行政院國家科學委員會專題研究計畫 期末報告

探討 AKR1C3、ROS 與 nm23-H1 於子宮頸癌細胞的表現及其與癌症轉移潛力、臨床病理因子及存活之關係

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公 開 資 訊 ：本計畫涉及專利或其他智慧財產權，2 年後可公開查詢

中 華 民 國 102 年 10 月 13 日

中 文 摘 要 ： 醛-酮還原酶家族成員 C3(AkR1C3)催化雄激素，雌激素和前列腺素代謝。許多癌症化療方面，AKR1C3 表現增加與抗順鉑藥物有關。過去研究指出，Lipoclin2 (LCN2)具有抑制宮頸癌轉移的能力。然而，AKR1C3 和 LCN2 之間的關係仍未有文獻提出討論。我們運用慢病毒 shRNA 系統釐清 AKR1C3 和 LCN2 在子宮頸癌細胞系的作用。藉由細胞傷口癒合實驗和 Boyden chamber 檢測抑制 AKR1C3 表現的 SiHa 和 Caski 細胞株，其細胞的遷移和侵襲能力下降。在抑制 LCN2 表現的細胞株，其 MMP-2 的 mRNA 表現量和活性都增加。我們的研究結果顯示，抑制 AKR1C3 表現，進而增加 LCN2 表現量和抑制子宮頸癌細胞轉移。因此，我們建議利用 AKR1C3 和 LCN2 作為癌症治療的分子標靶，以提高子宮頸癌患者臨床治療的預後結果。

中文關鍵詞： 醛-酮還原酶家族成員 C3、lipocalin 2、轉移和子宮頸癌

英 文 摘 要 ： Aldo-keto reductase family 1 member C3 (AkR1C3) catalyzes androgen, estrogen and prostaglandin metabolism. Up-regulated AKR1C3 is associated with cisplatin resistance in many cancer chemotherapy regimens. Lipocalin 2 (LCN2) inhibits cervical cancer metastasis. However, little is known about the involvement of and relationship between AKR1C3 and LCN2 in cancer development and progression. We investigated the roles of AKR1C3 and LCN2 using lentivirus shRNA system in cancer cell lines of uterine cervix. Silencing of AKR1C3 decreased cell migratory and invasive abilities of SiHa and Caski cells on wound healing and Boyden chamber assays. The mRNA level and activity of MMP-2 increased in silenced LCN2 cells. Our novel findings revealed that silencing of AKR1C3 increases the expression of LCN2 and inhibits cancer cell metastasis in cervical cancer. We suggest that AKR1C3 and LCN2 serve as molecular targets for cancer therapy to improve the clinical outcome of patients with cervical cancer.

英文關鍵詞： aldo-keto reductase family 1 member C3； lipocalin 2； metastasis； cervical cancer

行政院國家科學委員會專題研究計畫成果報告

探討 AKR1C3、ROS 與 nm23-H1 於子宮頸癌細胞的表現及其與癌症轉移潛力、臨床病理因子及存活之關係

Investigation of the expression of aldo-keto reductase family 1 member C3, reactive oxygen species and human non-metastatic clone 23 type 1 in cancer cells of uterine cervix and their relationships with clinicopatholigical variables and survival of cancer

計畫編號：NSC 101-2314-B-040-002
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計畫主持人：陳宣志
共同主持人：柯俊良和王博輝

中文摘要

醛-酮還原酶家族成員 C3(AkR1C3)催化雄激素，雌激素和前列腺素代謝。許多癌症化療方面，AKR1C3 表現增加與抗順鉑藥物有關。過去研究指出，Lipoclin2 (LCN2)具有抑制宮頸癌轉移的能力。然而，AKR1C3 和 LCN2 之間的關係仍未有文獻提出討論。我們運用慢病毒 shRNA 系統釐清 AKR1C3 和 LCN2 在子宮頸癌細胞系的作用。藉由細胞傷口癒合實驗和 Boyden chamber 檢測抑制 AKR1C3 表現的 SiHa 和 Caski 細胞株，其細胞的遷移和侵襲能力下降。在抑制 LCN2 表現的細胞株，其 MMP-2 的 mRNA 表現量和活性都增加。我們的研究結果顯示，抑制 AKR1C3 表現，進而增加 LCN2 表現量和抑制子宮頸癌細胞轉移。因此，我們建議利用 AKR1C3 和 LCN2 作為癌症治療的分子標靶，以提高子宮頸癌患者臨床治療的預後結果。

關鍵詞：醛-酮還原酶家族成員 C3、lipocalin 2、轉移和子宮頸癌

英文摘要

Aldo-keto reductase family 1 member C3 (AkR1C3) catalyzes androgen, estrogen and prostaglandin metabolism. Up-regulated AKR1C3 is associated with cisplatin resistance in many cancer chemotherapy regimens. Lipocalin 2 (LCN2) inhibits cervical cancer metastasis. However, little is known about the involvement of and relationship between AKR1C3 and LCN2 in cancer development and progression. We investigated the roles of AKR1C3 and LCN2 using lentivirus shRNA system in cancer cell lines of uterine cervix. Silencing of AKR1C3 decreased cell migratory and invasive abilities of SiHa and Caski cells on wound healing and Boyden chamber

assays. The mRNA level and activity of MMP-2 increased in silenced LCN2 cells. Our novel findings revealed that silencing of AKR1C3 increases the expression of LCN2 and inhibits cancer cell metastasis in cervical cancer. We suggest that AKR1C3 and LCN2 serve as molecular targets for cancer therapy to improve the clinical outcome of patients with cervical cancer.
Keywords: aldo-keto reductase family 1 member C3; lipocalin 2; metastasis; cervical cancer

前言、研究目的和文獻探討

Aldo-keto reductase family 1 member C3 (AkR1C3) is a member of the aldo-keto reductase family that is also known as type 5 17β-hydroxysteroid dehydrogenase (17β-HSD), which is a large superfamily of ~37 kDa enzymes [1]. AKR1C3 catalyzes NAD(P)H-dependent reduction of carbonyl groups on a wide variety of substrates and has diverse physiological roles. In humans, 13 isoforms of aldo-keto reductases have been found and among them 4 isotypes (AKR1C1-AKR1C4) reduce ketosteroids to hydroxysteroids [2]. AKR1C has been shown to modify steroid hormones and prostaglandins and has been implicated in the development of human cancer in several recent studies [3-5]. Overexpression of AKR1C3 has been demonstrated in many human cancers, such as breast and prostate cancers [6]. Furthermore, truncated human papillomavirus 16 (HPV16) E6 transactivates AKR1C3 expression in cervical cancer cells [7]. AKR1C3 exhibits cisplatin resistance in colon cancer and radio-resistance in lung cancer [8, 9]. However, the role of AKR1C3 in cancer of uterine cervix remains unclear.

LCN2, also called neutrophil gelatinase associated lipocalin (NGAL), is a 25 kDa secretory glycoprotein originally identified in human neutrophil granules [10]. LCN2 belongs to a large family of lipocalins, concerned with iron trafficking, induction of apoptosis, inflammation, cancer, innate immunity, cell proliferation and differentiation [11]. Our previous investigation demonstrated that increased LCN2 expression reduces migratory and invasive potentials of cancer cells of uterine cervix [12]. Moreover, cervical cancer patients with high levels of LCN2 have been shown to have significantly less deep stromal invasion of uterine cervix and significantly better overall survival.

Previous studies have indicated that AKR1C3 exerts anti-differentiation action, relying on its ability to perform 11β-ketoreduction of prostaglandin D_2 (PGD_2) to 11β-PGF2α, and prevents the ultimate generation of 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15d-PGJ_2), which inhibits NF-κB signaling and activates the peroxisome proliferator-activated receptor γ (PPAR-γ) [13]. Low expression of dihydrodiol dehydrogenase (DDH, AKR1C) in patients with squamous cell esophageal carcinoma correlates with

significantly lower incidence of tumor recurrence and better survival when compared with DDH overexpressed group [14].

When we consider the roles of AKR1C3 and LCN2 in metastatic phenotypes of cervical cancer and patient prognosis, it is necessary to delineate their correlations and implications in cervical cancer. We hypothesized that AKR1C3 and LCN2 are negatively regulated with the metastatic potentials of cancer cells of uterine cervix. The objectives of this study were to investigate whether AKR1C3 influences LCN2 in terms of cell motility, migration, invasiveness and cytoskeleton. Molecular interrogation of these biomarkers points to the need for therapeutic intervention and further definition of the clinical implications of AKR1C3 and LCN2 in cervical cancer.

In this study, the expression of LCN2 protein increased following silencing of AKR1C3 in SiHa and Caski cervical cancer cells. In addition, knockdown of LCN2 increased matrix metalloproteinase-2 (MMP2) expression. AKR1C3 impacts metastatic phenotypes. We demonstrated significant correlations among AKR1C3, LCN2 and MMP-2.

結果與討論

Results

shAKR1C3 up-regulates LCN2 expression in cervical cancer cells

To investigate whether LCN2 is affected by AKR1C3 expression, we first analyzed the protein levels of AKR1C3 in SiHa, HeLa, HeLa 229, Caski, Ect/E6E7 and C33a cells. AKR1C3 expressions were higher in SiHa, HeLa and HeLa 299 cells and LCN2 expressions were negative as compared to Caski and Ect/E6E7 cells (Fig. 1A). No AKR1C3 or LCN2 proteins were detected in C33a. To explore the negative regulation between AKR1C3 and LCN2, we analyzed the effect of shAKR1C3 in SiHa and Caski cervical cancer cells. When AKR1C3 gene was knocked down, the levels of LCN2 protein were elevated (Fig. 1B). Following Western blot, we used RT-PCR and real-time PCR to detect the mRNA levels of AKR1C3 and LCN2. The results were similar to those for protein levels, with AKR1C3 mRNA down-regulated and LCN2 up-regulated in shAKR1C3 cells (Fig. 1C). Next, we transfected the AKR1C3 gene into Caski cells and detected its impact on the expression of LCN2. The mRNA of LNC2 was significantly reduced in Caski flag-AKR1C3 No.6 and No.9 cell lines (p= 0.0002 and 0.016, respectively; Fig. 1D). From these findings, regulation of LCN2 expression by AKR1C3 occurs at transcriptional and translational levels in cervical cancer cells.

Silencing of AKR1C3 inhibits cell metastasis

To explore the role of AKR1C3 in the regulation of cell motility, we examined the

cell migration ability of SiHa shARK1C3 and Caski shAKR1C3 cells on wound healing assay. As shown in Fig. 2A, cell motility was significantly inhibited in SiHa shAKR1C3 #564 and #350 cells when compared with SiHa shLuc cells on 8 (p=0.044 and p=0.025, respectively), 24 (p=0.0037 and p=0.0017, respectively) and 32 hour cultures (p=0.01 and p=0.01, respectively). Cell motility was also significantly inhibited in Caski shAKR1C3 #564 and #350 cells in comparison with Caski shLuc cells on 8 (p=0.04 and p=0.04, respectively) and 32 hour cultures (p=0.01 and p=0.006, respectively) (Fig. 2B). Conversely, in Caski cells with overexpression of AKR1C3 gene, cell motility of Caski flag-AKR1C3 No.9 and No.6 was significantly increased when compared with Caski pcDNA cells on 8 h (p= 0.009 and p=0.012, respectively) and 24 h cultures (p=0.002and p=0.001, respectively) (Fig. 2C).

To confirm the role of AKR1C3 in cell migration and invasion *in vitro*, we performed Boyden chamber assays in which cells shifted from upper to lower chambers. These chambers were separated by cell-permeable membranes. We could not perform Boyden chamber assay for Caski cells due to the difficulty of these cells in passing through the polycarbonate membrane. Down-regulation of AKR1C3 in the parental SiHa cells led to significant reductions in migratory and invasive abilities of SiHa shAKR1C3 #564 and #350 cells (p=0.008 and p=0.012, Fig. 3A; p=0.001 and p=0.0005, Fig. 3B). These findings suggested that silencing of AKR1C3 inhibits cancer cell metastasis.

To assess whether AKR1C3 affects the distribution of F-actin, we used laser scanning confocal microscopy to track actin cytoskeleton. SiHa shLuc cells exhibited an elongated morphology with many long filaments throughout the cytoplasm. However, SiHa shAKR1C3 #564 and #350 cells displayed cobblestone-like appearance with decreased interior fibers (Fig. 4). Caski shAKR1C3 cell image is not shown as Caski shLuc cells appeared round.

Discussion

To the best of our knowledge, this is the first study to simultaneously investigate the implications of AKR1C3 and LCN2 in cancer of uterine cervix. AKR1C3 knockdown in SiHa and Caski cervical cancer cells results in increased expressions of LCN2 mRNA and protein. In addition, the expression of LCN2 mRNA is significantly reduced in AKR1C3 overexpressing No. 6 and No 9 Caski cancer cells. Moreover, LCN2 promoter activity increases following transfection of LCN2 promoter into shAKR1C3#564 and #350 of SiHa and Caski cancer cell lines. The influence on LCN2 by AKR1C3 may be initiated at the mRNA level.

When AKR1C3 is knocked down in SiHa or Caski cervical cancer cells, their

motility and invasive ability are reduced. When AKR1C3 is overexpressed, their motility increases. Human papillomaviruses (HPVs) are the causative agents of over 99% of cervical cancers and the HPV oncoproteins E6 and E7 are the primary viral factors responsible for initiation and progression of cervical cancer [15, 16]. Wanichwatanadecha et al. found that HPV oncoproteins cause upregulation of AKR1C3 in cervical cancer C33A cell line [7]. Dozmorow et al. demonstrated that AKR1C3 overexpression promotes aggressiveness of prostate cancer PC-3 cell line with subsequent IGF-1R and Akt activation followed by VEGF expression [17]. In addition, AKR1C3 has been reported to be overexpressed in a wide variety of cancers, including breast and prostate cancers and its expression increases with tumor aggressiveness [18-20]. Furthermore, AKR1C3 may exert its influence on the migration or invasion of cancer cells via its reverse relationship with LCN2. A previous study supports our findings that LCN2 overexpression inhibits migratory and invasive potentials of cervical cancer cells [12]. Addition of secreted LCN2 decreases migratory ability of cervical cancer cells and anti-LCN2 antibody restores it. In addition, LCN2 has been reported to suppress metastatic phenotypes of ovarian cancer, pancreatic cancer and keratinocyte cell lines [21-23].

When cells display dynamic extension and retraction, their plasma membranes develop protrusions called filopodia and lamellipodia. G-actin polymerizes to form F-actin filament and then further aggregates to form filopodia, lamellipodia and long filaments in the cytoplasm, which are fundamental to cell shape and motility events [24]. Mattila et al. reported that cancer cell metastasis leads to changes in and subsequent recombination of cytoskeleton [25] . In our study, shAKR1C3#564 and #530 SiHa cancer cell lines of uterine cervix had cobblestone-like appearance and decreased long filaments in the cytoplasm. This implies that when AKR1C3 is knocked down, LCN2 expression is elevated and the cytoskeleton is changed, resulting in suppressed migration and invasion. In agreement with our findings, Lee et al. demonstrated that LCN2 expression inhibits colon carcinoma cells from invading Matrigel *in vitro* [26]. In contrast, Yang et al. showed that MCF-7 human breast cancer cells have cobblestone-like appearance and strong cell-cell adhesion which is typical of epithelial phenotype, but LCN2 overexpressing MCF-7 cells exhibit an elongated morphology and increased cell migration[27]. We suggest that AKR1C3 enables changes in the cytoskeleton of cervical cancer cells with its function mediated via LCN2 [12] .

Cervical cancer was ranked 7th among causes of cancer death among Taiwanese women in 2011. Its age-standardized incidence rate in 2007 was 2.2 per 100,000 women as reported by the Bureau of Health Promotion of the Department of Health. Our study revealed that AKR1C3 is correlated with metastatic potentials of

cervical cancer cells based on our findings regarding migration, invasiveness, cytoskeleton and MMP-2 activity, as well as verification of our hypothesis. In addition, AKR1C3 affects the expression of LCN2. Therefore, we detected the expressions of AKR1C3 and LCN2 proteins for clinical application in cervical cancer, using cervical tissue microarrays. The AKR1C3 immunoreactivities were found to be significantly higher in cancerous tissue cores than in normal tissue cores. AKR1C3 has been reported to be up-regulated in squamous cell carcinoma of the head and neck [28]. High expression of AKR1C3 has also been found in primary acute myeloid leukemia cells [29]. High expression of AKR1C3 in cervical cancer is supported by the findings of Wanichwatanadecha et al. that HPV oncoproteins cause up-regulation of AKR1C3 [7] . In this study, we found that high AKR1C3 expression is linked with poor clinicopathological characteristics of cervical cancer, such as deep stromal invasion, large tumor diameter, more advanced stage, parametrial invasion and vaginal invasion. AKR1C3 also tends to be highly expressed in cases with pelvic lymph node metastasis. Therefore, it is reasonable that patients with high AKR1C3 expression have higher recurrence probability and lower survival. Ueda et al. found significant correlations for dihydrodiol dehydrogenase expression, FIGO stage, lymph node involvement and patient survival in cervical cancer [30]. Upregulation of AKR1C3 has been reported to be associated with poor prognosis in breast cancer [31, 32].

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圖表說明：

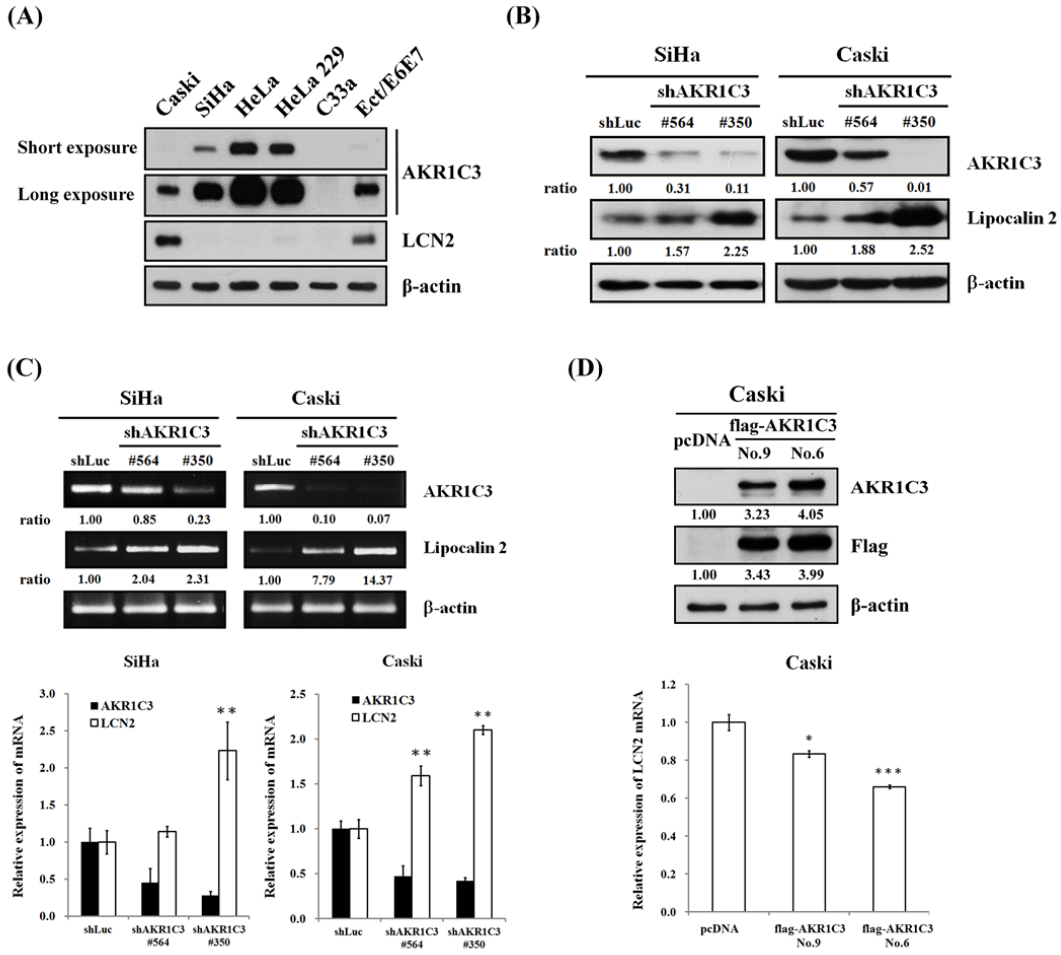

Figure 1. AKR1C3 negatively regulates LCN2 expression in cervical cancer cells. (A) The protein levels of AKR1C3 and LCN2 in Caski, SiHa, HeLa, HeLa 229, C33a and Ect/E6E7 cells. Thirty μg of total proteins were loaded onto each lane for Western

blot analysis. β-actin was used as the internal control. (B) The protein levels of AKR1C3 and LCN2 were detected by Western blot in SiHa or Caski cells with shAKR1C3 #564 or #350. β-actin was used as the internal control. The relative expressions of AKR1C3/β-actin and LCN2/β-actin are shown. (C) Top, AKR1C3 gene was knocked down in SiHa or Caski cells and mRNA levels of AKR1C3 and LCN2 were determined by RT-PCR. The relative expressions of AKR1C3/β-actin and LCN2/β-actin are shown. Bottom, quantitative real-time PCR analysis was used to detect AKR1C3 and LCN2 expressions with total RNA extracted from cells with shAKR1C3 #564 or #350. All values are normalized to the level of GAPDH and are the averages of three independent readings. (D) Caski flag-AKR1C3 cells were subjected to stable clone selection. Top, the protein levels of AKR1C3 and Flag were detected by Western blot. β-actin was used as the internal control. The relative expressions of AKR1C3/β-actin and Flag/β-actin are shown. Bottom, the mRNA levels of LCN2 in Caski flag-AKR1C3 No. 6 and No. 9 cells were analyzed by quantitative real-time PCR. All values are normalized to the level of GAPDH and are the averages of three independent readings. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$.

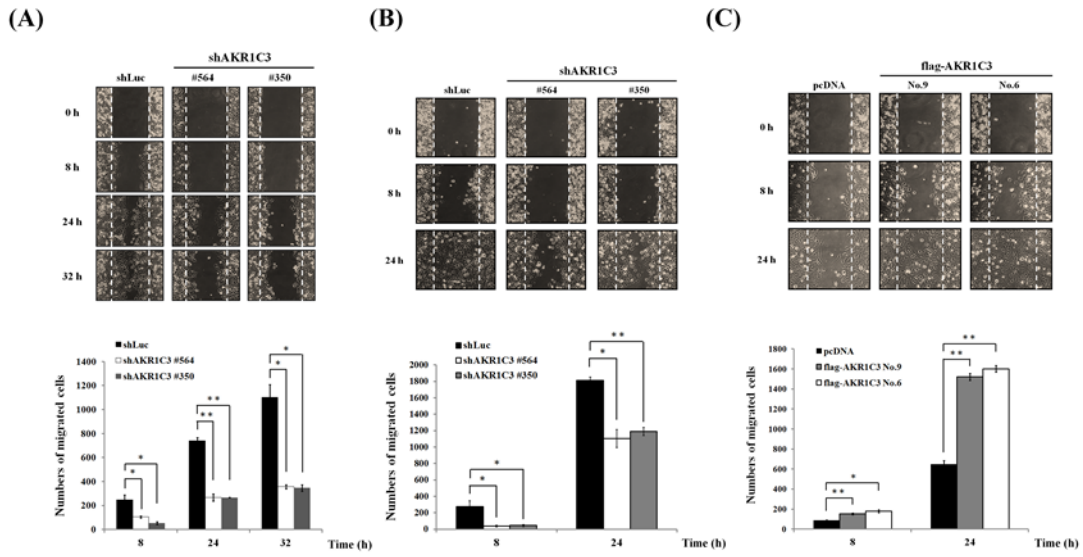

Figure 2. AKR1C3 affects cell motility of cervical cancer cells. Wound healing assays were performed on (A) SiHa shAKR1C3 (B) Caski shAKR1C3 and (C) Caski flag-AKR1C3 cells. Images were captured at the indicated times after the initial wound in the top panels (magnification, x100). The numbers of cells within the gap at the indicated times were calculated and are shown in the graphs in the bottom panels. All data are presented as the mean±SD of three independent experiments.

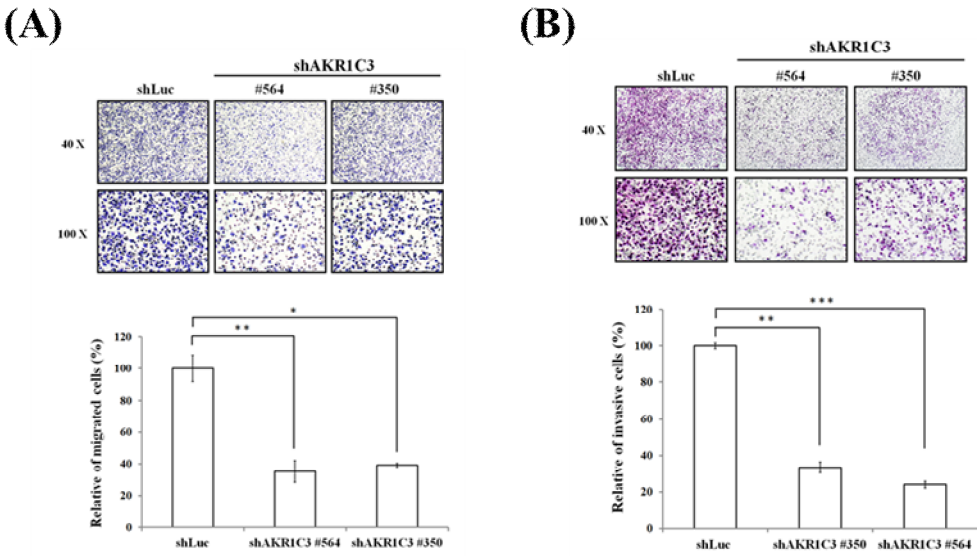

Figure3. SiHa cells were transduced with lentiviral expression vectors encoding shLuc , shAKR1C3 #564 or #350. Then, Boyden chamber assay was used to analyze cell (A) migration and (B) invasion. Representative images are shown at the top (magnification, x40 and x100) and the quantification of three randomly selected fields is shown at the bottom. Each bar represents the mean±SD of triplicate experiments.

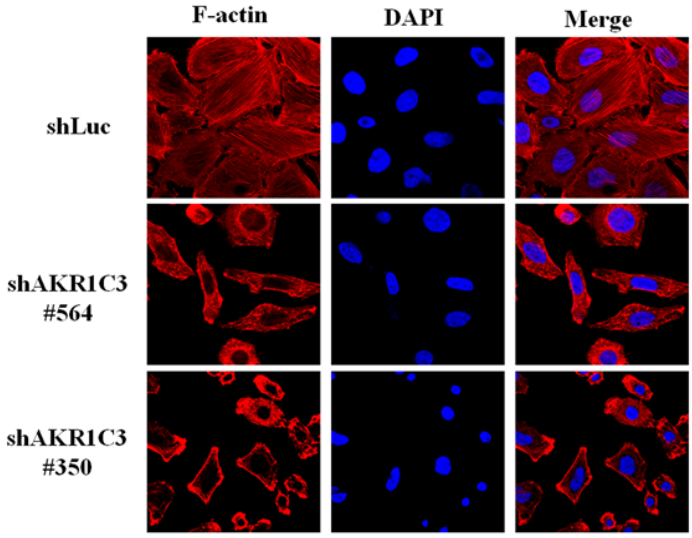

Figure 4. SiHa cells with shLuc exhibited elongated morphology and had many long filaments throughout the cytoplasm. shAKR1C3#564 or #350 of SiHa cervical cancer cells displayed cobblestone-like appearance. Interior fibers were significantly decreased in these cancer cells. SiHa cells were infected with lentivirus carrying shLuc, shAKR1C3 #564 or #350 and stained with immunofluorescence stain for F-actin (red) plus DAPI counterstaining for DNA (blue). Various cancer cells at about 5×10^4 per 24 wells were observed under confocal laser scanning microscope (Zeiss LSM 510 META) at 630 × magnifications for immunofluorescence. * $p< 0.05$,** $p < 0.01$, *** $p < 0.001$.

無研發成果推廣資料

101 年度專題研究計畫研究成果彙整表

計畫主持人：陳宣志	計畫編號：101-2314-B-040-002-
計畫名稱：探討 AKR1C3、ROS 與 nm23-H1 於子宮頸癌細胞的表現及其與癌症轉移潛力、臨床病理因子及存活之關係	

成果項目			量化			單位	備註（質化說明：如數個計畫共同成果、成果列為該期刊之封面故事...等）
			實際已達成數（被接受或已發表）	預期總達成數(含實際已達成數)	本計畫實際貢獻百分比		
國內	論文著作	期刊論文	0	1	100%	篇	
		研究報告/技術報告	0	0	100%		
		研討會論文	0	0	100%		
		專書	0	0	100%		
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（本國籍）	碩士生	1	0	20%	人次	
		博士生	2	0	60%		
		博士後研究員	0	0	100%		
		專任助理	0	0	100%		
國外	論文著作	期刊論文	0	0	100%	篇	
		研究報告/技術報告	0	0	100%		
		研討會論文	0	0	100%		
		專書	0	0	100%	章/本	
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（外國籍）	碩士生	0	0	100%	人次	
		博士生	0	0	100%		
		博士後研究員	0	0	100%		
		專任助理	0	0	100%		

其他成果 (無法以量化表達之成果如辦理學術活動、獲得獎項、重要國際合作、研究成果國際影響力及其他協助產業技術發展之具體效益事項等，請以文字敘述填列。)	抑制 AKR1C3 表現，進而增加 LCN2 表現量和抑制子宮頸癌細胞轉移。因此，我們建議利用 AKR1C3 和 LCN2 作為癌症治療的分子標靶，以提高子宮頸癌患者臨床治療的預後結果。

	成果項目	量化	名稱或內容性質簡述
科教處計畫加填項目	測驗工具(含質性與量性)	0	
	課程/模組	0	
	電腦及網路系統或工具	0	
	教材	0	
	舉辦之活動/競賽	0	
	研討會/工作坊	0	
	電子報、網站	0	
	計畫成果推廣之參與（閱聽）人數	0	

國科會補助專題研究計畫成果報告自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估

■達成目標

□未達成目標（請說明，以 100 字為限）

□實驗失敗

□因故實驗中斷

□其他原因

說明：

2. 研究成果在學術期刊發表或申請專利等情形：

論文：□已發表 ■未發表之文稿 □撰寫中 □無

專利：□已獲得 □申請中 ■無

技轉：□已技轉 □洽談中 ■無

其他：(以 100 字為限)

3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）(以 500 字為限）

抑制 AKR1C3 表現，進而增加 LCN2 表現量和抑制子宮頸癌細胞轉移。因此，我們建議利用 AKR1C3 和 LCN2 作為癌症治療的分子標靶，以提高子宮頸癌患者臨床治療的預後結果。