國立臺灣大學醫學院附設醫院

研究計畫書

□藥品　□醫療器材　□醫療技術　V其他

一、計畫名稱：

中文: 標定巨噬細胞與癌細胞融合機制以改善癌症纖維增生

英文：Targeting fusion machinery between macrophages and cancer cells to ameliorate cancer desmoplasia

二、主要主持人：姚明醫師

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四、摘要

臨床及臨床前研究顯示，癌細胞能與血球細胞融合，而獲得移動，增生，與藥物抗性的特性. 我們以往在小鼠模式研究，發現腸道間質細胞與巨噬細胞融合，導致慢性腸道纖維化. 我們認為，與巨噬細胞之融合癌細胞，可能導致腫瘤纖維化與惡化.

為研究與血球細胞融合癌細胞，我們以 cre-loxP 系統，或性別錯配方式，建立小鼠腫瘤模型; 也建立由 ROSA mice 骨髓，移植給予 pdx-cre-Kras $^{LSL\text{-}G12D}$ (KC) mice 的研究模型. 融合細胞與巨噬細胞生物標記，以免疫螢光染色和流式細胞儀偵測.

在實驗室中，我們以 cre-loxP 系統，或雙螢光染色方式，評估胰臟癌細胞與巨噬細胞，共培養後的細胞融合現象. 我們將會檢測融合細胞的增生，移動，與藥物抗性. 融合與未融合的癌細胞基因型態，將以 Affymetrix 微陣列分析.

在小鼠腫瘤模式中，胰臟癌細胞與宿主小鼠細胞融合機率，在腫瘤植入 1 到 5 周後，為 1.6 to 4.86%. 在 KC 小鼠模型，於骨髓移植後 7-16 周，融合細胞比率為 0.62 to 26.49%. 以脂質體氯磷酸鹽去除巨噬細胞，小鼠的腫瘤生長減緩，細胞融合比率降低. Masson 三色染色，與免疫印跡實驗顯示，腫瘤纖維化狀態與細胞融合程度相關.

在實驗室中共培養巨噬細胞與胰臟癌細胞，顯示 24 到 72 小時內細胞融合比率 0.64 to 1.12%. 在小鼠骨髓細胞中去除 CD11b(+)細胞, 明顯降低與癌細胞細胞融合比率. 融合癌細胞較野生胰臟癌細胞，顯示較高的移行，增生能力; 以及對放射照射的抗性. 基因分析顯示 degradation of the extracellular matrix, 以及 signal regulatory protein (SIRP)訊息傳遞路徑, 在融合細胞, 較未融合細胞明顯上升. 核糖核酸與蛋白測量, 顯示細胞外基質, 纖維化, 及 SIRP 分子表現量，在融合細胞較高.

綜合言之, 與巨噬細胞融合的胰臟癌細胞, 與腫瘤纖維化以及癌症惡化相關. SIRP 訊息傳遞路徑，是具有潛力破壞細胞融合，改善胰臟癌纖維化與惡化的標的.

我們將收集以往接受骨髓移植之後，發生惡性腫瘤的病理組織. 評估其癌細胞中，是否含有來自捐髓者的基因. 如:Y 染色體, 或短串聯重複(short tandem repeats) 研究… 以證明臨床上，癌細胞與巨噬細胞融合的現象. 預計由病理部, 收集 20 位符合條件病患之腫瘤檢體切片, 每位 10 片各 4-8μm.

另將收集健康人,或多血症病患之周邊單核球細胞,作為實驗室中,與癌細胞株共培養,篩選純化融合癌細胞, 作為對於融合癌細胞之增生,輻射敏感性,轉移能力… 等等生物特性研究.預計由檢醫部血庫,收集30位健康捐髓者或多血症病患之周邊血液單核球細胞,一次10-20ml.

Abstract

Clinical and preclinical evidence reveal that cancer cells may fuse with hematopoietic cells to obtain properties including migration, proliferation and drug resistance. Our previous studies in murine model revealed fusion hybrids between intestine stromal cells and macrophages contribute to chronic intestine fibrosis. We hypothesize that cancer cell-macrophage fusion hybrids may lead to pancreatic cancer desmoplasia and progression.

Murine tumor models using cre-loxP or gender-mismatched xenografts as well as pdx-cre-Kras$^{LSL-G12D}$ mice after bone marrow transplantation from reporter ROSA mice were established. Fusion hybrids and macrophage markers were detected using immunofluorescence staining and flowcytometry.

In vitro co-culture using cre-loxP or dual fluorescence methods of pancreatic cancer cells with macrophages was used to evaluate the frequency of fusion phenomenon. The proliferative, migratory and resistant phenotypes of purified fusion hybrids were measured. Differentially expressed genes between fusion hybrids and non-fused cancer cells were compared by Affymetrix microarray analysis.

Fusion between pancreatic cancer and host derived cells in murine xenograft model ranges from 1.6 to 4.86%, 1-5 weeks after tumor implantation. In KC murine model, fusion hybrids of 0.62 to 26.49% was noted 7-16 weeks after BM transplantation from ROSA mice. Depleting macrophage using liposomal clodronate in mice with tumor xenograft showed delayed tumor growth and reduced fusion hybrids. Masson trichrome stain and immunoblots revealed the fibrosis status of tumors were correlated with the amount of fusion hybrids.

In vitro co-culture of macrophages with pancreatic cancer cells showed spontaneous fusion of 0.64 to 1.12% in 24 to 72 hrs. Depleting CD11b(+) cells from BM cells decreased the level of fusion phenomenon significantly. Fusion hybrids revealed enhanced migratory and proliferative ability as well as resistance to radiation cytotoxicity compared with wild type pancreatic cancer cells. Gene set enrichment analysis showed the degradation of the extracellular matrix (ECM) and signal regulatory protein (SIRP) signal pathway were significantly upregulated in fusion hybrids compared with non-fused pancreatic cancer cells. The expression level of ECM, fibrosis, SIRP molecules were elevated at RNA and protein levels in fusion hybrids.

In summary, fusion between pancreatic cancer cells and macrophages contribute to cancer desmoplasia and malignant progression of pancreatic cancer. SIRP signal pathway is a potential target to disrupt fusion phenomenon and ameliorate pancreatic cancer fibrosis and progression.

We are going to collect tumor tissues from cancer patients who received allograftic bone marrow transplantation before. We will evaluate Y chromosome or short tandem repeats to

identify donor- derived genes in cancer cells and demonstrate the clinical evidence of fusion between cancer cells and macrophages. The tumor tissues will be collected from the Pathology Department. Ten slides of 4-8μm will be collected from twenty patients enrolled according to the inclusion criteria.

We will collect peripheral mononuclear cells from healthy volunteer (eg. Donors for bone marrow transplantation) or hyperemia patients. The mononuclear cells will be induced to differentiate into macrophages and will be co-cultured with cancer cells in order to purify fusion hybrids. The fusion hybrids between cancer cells and macrophages will be evaluated for biologic characters including proliferation, radio-sensitivity, migration etc. We planned to collect blood samples from Department of Laboratory Medicine, Blood bank. Thirty subjects of healthy volunteer or hyperemia patients will be enrolled. Ten to 20ml peripheral blood will be collected from each subjects for one time.

五、研究主題說明

癌症纖維增生，尤其在胰臟癌，是導致癌症惡化，對治療產生抗藥性的主要原因之一．目前針對剔除被癌症活化之纖維細胞的治療，效果不如預期，反而會導致更具侵略性的癌症，吸引抑制免疫反應的Ｔ細胞，大量湧入腫瘤微環境.

考量巨噬細胞在發炎反應，纖維化，癌症發生，與惡化的角色，許多針對巨噬細胞剔除或極化的治療發展，得到具有潛力的臨床前期結果．研究顯示巨噬細胞與癌細胞的細胞融合，是導致腫瘤多樣性的動力，引起抗藥性與癌症惡化．但對於巨噬細胞與癌細胞的細胞融合，在癌症纖維增生的研究很少，為改善胰臟癌治療效果，提高醫療經費經濟效益與病患生活品質，我們將發展以巨噬細胞為主的治療策略，改善癌症纖維化環境，達到預防腫瘤新生、惡化，以及提高治療效果的目的.

我們將前瞻性收集健康受試者之周邊單核血球，在實驗室中分化為巨噬細胞，再與癌細胞共培養，挑選出與巨噬細胞融合的癌細胞，進行細胞與動物實驗，檢測融合細胞之生物特性，包括纖維化激素分泌.

另外將回溯性收集接受骨髓移植後，罹患惡性腫瘤的病患組織，檢測腫瘤中是否含有來自捐髓者的基因，包括巨噬細胞特性基因，以及與巨噬細胞融合的癌細胞表現量，並與腫瘤纖維化程度進行比對.

六 研究目的及背景說明（包括學理根據及有關文獻報告）

Desmoplasia has been shown to exert mechanical forces and create a biochemical intra-tumoral immunity and influence the development and progression of a malignancy.[1] Recent reports suggest a significant negative correlation between cancer patient survival and extracellular matrix deposition in primary tumors; and concomitant stromal targeting may enhance therapeutic outcomes in cancer patients.[2]

In defining the source of the dense stroma, it is generally believed that over-activated CAFs

could produce extracellular matrix (ECM) proteins, the major component of the dense stroma. Anti-fibrosis drugs including those depleting CAFs or collagen, sonic hedgehog inhibitor, antibodies against LOX/LOX2, were under clinical trials.[1] However, CAF-depleted tumors displayed a more aggressive phenotype and alteration in regulatory T cells, raising the possibility that CAF-targeting strategies may have both beneficial and detrimental effects.[2]

Considering the functional significance of macrophages in inflammation, desmoplasia, cancer initiation and progression, mounting studies had conducted to evaluate the efficiency of anti-macrophage as a novel strategy against cancer.[3] Accordingly, the anti-macrophage strategies include the inhibition of the monocytes recruitment as well as transformation, and the ablation the macrophages directly. Given the multifaceted roles of maintaining homeostasis, the systemic depletion of macrophages may lead to increased infections or impaired ability of tissue-resident cells to carry out their normal function. Thus, the identification of tumor associated macrophage (TAM)-specific markers will enable the development of more sophisticated therapies that can be targeted specifically to tumors without affecting the function of other tissue-resident immune cells.[4]

Recent studies had identified numerous factors such as platelet-derived growth factor (PDGF) and transforming growth factor β1 (TGFβ1) released by TAMs, especially bone marrow (BM)-derived ones,[5] involving in the process of activating CAFs. In addition, there were TAMs-derived pro-fibrotic factors, such as connective tissue growth factor (CTGF), chemokine ligand 17 (CCL17), CCL22 and reactive oxygen species (ROS). Similar observation was found in TAMs-derived matrix metallopeptidase 9 (MMP9) which involved in the stroma turnover by degrading the ECM proteins.[6] However, rare study investigates the role of fusion between macrophages and cancer cells in desmoplasia and tumor progression as well as the strategy targeting fusion machinery of macrophages with cancer cells.

Several lines of evidence support that cell fusion between cancer cells and leukocytes, majorly macrophages, is one of the explanations for tumor resistance and progression.[7] Recent genotyping of a metastatic melanoma to the brain that arose following allogeneic BM transplantation supports, first time clinically, fusion between a BM derived cell and a tumor cell playing a role in the origin of metastasis. Fusion hybrids enumerated in peripheral blood of pancreatic cancer patients were reported to correlate with disease stage and predict overall survival.[8] Furthermore, general inflammatory responses were demonstrated to increase the number of fusion events.[9] While the fusion events were demonstrated in animal and human, and were correlated with cancer resistance and metastasis,[7] little is known regarding key questions such as the mechanisms through which macrophage-cancer cell fusion and subsequent genomic hybridization occurs in vivo; potential survival advantages of hybrids; reprogramming of fibrotic and immunologic tumor microenvironment by hybrids; and the development of novel strategies to target fusion machinery.

The majority of macrophages, including tissue macrophages in normal tissue and TAMs, are derived from bone marrow (BM).[5] Our preliminary study, using irradiated murine intestine model,

suggested parallel between the development of cell fusion between BM-derived monocytes and intestine stromal cells with radiation-induced fibrosis.[10] Using cre-loxP system and gender mismatched BM transplantation, we demonstrated that BM-derived CD11b(+) myelo-monocytic cells/macrophages were the major fusion partner to damaged intestine stromal cells after radiation. The fusion events in irradiated intestine stroma correlated with chronic intestine fibrosis with enhanced TGFβ transcripts and collagen deposition. With macrophage deletion by liposomal chlodronate or conditional CD11b knock-out, we can ameliorate radiation induced chronic intestine fibrosis.[10]

The main property of cancer cells that makes them malignant is the ability to produce diverse progeny. Fusion between cancer cells and TAMs can be an engine of genomic and epigenetic variability that has a potential to make cells with new properties at a rate exceeding that achievable by random mutation.[11] Previous reports revealed cell fusion contribute to drug resistance and can promote the ability of cancer cells to metastasize.[7]

Fibrosis and inflammation are hallmarks of tumor desmoplasia. TAMs, majorly derived from bone marrow,[5] act as an essential connecting moiety between inflammation and cancer via secretion of pro-inflammatory cytokines/chemokines. The M2 polarized macrophages, which constitute majority of the macrophages in tumor microenvironment, secrete IL10 and other cytokines that mediate T helper 2 (Th-2) responses and are responsible for malignant tumor tansformation and inhibit antitumor immune response mediated by T cells. TAMs were reported to play a phenomenal role in enhancing mesenchymal phenotype of cancer cells via TGFβ. The lipopolysaccharide (LPS) receptor toll-like receptor 4 (TLR4) on the surface of TAMs has been implicated in a role in the EMT via TLR4/Interleukin 10 (IL10) cascade.[12] The level of secreted protein acidic and cysteine-rich (*SPARC)* gene, a regulator of EMT, was reported to be 3-4 fold higher in hybrids of BM-derived cell and melanoma.[13]

Analysis of microenvironment microarray assay revealed that fusion hybrids of colon cancer cells and macrophages exhibited a combination of biases, reflecting properties of both parental cells, including fibronectin, collagen XXIII, vitronection, potentially providing a broader desmoplastic process in different microenvironment.[8] Our preliminary results of differential expression of genes between fusion hybrids of cancer cells and macrophages with non-fused cancer cells show significant elevation of signal pathways involving inflammation, extracellular matrix degradation and mesenchymal phenotypes etc. Besides, prominent increase in signal regulatory protein (SIRP) family members, a cell-cell communicating system, were also found.

Current treatment result of cancer with desmoplasia is unsatisfactory. It is generally believed that desmoplastic stroma provides cancer cells with cytokines/chemokines to suppress immune surveillance and to enhance malignant progression. Recent clinical trials revealed direct targeting desmplasia using sonic hedgehog inhibitor lead to a more aggressive phenotype with increased regulatory T cells.[1,14] On the other hand, depleting macrophage or its recruitment from circulation revealed to be effective in reducing pro-fibrotic cytokines/chemokines and CAFs activity; however,

with significant normal tissue toxicity.[4]

From previous investigation and our study, fusion hybrids between cancer cells and macrophages appeared to play important role in promoting cancer desmplasia as well as malignant progression.[8] We proposed to develop an alternative method to ameliorate cancer desmoplasia by focusing on novel targets interfering fusion between cancer cells and macrophages. The potential candidates were revealed by our preliminary microarray study. eg. Signal regulatory protein (SIRP) signal pathway including SIRPα, a macrophage fusion receptor,[15] which was nearly 5 folds differentially expressed in fusion hybrids. Furthermore, the down-stream molecules including DAP12 (DNAX activating protein), a general macrophage fusion regulator, and protein tyrosine phosphatase (PTPN6), protein tyrosine kinase (PTK2B) were increased 8 folds and 4 folds, respectively, in fusion hybrids.

We proposed to target on SIRPα and other potential candidate molecules with neutralizing antibodies or silencing mRNA to reduce the incidence of fusion phenomenon in cancer with desmoplasia. By reducing the frequency of tumor heterogeneity and the fibrotic phenotype, we would like to improve the therapeutic outcome of cancer with desmoplasia. The combination effect of anti-fusion agents with other anti-fibrosis agents or immunologic therapy will also be explored

七 研究方法與程序

(1) To demonstrate tumor fibrosis and fusion between bone marrow derived monocytes and cancer cells in human specimen and in murine model.

Murin model

pdx-Cre-Kras-mutated (KC) mice of 10~12 wk-old, a spontaneous pancreatic adenocarcinoma murine model, will be given whole body irradiation followed by bone marrow transplantation from ROSA mice (Gt(ROSA)26Sor $^{tm4(ACTB-tdTomato,-EGFP)Luo}$/JNarl). U937(fl-dsRed-fl-eGFP). Mice will be sacrificed at 16~26wk-old. Pancreas tissues will be evaluated for fibrosis status using masson trichrome stain, immunoblots and qPCR measurement of fibrosis and extracellular matrix proteins. Fusion between pancreatic cancer cells with bone marrow derived cells will be identified as cells expressing green fluorescence due to DNA recombination. Fusion hybrids will be quantified and further immune-stained with macrophage, lymphocyte markers.

Human specimen

Tumor tissue specimens of patients receiving bone marrow transplantation from mismatched gender (eg. female receiving bone marrow cells from male donor) in previous will be collected. Cancer cells will be evaluated with Y chromosome expression using fluorescence in situ hybridization. Tumor fibrosis score will be measured after Masson trichrome stain.

(2) To evaluate the functions and the expression of fibrosis, and inflammatory molecules after fusion between cancer cells and macrophages in vitro.

Cell culture

Cancer cells including Pan02 (mouse pancreatic epithelial cancer cells) and Panc-1 (human pancreatic adenocarcinoma cells) were cultured in Dulbecco'a modified Eagle's medium (DMEM) + 10% serum. Stable cancer cell lines, Pan02(Cre), Pan02(H2B-YFP/Cre), Panc-1-EGFP were generated by retroviral transduction and polyclonal populations were selected by antibiotic resistance and flow-sorted for bright fluorescence as appropriate. Primary macrophages derivation was conducted from the bone marrow of ROSA mice. U937-loxP-dsRed-loxP cells were generated by stably expressing a MSCVLPdsRed-LoxP-eGFP-PURO construct (Addgene#32702) into the parental U937 cells**. Primary monocytes derived from healthy peripheral blood donors will be collected after CD14+ magnetic beads selection.** To elicit macrophages, cells were cultured for 6 days in DMEM + 15% serum supplemented with sodium pyruvate, nonessential amino acids and CSF1(25ng/ml). 25ng/ml GM-CSF or 50ng/ml M-CSF was used to promote M1- or M2-like polarization.

Co-culture to generate fusion hybrids

Cell fusion hybrid generating co-cultures were established in macrophage-derivation media without CSF1 for 4 days. Pancreatic cancer cells and macrophages with stably expressed fluorescence or labelled with fluorescent dyes (CytoTell^{TM}) were co-seeded at a 1:2 ratio at low density. Hybrid cells were FACS-isolated for appropriate fusion markers on a FACS sorters. Low-passage hybrid isolates were established; functional experiments were conducted on 8 to 20 hybrid isolates. Live imaging of co-cultured cells was performed using an automated microscope system and associated software.

Invasion analysis

Cellular invasion was measured in a growth factor-reduced Matrigel invasion chamber with 8 μm pores. Cells ($3x10^5$) in a medium containing 0.1% FBS were placed into each Boyden chamber. The medium containing 10% FBS was placed in the lower chamber to facilitate chemotaxis. Invasion assays were run for 15 hours, and then cells that passed through the Matrigel membrane were stained with 0.09% crystal violet/10% ethanol. After extraction by elution buffer, the stain was measured at 560nm. Representative images of invaded cells were taken by microscope.

Proliferation assay

A suspension of hybrid or wild type cancer cells in DMEM+4% FBS was added to each well of 96-well plates. Plates were cultured in a humidified incubator for 72 hours, after which 5 μl of MTS reagent was added to each well. Two hours later, absorbance at 490nm was read with a plate reader.

Immunoblots

Hybrid or wild type cancer cells were collected and lysed in protein lysis buffer

containing a protease inhibitor cocktail. The supernatants were centrifuged at 4°C for 15 min at 13000rpm. Twenty micrograms of total protein were loaded into each well of a sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel and separated. Protein was transferred onto a polyvinylidene fluoride membrane, and membranes were incubated in blocking solution at room temperature for 1 hr, followed by incubation with the indicated primary antibodies (antibodies to fibronectin, TGFβ1, collagens, Interleukin 1 β, etc) at 4°C overnight. The membranes were washed in TBS-T and incubated at room temperature with peroxidase-conjugated secondary antibodies for 1 hr. Signals were detected using the enhanced chemiluminescence assay.

(3) To demonstrate the development of desmoplasia and fusion hybrids between cancer cells and bone marrow-derived macrophages during cancer progression in murine model

Mice and bone marrow transplantation

The following strains were used in the described studies: C57BL/6J mice, ROSA mice [Gt(ROSA)26Sor$^{tm4(ACTB-tdTomato,-EGFP)Luo}$/JNarl], and KC mice [KRAS$^{mut/+}$/Cre$^{tg/0}$], Kras mice [KRAS $^{mut/+}$]. For tumor growth, 8 to 12 week-old C57BL/6J mice were injected with 5x10^4 cells subcutaneously. Length and width of palpable tumors were measured three times weekly with calipers until tumors reached a maximum diameter of 2 cm. Tumors were surgically removed and animals were sacrificed during tumor removal. For growth of tumor at metastatic sites, 1x10^6 cells were injected into the spleen. Livers were analyzed 3 weeks later for tumor burden by H&E stain.

To evaluate fusion hybrids during tumorigenesis, two sets of experiment were conducted. (1) ROSA mice at 8 to 10 week-old were subcutaneously injected with 5x10^4 cancer cells stably with or without expressing cre recombinase. (2) KC or Kras mice were irradiated at 8 to 10 week-old followed, within 24 hr, by bone marrow transplantation (BMT) from ROSA mice. Mice were sacrificed at various time points after BMT to evaluate pancreatic tissues. H&E and immunofluorescence stain were performed to measure the level of pancreatic malignancy and fusion hybrids.

Immunofluorescence analysis

Primary tumors or livers were fixed in 10% buffered formalin, frozen in optimum cutting temperature (OCT), and 4μm sections were obtained. Tumors were incubated with antibodies for GFP followed by detection with fluorescent secondary antibody. Nuclei were counterstained with Hoechst. Slides were digitally scanned and analyzed. Confocal images were acquired with an Olympus confocal microscope.

Tumors from KC or Kras mice were fixed in 4% paraformaldehyde for 2 hours at 20°C, washed and cryopreserved in 30% sucrose for 16 hr at 4°C and then embedded in OCT. Sections were cut to 4μm thickness, baked for 30 min at 37°C, then subjected to antigen retrieval under standard conditions, blocked with DAKO protein block

serum-free, and incubated for 16 hr at 4°C with primary antibodies, anti-dsRed, anti-YFP, and anti-GFP in background-reducing antibody diluent. Fluorescent-tagged secondary antibodies were applied, and then sections were mounted in a ProLong Gold antifade reagent. Antibody specificity was determined by immunostaining healthy livers of non-tumor bearing mice and performing secondary antibody only controls.

(4) To correlate desmoplasia with fusion hybrids during cancer progression by macrophage depletion in murine model

Pravastatin, and clodronate treatment

To suppress cancer desmoplasia, mice were given pravastatin, a Rho pathway inhibitor, 30mg/kg/day in drinking water for 10 weeks starting two weeks before tumor implantation. To deplete macrophages, Liposomal Clodronate 2.5mg/ml in PBS 100μL/mouse, was injected once every three days for two weeks before sacrifice.

Immunohistochemical analysis

Sections were stained with hematoxylin-eosin as well as Masson Goldner trichrome staining to evaluate collagen deposition. Fusion hybrids were measured by immunofluorescence staining at serial histology section and correlated with Masson trichrome staining.

(5) To validate in vivo-derived cell fusion hybrids

For demonstration that tumor cells can fuse with myeloid cells, Pan02(fl-dsRed-fl-eGFP) cells were injected intradermally into 8 to 10 wk-old Cd11b-DTR mice [B6.FVB-Tg(ITGAM-DTR/EGFP)34Lan/J]. Mice were given diphtheria toxin was given 10ng/g i.p. every three days for two weeks before sacrifice. When tumors reached 1cm^3, primary tumors were removed for immunofluorescence analysis.

Tumors were diced and digested for 30min at 37°C in DMEM + collagenase A(2mg/ml) + DNase under stirring conditions. Digested tumor cells were filtered through a 40μm filter and washed with PBS. Cells were washed and resuspended in FACS buffer. Cells were incubated in PBS containing LIVE/DEAD Fixable Aqua with Fc Receptor binding inhibitor. Cells were then incubated in FACS buffer for 30 min with fluorescence labelled primary antibodies including GFP, DsRed, cytokeratin, CD45, CSF1R, F4/80. FACS machine was used for analyses. The level of fusion hybrids expressing macrophage markers, unfused cancer cells were evaluated.

(6) To conduct karyotype and gene expression analysis of fusion hybrids

Karyotype analyses

Chromosome spreads from cells in S phase were prepared from cells treated for >12 hr with colcemid (100ng/ml) to induce mitotic arrest. DNA was visualized by staining with DAPI. Images of stained fixed cells and chromosome spreads were acquired by inverted microscope. Chromosomes were counted manually. A minimum of 20 cells were analyzed in each experiment.

Gene expression analysis

Microarray analysis was performed with gene chips of Affymetrix and data were analyzed using software to identify relative expression differences between cell types (wild type cancer cells, macrophages, non-fused cancer cells or fusion hybrids) and produce gene ontology (GO) analyses. GO category enrichment was calculated using the GOstats R package and visualized using functions from the GOplot R package.

(7) To validate the differential expressed signal molecules between fusion hybrids and non-fused cancer cells.

Polymerase chain reaction

DNA was extracted from wild type cancer cells and fusion hybrids and incubated in lysis buffer followed by neutralization with neutralization buffer. Primers of forward and reverse sequences were prepared according to the candidate molecules (eg SIRPα, DAP12 etc.). Reactions were run with a 60^{o}C annealing temperature.

Immunoblots

As described previously. The primary antibodies will be candidate signal molecules of fusion machinery eg. SIRPα, DAP12 etc.

(8) To evaluate the efficacy of small molecules interfering fusion machinery in suppressing fibrosis development in murine model.

In vitro fusion assay to evaluate anti-fusion agents

Freshly isolated macrophages from ROSA mice or U937(fl-dsRed-fl-eGFP) were co-cultured with Pan02(H2B-YFP/Cre) or Panc-1-EGFP cells supplemented with anti-SIRPα neutralizing antibody, PTK2B inhibitor at various concentrations. The cells were examined daily for 4 days. The level of fusion was graded by FACS of GFP. Expression of fibrosis related molecules including TGFβ, fibronectin were measured by immunoblots and PCR described previously.

Murine model to evaluate anti-fusion agents

Mice were injected i.p. with normal IgG, anti-SIRPα antibody three times a week beginning immediately after the establishment of murine models of primary tumor and liver metastatic tumor described previously. Tumors were measured with digital calipers and tumor volume was calculated. Mice were sacrificed to obtain primary and metastatic tumors for immunofluorescence study to detect the level of fusion hybrids. Tumor tissues were prepared for immunoblots and PCR to evaluate fibrosis status.

(9) To evaluate the combination of fusion interfering agents and other agents in ameliorating cancer progression.

Pan02(H2B-YFP/Cre) or Panc-1-EGFP cells were injected subcutaneously into the flanks of 8 week-old ROSA mice. The mice were injected i.p. with normal IgG or anti-SIRPα, each with or without anti-PD1 antibody, twice a week beginning immediately after tumor implantation. Tumor growth were measured. Mice were sacrificed to

evaluate the level of fusion hybrids and fibrosis as described previously. The combination of anti-SIRPα with chemotherapy, eg. Gemcitabine, will be explored as well.

八 受試者選擇標準 (檢體提供者)：

預計收案人數：50 人

身分：■病人：■健檢人：■其他： 捐髓, 放血者...

類別：前瞻性收集周邊單核血球: 30 人; 健檢, 捐髓, 放血者...

回溯性收集惡性腫瘤組織: 20 人; 惡性腫瘤且曾接受骨髓移植之病患.

九、研究設計與流程（與臨床研究相關部份）：

1. 前瞻性收集周邊單核血球

由受試者周邊血管抽血, 收集約 10-20ml, 置於添加抗凝血劑試管中, 再進行後續處理.

納入條件(參加本研究的條件):

(1) 於進案一周內,有正常的周邊血液檢測結果.

(2) 若為捐髓者,可以為接受血球刺激或生長激素之後.

(3) 可以為血紅素偏高的放血者.

(4) 年齡 20-70歲.

排除條件(若您有下列任一情況，您將無法參加本研究):

(1)有惡性腫瘤,且無復發狀態少於五年者.

(2)有重大心血管疾病, 免疫疾病, 懷孕.

(3)長期服用免疫抑制劑或類固醇者.

2. 回溯性收集惡性腫瘤組織

納入條件

(1) 曾接受骨髓移植, 且骨髓移植完成之後發生固態腫瘤.

(2) 曾接受切片或手術切除腫瘤, 檢體儲存於病理部之剩餘腫瘤組織, 約 4-8μm 厚, 10 片.

排除條件

(1)無.

十、計畫預定進度：

自研究倫理委員會核准後，開始收集檢體 3 年, 包括前瞻收集周邊血液 30 名, 回溯收集腫瘤組織 20 名. 檢體將保存 10 年(或檢體保存至 2032 年)。

十一、研究對象權益之保障、同意之方式及內容：

於門診或病房進行口頭告知說明，加入本研究, 接收抽血, 可能發生抽血部位少量出血, 紅腫瘀血. 極少情況有暈眩的情形.只需靜坐一段時間, 按壓抽血傷口止血, 局

部適度冰敷即可. 若仍不適, 可以詢求醫護協助.並由檢體提供者簽署同意書。

我們將依法, 把任何可辨識身分之記錄, 與個人隱私資料視為機密來處理，不會公開。研究人員將以一個研究代碼代表受試者身分，此代碼不會顯示其姓名、國民身分證統一編號、住址等可識別資料。如果發表研究結果，受試者的身分仍將保密。

其餘參照同意書。

十二、研究人力及相關設備需求。

1. 收集臨床醫療剩餘檢體腫瘤組織，借助病理部臨床醫事人員。

   工作職責: 由計畫主持人姚明醫師, 以核可 IRB 向台大醫院病理部, 申請組織切片. 依照病理部規範,進行檢體回溯收集.

2. 其他 收集周邊血液，借助臨床醫事人員

   工作職責: 由計畫主持人姚明醫師或共同主持人羅仕錡醫師解釋研究目的流程後, 由計畫主持人委託臨床研究護士協助收集受試者同意書.在檢醫部臨床工作人員協助下, 收集周邊血液, 由研究護士初步處理血液檢體, 並冷凍儲存, 以利計畫主持人姚明醫師或共同主持人常慧如醫師後續研究所用.

3. 相關設備需求: 離心機, 4°C 冰箱, 周邊血液單採機.

十三、研究經費需求及其來源。

經費來源：預計由國衛院經費支持。**包括 IRB 審查, 病理檢體收集, 周邊血液收及耗材儲存費用 約 NTD 100,000 元**

十四、預期成果及主要效益。

前瞻收集周邊血液, 將抽取單核白血球, 分化為巨噬細胞, 於實驗室中與癌細胞進行共培養, 並篩選融合細胞, 研究巨噬細胞與癌細胞之交互作用, 及融合細胞之生物特性 包括纖維化激素表現.

回溯性收集腫瘤組織切片, 將進行免疫螢光染色, 偵測來自捐髓者的基因表現, 與腫瘤纖維化程度.

目前無法預測可能的研究成果。當您的檢體使用於研究時，我們將不會通知您研究結果或檢體的檢測結果。一般而言，研究所做的檢測分析，大多還不能運用於醫療照護；但若研究人員認為研究分析的結果，有助於您的醫療照護，也可能會通知您。

十五、研發成果之歸屬及運用。

由收集的病理及周邊血液臨床檢體, 所產生之研發成果, 歸屬台大醫院與國衛院共同所有，未來可能將成果發表學術論文等運用

十六、研究人員利益衝突事項之揭露。

無利益衝突事項

十七、 參考資料

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