行政院國家科學委員會專題研究計畫　成果報告

以基因分析評估參與卵巢轉移能力的基因(2/2)

計畫類別：個別型計畫
計畫編號：NSC91-2314-B-002-315-
執行期間：91 年 08 月 01 日至 92 年 07 月 31 日
執行單位：國立臺灣大學醫學院婦產科

計畫主持人：鄭文芳

計畫參與人員：鄭文芳　謝長堯　謝豐舟 陳祈安 李建南 蘇怡&#23509；

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附件一

行政院國家科學委員會補助專題研究計畫 ■ 成 果 報 告 □期中進度報告

以基因分析評估參與卵巢轉移能力的基因(2/2)

計畫類別：■ 個別型計畫　　□ 整合型計畫
計畫編號：NSC 91－2314－B－001－315－
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共同主持人：謝長堯
計畫參與人員：

成果報告類型(依經費核定清單規定繳交)：□精簡報告　■完整報告

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執行單位：國立臺灣大學醫學院婦產科
中　華　民　國　　年　　月　　日

一、中文摘要

關鍵詞：卵巢癌，轉移，動物模式，分示法，去氧核糖核酸分析

卵巢癌在婦女惡性腫瘤中是死亡率排名第一位的疾病。卵巢癌的發生率在最 近十年逐年上升，卵巢癌在台灣地區婦女惡化腫瘤發生率排名為第九位的疾病。以上數據顯示卵巢癌實在是一個值得重視的疾病，但在國內僅有少數的卵巢癌研究。雖然積極的減積手術和化學治療藥品已被廣泛地使用，近二十年來卵巢癌病人的總存活只有稍為地改變。治療卵巢癌的基本問題在於一旦它的散佈超過了卵巢，卵巢癌的控制及治療將相當的困難。超過百分之七十的卵巢癌病人在被診療時已是晚期。因此研究卵巢癌轉移的機制將有助於我們了解這個疾病和發展新的卵巢癌治療方針。

在癌症進展中最傷害的改變是它從局部生長的腫瘤轉變為轉移的殺手。這個轉變被相信包含了很多的改變來允許腫瘤細胞完成轉移所須的一系列事件。僅有相當少的基因已被證實涉及此一系列事件。我們建立和定性一株腹腔內腹水生成的腫瘤細胞株在鼷鼠動物模式再。我們進一步利用分式法比較高轉移細胞株和低轉移細胞株之間基因的差異。我們未來將進一步研究這些和轉移相關的基因。

二、英文摘要

Keywords：ovarian cancer, metastasis, animal model, differential display, DNA analysis

Ovarian cancer is the first mortality rate of gynecologic malignancies. The incidence of ovarian cancer increased in recent 10 years and it has become the ninth cause of malignancies in the women in Taiwan. From the above-mentioned data, ovarian cancer indeed is a disease that should be respected, however, there were only few of research work focusing on it in Taiwan. Despite the widespread use of aggressive cytoreductive surgery and the introduction of chemotherapy regimens, the overall survival has changed little over the last two decades. The basic problem in treating epithelial ovarian cancer is that once it has spread beyond the ovary, it is exceedingly difficult to control and ultimately to cure. More than 70% of ovarian cancer patients were advanced stage when diagnosed. To study the mechanisms of metastasis of ovarian cancer will help us understand this disease and develop new treatment strategies for ovarian cancer in the future.

The most damaging change during cancer progression is the switch from a locally growing tumor to a metastatic killer. This switch is believed to involve numerous alternations that allow tumor cells to complete the complex series of events needed for metastasis. Relatively few genes have been implicated in these events. We established and characterized an ascitogenic itnraperitoneal tumor cell line-WF3 in the mouse model. We further performed microarray to compare the gene profiling between high and low metastatic cell lines. These metastsis-related genes will be evaluated in our future study.

三、計畫原由與目的

Ovarian cancer is the first mortality rate of gynecologic malignancies (1-3) and became a more and more important disease in recent years (4-6). From the above mentione, ovarian cancer indeed is a disease that should be respected, however, there were only few of research work focusing on it. Despite the widespread use of aggressive cytoreductive surgery and the introduction of chemotherapy regimens, the overall survival has changed little over the last two

decades (7, 8). The basic problem in treating epithelial ovarian cancer is that once it has spread beyond the ovary, it is exceedingly difficult to control and ultimately to cure. More than 70% of ovarian cancer patients were advanced stage when diagnosed (9-11). To study the mechanisms of metastasis of ovarian cancer will help us understand this disease and develop new treatment strategies for ovarian cancer in the future.

The most damaging change during cancer progression is the switch from a locally growing tumor to a metastatic killer. This switch is believed to involve numerous alternations that allow tumor cells to complete the complex series of events needed for metastasis (12, 13). Relatively few genes have been implicated in these events (14-17). We have established an ascitogenic itnraperitoneal tumor cell line-WF3 in the mouse model. By applying this cell line, we designed this two-year project including animal model and human specimen to research the molecular mechanisms of ovarian cancer mestastasis. In the mice model, we are going to use an in vivo selection scheme to select highly metastatic WF-3 cell clones. In the part of human tumor tissues, we will also collect the ovarian cancer tissues from the original sites and metastatic sites. By analyzing these tumor cells from animal model and tumor tissues from the cancer patients on the techniques of differential display of DNA analysis, we can have the chance to define a pattern of gene expression that correlates with progression to a metastatic phenotype.

四、實驗方法

Construction of Mouse Tumor Cells by Cotransformation of HPV-16 E6 and E7 and Activated *ras* Oncogene

C57BL/6 mouse peritoneal cells were collected by peritoneal washing by 1XHBSS. The primary single cell suspension were cultured *in vitro* in RPMI1640, supplemented with 10% fetal calf serum, 50 units/ml penicillin/streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, and 2 mM nonessential amino acids, and grown at 37^0C with 5% CO_2. Transduction of HPV-16 E6 and E7 genes into primary peritoneal cells was performed with the LXSN16E6E7 retroviral vector, a generous gift from Denise A. Galloway (Fred Hutchinson Cancer Research Center, Seatle, WA) (26). The HPV-16 E6 and E7 containing LXSN16E6E7 were used to infect CRIP cells to generate recombinant virus with a wide host range. The primary peritoneal cells were immortalized by transduction as described previously (26). Following transduction, the retroviral supernatant was removed, and the cells were grown in G418 (0.4 mg/ml) culture medium for an additional 3 days to allow for integration and expression of the recombinant retroviral genes. The immortalized lung (E6+E7) cells were then trnasduced with pVEJB expressing activated human c-Ha-*ras* gene, kindly provided by Chi V. Dang (The John Hopkins Hospital, Baltimore, MD), and selected with G418 (0.4 mg/ml) and hygromycin (0.2 mg/ml).

Histological and Pathological Features of WF-3

$5x10^4$ WF-3 tumor cells were injected in C57BL/6 mice intraperitoneally (i.p.). 5 to 6 weeks later, the mice were sacrificed and the invasiveness of the tumor was examined grossly. Then, evisceration was performed. The removed internal organs were fixed with 4% buffered formaldehyde and histological sections were done and stained with routine hematoxylin-eosin stain. The slides were observed under light microscope.

Cytologic Examination of WF-3

$5x10^4$ WF-3 tumor cells were injected in C57BL/6 mice i.p.. The mice were sacrificed 5 to 6 weeks later. The ascites was aspirated, centrifuged onto the slides, and fixed in 95% alcohol and stained with Papanicolaou stain. The slides were then observed under light microscope.

Immunocytological Stainings of Cytokeratin

WF-3 cells were cultured in culture chamber slides for two days and fixed with 4% buffered formaldehyde for 1 hr and washed twice with PBS. The slides were incubated with 3% H_2O_2 in methanol for 10 min at room temperature in order to eliminate endogenous peroxidase. The slides were incubated with mouse IgG blocking reagent (Vector, Burlingam, CA) for 1 hr and then washed twice with PBS. The slides were then incubated with MOM diluent (Vector, Burlingam, CA) for 5 min. Subsequently, tip off the excess MOM diluent and followed by incubation of mouse anti-mouse cytokeratin monoclonal antibody, (1:300, Sigma, Saint Loius, Missouri) for 30 min at room temperature in a humidified chamber. After rinsed 3 times in PBS for 5 min, the slides were incubated with biotinylated anti-mouse IgG (Vector, Burlingam, CA) for 30 min at room temperature. The slides were rinsed 3 times in PBS for 5 min. Then, the slides were incubated with avidin-biotin complex (Vector, Burlingam, CA) for 30 min at room temp. The slides were then rinsed 3 times in PBS for 5 min. Followed by adding DAB substrate solution (DAKO, Carpinteria, CA) and incubated for 5 min at room temperature. Finally, the slides were washed with distilled water and counterstained with Mayer's hematoxylin. The stained slides were then dehydrated, mounted with Permount (FisherScientific, Fair Lawn, New Jersey) and observed under light microscope.

MHC Class I and Class II Expressions of WF-3

WF3 tumor cells were harvested by trypsinization, washed, and resuspended in FACSCAN buffer. Anti-H-$2K^b$/H-$2D^b$ monoclonal antibody (Clone 28-8-6, PharMingen, San Diego, CA) or Anti-I-A^b monoclonal antibody (Clone 25-9-17, PharMingen, San Diego, CA) were added and incubated 30 min on ice. After washing with FACSCAN buffer twice, FITC-conjugated goat anti-mouse antibody (Jackson ImmunoResearch Lab. Inc., West Grove, PA) was added and incubated 20 min on ice. The samples were resuspended in FACSCAN buffer. Analysis was performed on a Becton-Dickinson FACScan with CELLQuest software (Becton Dickinson Immunocytometry System, Mountain View, CA).

In Vivo Tumor Growth Kinetics of Tumor Cell Lines

$5x10^4$ WF-0, WF-3, or WF-3L3 tumor cells were injected in C57BL/6 mice subcutaneously. Mice were monitored regularly for tumor growth and overall survival. The data were used to plot graphs of tumor growth kinetics. Some animals were sacrificed, and the tumor nodules were processed to check the cytologic examinations, MHC class I and II expression, cytokeratin expression, and HPV-16 E7 expression as described earlier.

RNA Preparation, Microarray, and Data Analysis

Poly$(A)^+$ RNA from approximately $1x10^7$ WF-0 and WF-3 were isolated according to the manufacturer's protocol using a FastTrack 2.0 mRNA isolation kit (Invitrogen, Carlsbad, CA). cDNA generation, hybridization, and data collection were performed by Incyte Genomics. In brief, alterations in gene expression were evaluated by reverse transcription of poly$(A)^+$ RNAs in the presence of Cy3 or Cy5 fluorescent labeling dyes followed by hybridization to a mouse

GEM2 (UniGene 1) microarray chip. Each chip displays a total of 8734 elements of which 7854 are unique genes/clusters. These unique gene/clusters can be further defined as 3205 annotated and 4649 unannotated sequences. Subsets of genes were selected for further study based on differential Cy3/Cy5 expression ratios that were $\geq$ 1.7 in response to Ab cross-linking treatment. Differential expression of representative selected genes was confirmed by RT-PCR and/or Northern hybridization. Definition of terms for the gene chip can be found at http://reagents.incyte.com/.

四、結果與討論

WF-3腫瘤細胞株的組織學和病理學特徵

$5x10^4$ 的 WF-3 腫瘤細胞以腹腔內注射的方式注射至 C57BL/6 鼷鼠的腹腔中。五至六個星期後鼷鼠被犧牲掉檢查巨觀上腫瘤的侵犯力之後，鼷鼠的內部將做進一步的檢查。被移除的內臟器官被固定、切片和染色。在外觀上鼷鼠的大小變大且腹部澎出。腹腔中充滿血色的腹水(約 2 毫升) 。許多易碎的灰白色大小為 1.2 X 1.0 X 0.8 公分的腫瘤散生在腸膜區域。腫瘤也會侵犯鄰近的器官，包括肝臟(圖 1A)、胰臟(圖 1B)、小腸(圖 1C)、大腸(圖 1C)及橫隔膜(圖 1D)。

在顯微鏡下，腫瘤是由奇異地未分化的腫瘤細胞群所組成的。腫瘤細胞有多型行性的變化、高比例的細胞質核比和高染色質(圖 2A)。腫瘤細胞群出現兩種生長型態，為一種多角型細胞的惡化上皮腫瘤成份混合另一種梭狀腫瘤細胞束的惡性肉瘤成份(圖 2B 和 2C)。同時可以見到腫瘤乳突狀生長結構(圖 2D)。

WF-3 腫瘤細胞株的細胞學檢查

WF-3 腫瘤細胞株的細胞學檢查是吸取腹水，離心將腹水中的細胞被置於玻片上，以 95% 酒精固定和 Papanicolaou 染色法染色。如圖 3 所示，有多型行性變化的腫瘤細胞，腫瘤細胞顯示高染色質和顯著的核仁。

WF-3 腫瘤細胞株的 Cytokeratin 化學組織免疫染色

4%福馬林液固定石蠟包埋的 WF-3 腫瘤組織切片, 先去石蠟再浸入 PBS 中。Cytokeratin 染色的過程如上所述。WF-3 腫瘤細胞的 Cytokeratin 化學組織免疫染不論是惡化上皮腫瘤成份或是惡性肉瘤成份皆為正反應。(圖 4A 和 4B) 。

WF-3 腫瘤細胞株的 MHC Class I 和 Class II 的表達

測量 WF-3 腫瘤細胞株的 MHC Class I 和 Class II 的表達的方法和步驟如上所述。如圖 5A 所示，WF-3 腫瘤細胞表達 MHC class I ，但卻不表達 MHC class II (圖 5B) 。

WF-3 腫瘤細胞株的腫瘤生長動態

以 $1x10^4$、$5x10^4$、$1x10^5$ 或 $1x10^6$ 不同數目的 WF-3 腫瘤細胞以腹腔內注射的方式注射至 C57BL/6 鼷鼠的腹腔中。鼷鼠定期地監視腹水的生成和其存活時間。這些資料被繪置成腫瘤生長動態的圖。如圖 6A 和 6B 所示，所有注射 $5x10^4$、$1x10^5$ 或 $1x10^6$ 腫瘤細胞的鼷鼠在 30 天內會產生腹水，在 90 天內死亡。20%接受 $1x10^4$ 腫瘤細胞的鼷鼠無腹水產生且存活超過 90 天。 我們進一步將 $5x10^4$ 的 WF-3 腫瘤細胞以皮下注射的方式注射至 C57BL/6 鼷鼠右後腿皮下組織處。在適當時間犧牲鼷鼠檢查 WF-3 肺部轉移的能力。WF-3 具有比 WF-0 較佳的轉移能力(圖 7A 和 7B)。

WF-3 腫瘤細胞株的轉移能力

我們進一步將 $5x10^4$ 的 WF-3 腫瘤細胞以皮下注射的方式注射至 C57BL/6 鼷鼠右後腿皮下組織處。在適當時間犧牲鼷鼠檢查 WF-3 肺部轉移的能力。WF-3 具有比 WF-0 較佳的

轉移至肺臟的能力(圖 7A 和 7B)。

WF-3 和 WF-0 表現差異基因

WF-3 和 WF-0 以分式法顯示之表現差異基因如表一所示。

附圖說明

圖一: WF-3 腫瘤細胞株的組織學和病理學特徵。$5x10^4$ 的 WF-3 腫瘤細胞以腹腔內注射的方式注射至 C57BL/6 鼷鼠的腹腔中。五至六個星期後鼷鼠被犧牲掉檢查巨觀上腫瘤的侵犯力之後，鼷鼠的內部將做進一步的檢查。被摘除的內臟器官以 4%福馬林液固定，再加以組織切片和以 hematoxylin-eosin 染色。這些組織將在光學顯微鏡下觀察。許多易碎的灰白色大小為 1.2 X 1.0 X 0.8 公分的腫瘤散生在腸膜區域。腫瘤也會侵犯鄰近的器官，包括肝臟(A)、胰臟(B)、小腸(C)、大腸(C)及橫隔膜(D)。(Hematoxylin eosin stain 10X10)

圖二: WF-3 腫瘤細胞株的微觀特徵。WF-3 腫瘤細胞注射的方式注射至 C57BL/6 鼷鼠的腹腔和動物組織切片的處理步驟如圖一所述，以觀察 WF-3 腫瘤細胞株的微觀特徵。(A) 在顯微鏡下，腫瘤是由奇異地未分化的腫瘤細胞群所組成的。腫瘤細胞有多型行性的變化、高比例的細胞質核比和高染色質。腫瘤細胞群出現兩種生長型態，為一種多角型細胞的惡化上皮腫瘤成份混合另一種梭狀腫瘤細胞束的惡性肉瘤成份。同時可以見到腫瘤乳突狀生長結構。(B) WF-3 腫瘤的惡性肉瘤成份。(C) WF-3 腫瘤的惡化上皮腫瘤成份。

圖三: WF-3 腫瘤細胞株的細胞學檢查。$5x10^4$ 的 WF-3 腫瘤細胞以腹腔內注射的方式注射至 C57BL/6 鼷鼠的腹腔中，五至六個星期後鼷鼠被犧牲掉。WF-3 腫瘤細胞株的細胞學檢查是吸取腹水，離心將腹水中的細胞被置於玻片上，以 95% 酒精固定和 Papanicolaou 染色法染色。如圖所示，有多型行性變化的腫瘤細胞，腫瘤細胞顯示高染色質和顯著的核仁。(40X10)

圖四: WF-3 腫瘤細胞株的 Cytokeratin 化學組織免疫染色。WF-3 腫瘤細胞注射的方式注射至 C57BL/6 鼷鼠的腹腔和動物組織切片的處理步驟如圖一所述。4%福馬林液固定石蠟包埋的 WF-3 腫瘤組織切片，先去石蠟再浸入 PBS 中切片浸泡於室溫的 3% H_2O_2/甲醇中。切片接著浸泡於鼷鼠的 IgG 阻斷試劑中。切片再浸泡於 MOM diluent 中。再將切片浸泡於抗鼷鼠 cytokeratin 單株抗體中(1:300, Sigma, Saint Loius, Missouri) 。切片再浸泡於 biotinylated 抗鼷鼠 IgG。切片再浸泡於 avidin-biotin complex。再加入 DAB 受質溶液(DAKO, Carpinteria, CA)。最後切片以清水沖洗和以 Mayer's hematoxylin 作做背景染色。染色後的切片經脫水以 Permount 。WF-3 腫瘤細胞的 Cytokeratin 化學組織免疫染不論是(A)惡性肉瘤成份或是(A)惡化上皮腫瘤成份皆為正反應。

圖五: WF-3 腫瘤細胞株的 MHC Class I 和 Class II 的表達。WF-3 腫瘤細胞以抗 $H\text{-}2K^b$/$H\text{-}2D^b$ 單株抗體或抗 $I\text{-}A^b$ 單株抗體作用。再以 FITC-conjugated goat anti-mouse 抗體作用。WF-3 腫瘤細胞最後置於緩衝液中，以流式細胞儀分析。WF-3 腫瘤細胞表達(A) MHC class I ，(B) 不表達 MHC class II。

圖六: WF-3 腫瘤細胞株的腫瘤生長動態。以 $1x10^4$、$5x10^4$、$1x10^5$ 或 $1x10^6$ 不同數目的 WF-3 腫瘤細胞以腹腔內注射的方式注射至 C57BL/6 鼷鼠的腹腔中。鼷鼠定期地監視腹水的生成和其存活時間。這些資料被繪置成腫瘤生長動態的圖。(A)腹水生成情形。所有注射 $5x10^4$、$1x10^5$ 或 $1x10^6$ 腫瘤細胞的鼷鼠在 30 天內會產生腹水，且在 30 天內死亡。(B)存活情形。20% 接受 $1x10^4$ 腫瘤細胞的鼷鼠無腹水產生且存活超過 90 天。

圖七: WF-3 腫瘤細胞株的轉移能力。我們進一步將 $5x10^4$ 的 WF-3 腫瘤細胞以皮下注射的方式注射至 C57BL/6 鼷鼠右後腿皮下組織處。在適當時間犧牲鼷鼠檢查 WF-3 肺部轉移的能

力。WF-3 具有比 WF-0 較佳的轉移至肺臟的能力(圖 7A 和 7B)。

五、計畫成果自評

我們建立的腫瘤細胞株-WF-3，它的腫瘤細胞生物學和人類的卵巢上皮細胞癌的腫瘤細胞生物學十分的相像，也成功地建立了卵巢癌的動物模式。卵巢癌動物模式的建立，將有助於卵巢癌的研究，諸如卵巢癌的發生學、腫瘤基因學、卵巢癌的預防及發展各種治療卵巢癌的策略。這些研究工作將能對卵巢癌的研究有重要的幫助。

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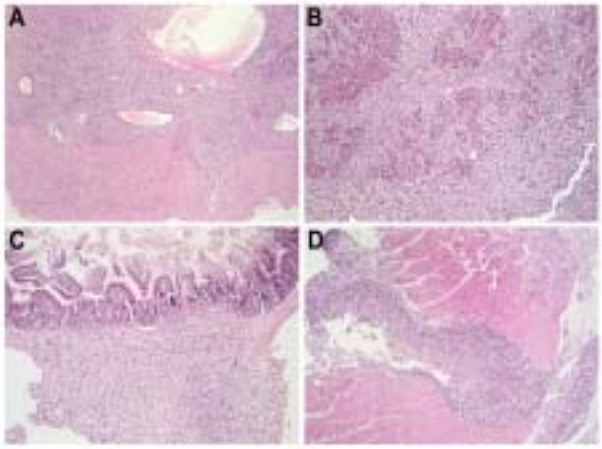
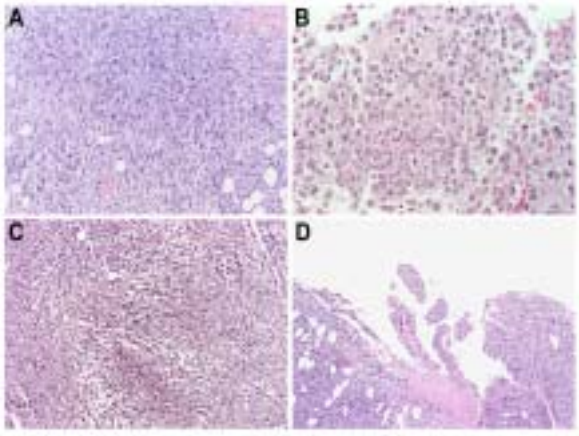
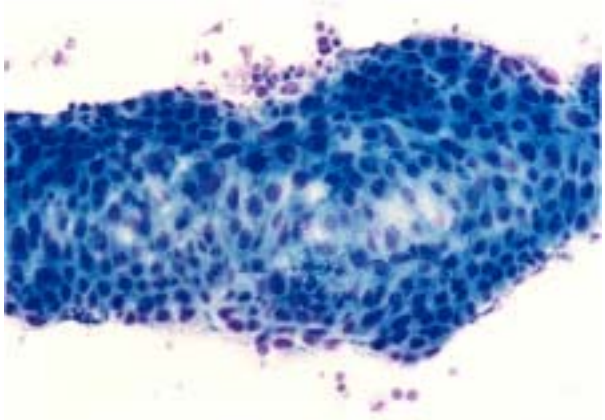
附圖一



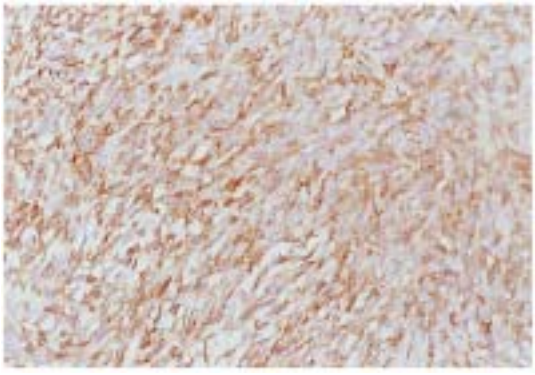
附圖二



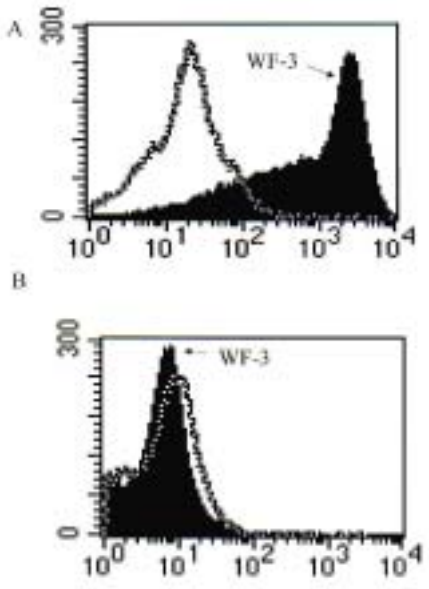
附圖三



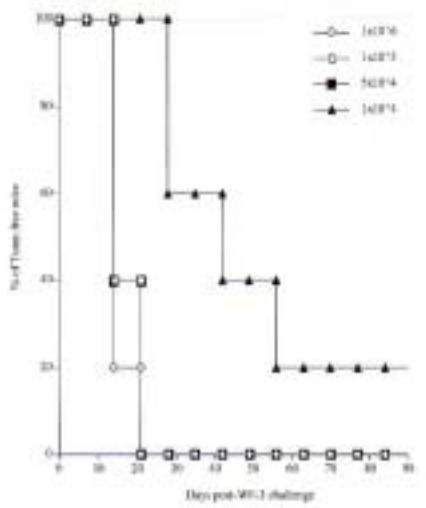
附圖四



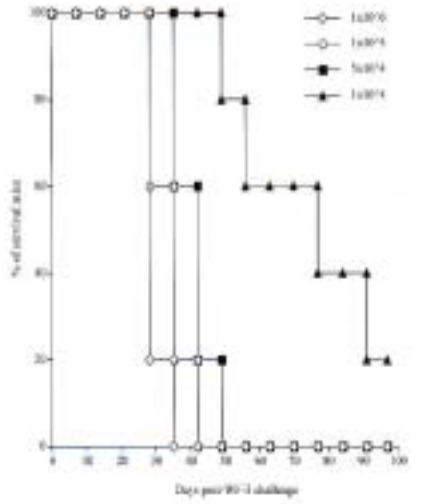
附圖五



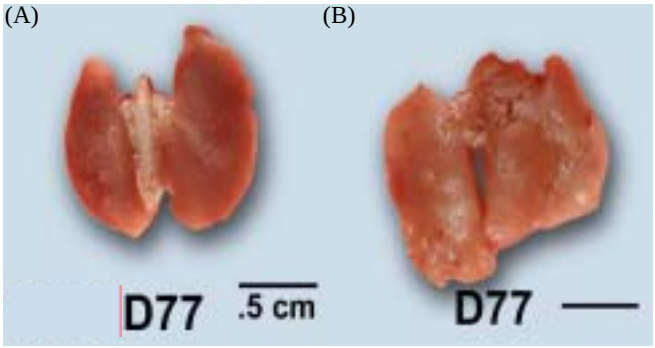
附圖六 A



附圖六 B



附圖七



表一 Enhanced gene expression in WF-3/WF-0

	Gene name	WF-0	WF-3
1	ESTs	1	5.8
2	lectin, galactose binding, soluble 3	1	5.6
3	RIKEN cDNA 1300019I03 gene	1	4.7
4	ESTs	1	4.6
5	high mobility group AT-hook 1	1	4.5
6	ESTs	1	4.5
7		1	4.4
8	inter-alpha trypsin inhibitor, heavy chain 2 potassium intermediate/small conductance calcium-activated channel, subfamily N, member 4	1	4.4
9	ESTs	1	4.3
10	ESTs	1	4.3
11		1	4.1
	insulin-like growth factor binding protein 4		
12	ESTs	1	4.0
13	glutathione S-transferase like	1	3.8
14	Complement factor H precursor	1	3.7
15	ESTs	1	3.4
16	ESTs	1	3.3
17	tissue inhibitor of metalloproteinase	1	3.2
18	cytoplasmic polyadenylation element binding protein	1	3.2
19	EST	1	3.2
20	ESTs	1	3.2
21	heat shock protein, 60 kDa	1	3.1
22	ESTs	1	3.1
23	ESTs	1	3.0
24	RIKEN cDNA 1700095N21 gene	1	3.0
25	ESTs	1	3.0
26		1	3.0
	epidermal growth factor-containing fibulin-like extracellular matrix protein 1		
27	tripartite motif protein TRIM2	1	3.0
28	ESTs	1	3.0
29	programmed cell death 4	1	3.0
30	RIKEN cDNA 2700078F24 gene	1	2.9
31	DNA segment, Chr 8, ERATO Doi 580,	1	2.9

	expressed		
32	sequestosome 1	1	2.8
33	DNA segment, Chr 4, Wayne State University 125, expressed	1	2.8
34	RIKEN cDNA 1810033A19 gene	1	2.8
35	RIKEN cDNA 3230402K17 gene	1	2.8
36	secreted phosphoprotein 1	1	2.8
37	glutamine fructose-6-phosphate transaminase 2	1	2.7
38	ESTs	1	2.6
39	ESTs	1	2.6
40	RIKEN cDNA 2410004J23 gene	1	2.6
41	ESTs	1	2.6
42	phenylalanine-tRNA synthetase-like	1	2.6
43	SIK similar protein	1	2.5