaling pathway genes up-regulated by 14-3-3ζ overexpression.

Myc, CyclinD1, Twist and Slug are Wnt target genes (22-23). We carried out quantitative real-time PCR to analyze the effect of 14-3-3ζ on transcriptional activities of target genes.

As shown in Fig.8, it demonstrates that the RNA expression of Myc, CyclinD1, Twist and Slug in 14-3-3ζ stable clones was significantly higher than the Mock control.

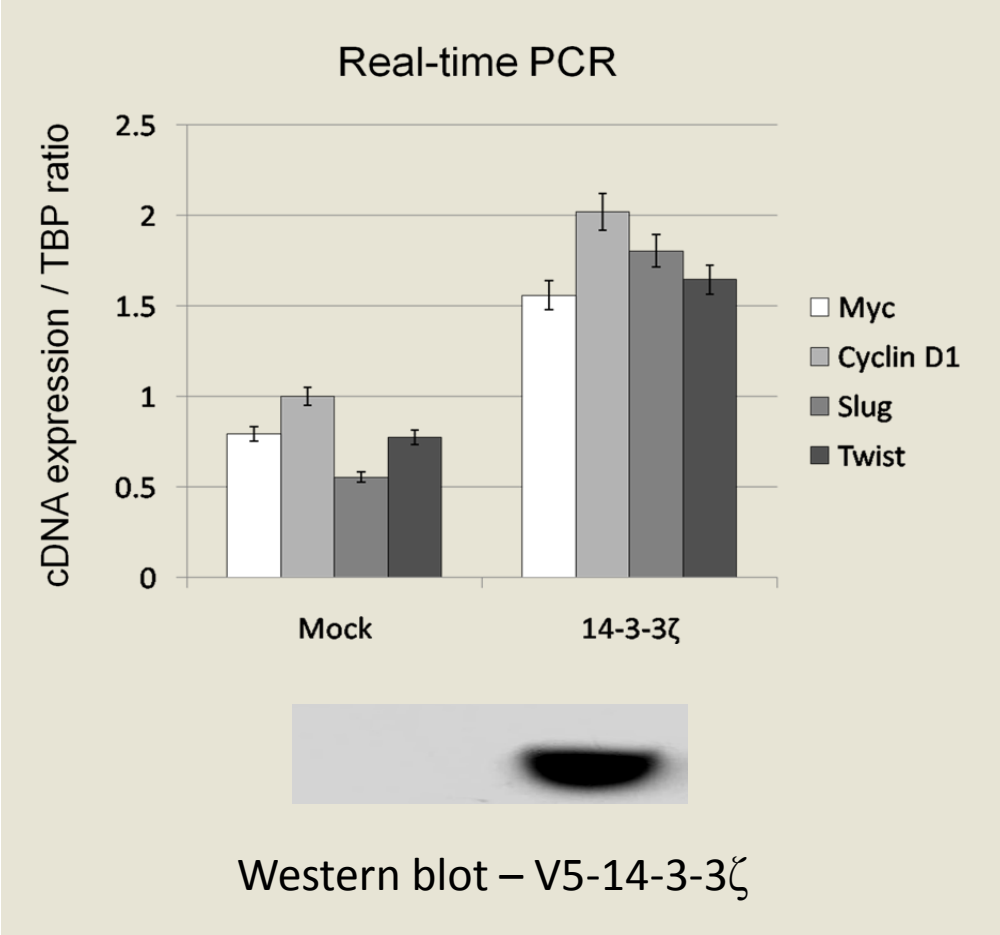


Fig.8 The expression of Myc, CCDN1, Slug and Twist in mock cell and 14-3-3ζ stable clone determined by Q-RTPCR. TBP served as an internal control. 14-3-3ζ was immunoblotted to ensure the stable expression of 14-3-3ζ stable cell line.

5. Overexpression of 14-3-3ζ in lung cancer cells induced the metastatic activity.

A. Cell proliferation assay

Because the higher transcriptional activities of Myc and CCDN1 as shown in Fig.8, the effects of 14-3-3ζ on the proliferation activities of stably expressed clones were studied by thiazolyl blue tetrazolium bromide (MTT) assay. After 72 hours, the data shows that overexpression of 14-3-3ζ induces the higher proliferation activity in stably expressed clone than that of low malignant lung adenocarcinoma cell lines CL1-0 (Fig.9).

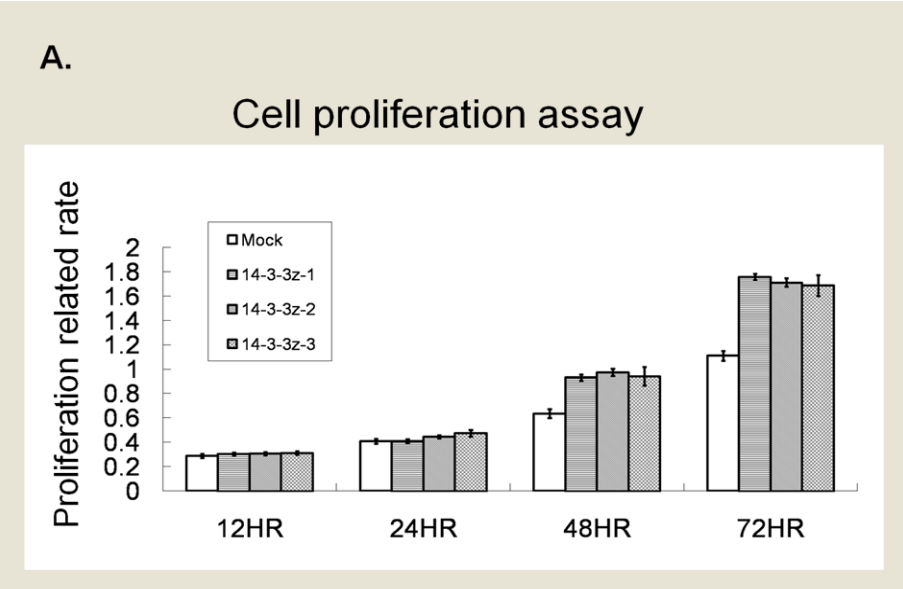


Fig.9 Cell proliferation rates assessed by MTT assay in CL1-0 and 14-3-3ζ stable clones (14-3-3ζ-1, 14-3-3ζ-2, 14-3-3ζ-3).

B. Colony formation assay

The stimulatory effects of 14-3-3ζ on anchorage independent growth was shown by increased colony formation of 14-3-3ζ stably expressed cell line (black bars) compared with that of mock cells (white bars) measured by soft agar assay (Fig.10).

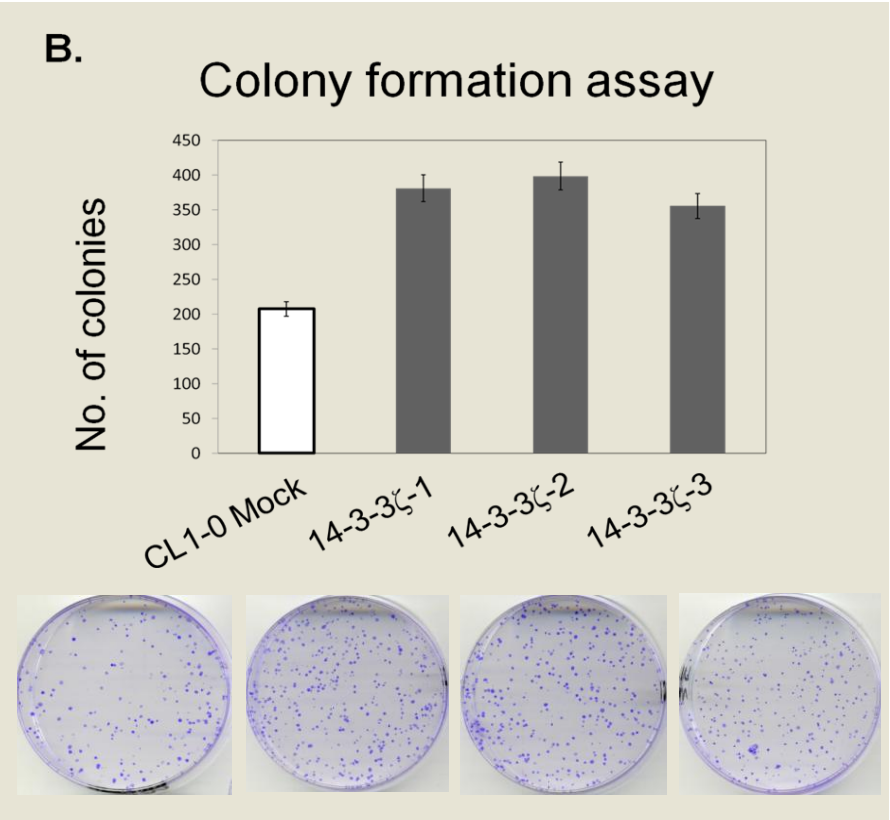


Fig.10 Colony formation measured by soft agar assay in CL1-0 and 14-3-3ζ stable clones (14-3-3ζ-1, 14-3-3ζ-2, 14-3-3ζ-3).

C. Cell migration assay

Due to the up-regulation of Slug and Twist in 14-3-3ζ stably expressed clone, we studied the cell migratory ability on mock cells and 14-3-3ζ stably expressed clone. The results suggest that overexpression of 14-3-3ζ induced the high migration of low malignant lung cancer cells (Fig.11).

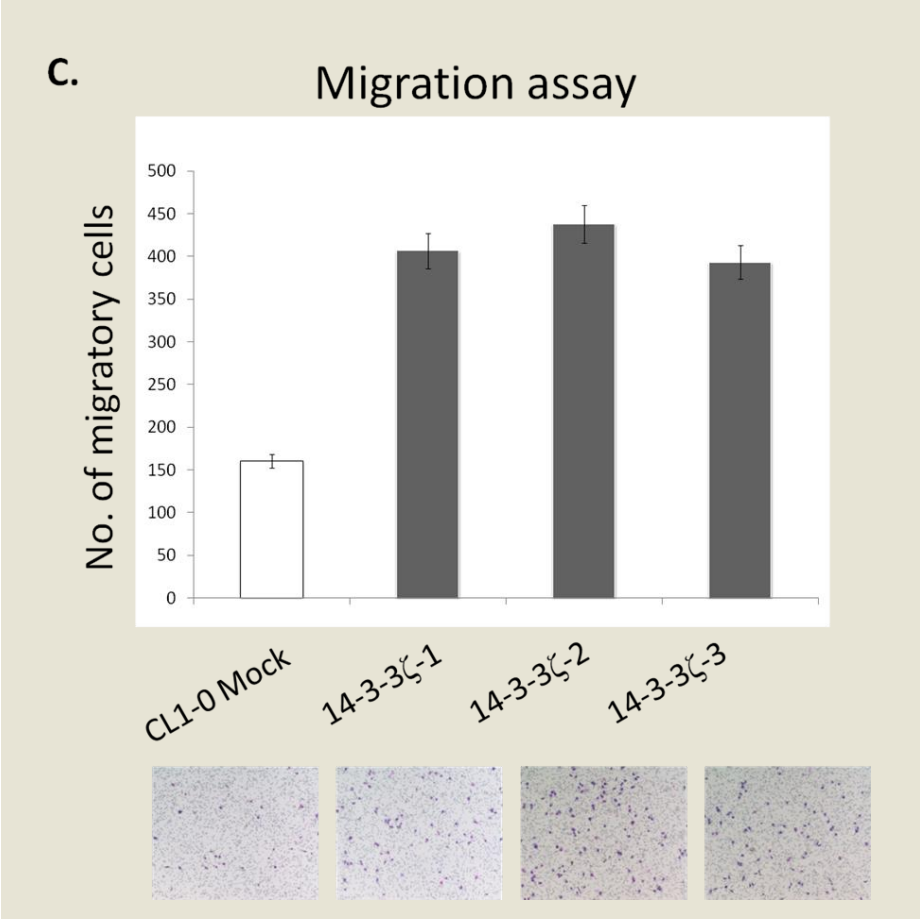


Fig.11 Cell migratory ability detected by Boyden chamber assay in CL1-0 and 14-3-3ζ stably expressed clones (14-3-3ζ-1, 14-3-3ζ-2, 14-3-3ζ-3).

D. In vitro Invasion assay

The activation of invasion on 14-3-3ζ stable clone was shown at Fig.12. We can see that over two-fold invasion activity were observed in 14-3-3ζ-1, 14-3-3ζ-2 and 14-3-3ζ-3 compared with Mock control.

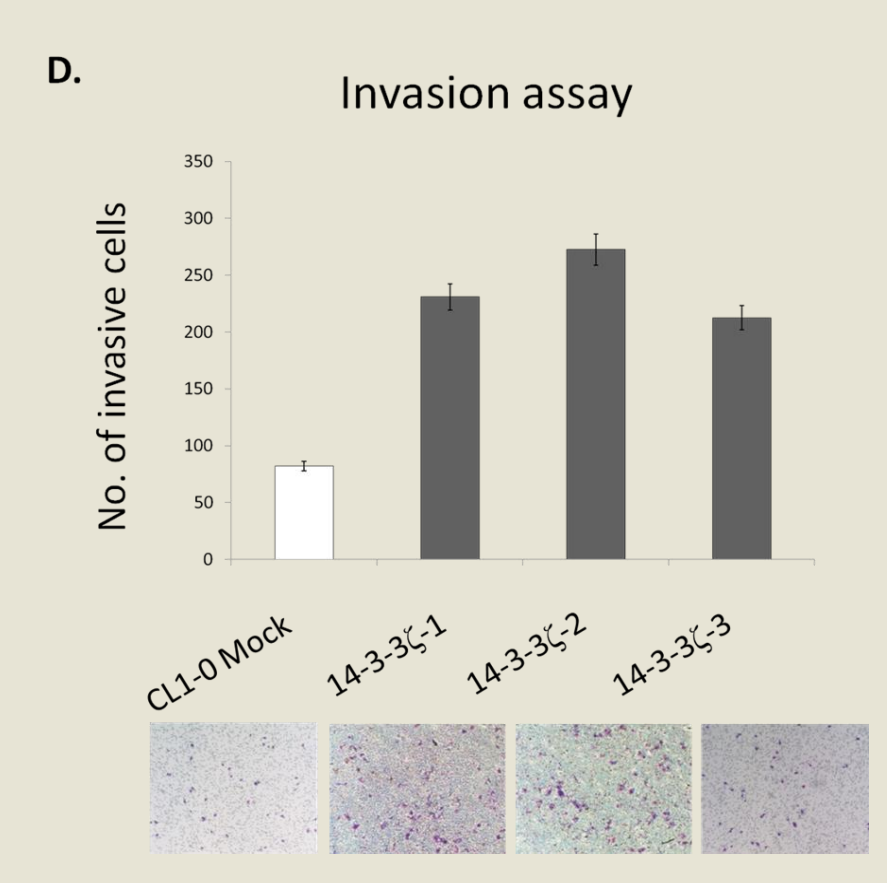


Fig.12 In vitro invasive activity examined by Boyden chamber assay coating with Matrigel in CL1-0 and 14-3-3ζ stable clones (14-3-3ζ-1, 14-3-3ζ-2, 14-3-3ζ-3).

Discussion

14-3-3ζ is a highly conserved mammalian gene, which is one of the 14-3-3 family. 14-3-3 family has 7 defined isoforms, including α/β, ε, η, γ, τ/θ, ζ/δ and σ found in brain in 1962, all of them are expressed in eukaryotic cells. 14-3-3 family functions as an adaptor, scaffold or coordinator in assembling signaling complexes which plays a critical role in the mediation of central physiological actions, such as cell survival signaling, cell cycle control, cytoskeletal regulation and apoptotic cell death (17-21). Each isoforms has different functions in cancer disease. Reduced 14-3-3β is associated with decreased VEGF production, inhibition of angiogenesis, and much decreased tumor size (22), and it was also demonstrated that overexpression of 14-3-3β promotes MAPK dependent tumor formation in nude mice (23); 14-3-3σ (Stratifin) prevents apoptosis through sequestration of Bax (24); more importantly, 14-3-3ζ has been reported involved in many oncogenic transformations, such as an interaction with EGF receptor (25), a down-regulation of p53 (26), a preventer to cell adhesion (27) and also an overexpression in high migratory lung cancer cell model determined by cDNA microarray (5).

Based on the report in 2004 (7), 2007 (8) and 2008 (34), 14-3-3ζ was demonstrated having a strong interaction with β-catenin. To examine its association with β-catenin in lung cancer, the immunohistochemistry was performed (Fig.3, 4), and results showed that in the tumor tissue of III A NSCLC patients, both of 14-3-3ζ and β-catenin have a significantly increasing expression, and the nuclear localization. It indicates that 14-3-3ζ is positively correlated with β-catenin in the pathological consequences of NSCLC patients.

β-catenin is a critical switch between normal and abnormal performance of cells. In normal cells, β-catenin is usually phosphorylated and ubiquitinated and then degraded by proteosome when it loses contact to E-cadherin. While β-catenin is not degraded for abnormal resources, it binds to TCF4 (T cell factor 4) and translocates into the nucleus, activating Wnt target genes which promote cell growth and migration (9-15).

To further investigate 14-3-3ζ's effects on the metastasis of lung adenocarcinoma, we constructed 14-3-3ζ-overexpressing of low malignant lung adenocarcinoma cells compared with mock control as described previously (Fig.2). The immunoblotting assay (Fig. 5) and immunoflourscence stain (Fig.6) indicate that 14-3-3ζ induces the expression and nuclear translocation of β-catenin and TCF4. Interestingly, we also found that there's the higher expression of 14-3-3ζ in the nucleus of 14-3-3ζ stably expressed cell lines.

The nuclear function of 14-3-3ζ is seldom reported, so we are curious about its regulation in the nucleus. In the activation of Wnt/β-catenin signaling cascade, β-catenin displaces Groucho repressors in the nucleus as a switch through binding to TCF/LEF transcription factor, coactivating Wnt downstream genes (16). It suggests that 14-3-3ζ might participate in the interaction of transcription factor complexes. The hypothesis was strongly supported by co-immnoprecipitation analysis (Fig.7) which implicates that 14-3-3ζ has a strong interaction with β-catenin and TCF4 in the nucleus.

Since interacting with TCF4 in the nucleus, β-catenin can up-regulate several oncogenes, for example, Myc, CCDN1, Twist, Slug (28-29). Myc and CCDN1 facilitates the cell proliferation; whereas Twist and Slug promotes the cell invasion and migration. Indeed, we observed that 14-3-3ζ was able to induce the expression of Myc, cyclinD1, Twist and Slug genes as demorstated by Q-RTPCR (Fig. 8).

Furthermore, we examined the effect of 14-3-3ζ on cell proliferation, colony formation, in vitro migration and cell invasion activity. The results (Fig.9-12) suggest that 14-3-3ζ strongly enhanced the low malignant lung cancer cells proliferation, colony formation, migration and invasion activity of 14-3-3ζ stably expressed cell lines than that of mock control cell lines.

Conclusion

Taken together, our data suggests that 14-3-3ζ plays an important role in the metastasis of lung adenocarcinoma. The results reveal that 14-3-3ζ not only induces the expression of β-catenin, but also forms a complex with β-catenin and TCF4 in the nucleus. Additionally, the up-regulation of 14-3-3ζ on several Wnt target genes, like Myc and CCDN1 was demonstrated, which facilitates the metastatic performance on the low malignant lung cancer cells.

Recently, more findings of 14-3-3ζ about lung cancer were reported. Zang *et al.* demonstrated that 14-3-3ζ is significantly associated with poor differentiation and progression in NSCLC (30); Li *et al.* validated that anchorage-independent growth of lung cancer cells requires the presence of 14-3-3ζ through anoikis activation (31).

Furthermore, overexpression of 14-3-3ζ was also confirmed previously in breast (32), head and neck (33-35), oral (36), ovarian (37) cancer. These findings indicate that 14-3-3ζ aberration has a huge influence on various types of cancer. Therefore, targeting 14-3-3ζ may provide an effective approach for cancer therapy.

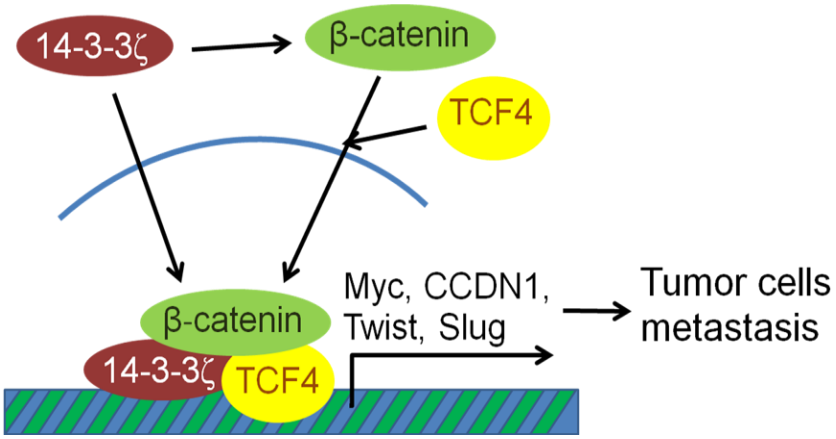


Fig.13 Possible mechanism
14-3-3ζ promotes the metastasis of low malignant lung adenocarcinoma by activating Wnt signaling pathway.

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