科技部補助專題研究計畫成果報告
期末報告

Gallic acid 對腫瘤微環境中Slit2-Exon15剪接變異型表現的影響(第2年)

計 畫 類 別 ： 個別型計畫
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執 行 單 位 ： 中山醫學大學醫學研究所

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中 華 民 國 104 年 10 月 26 日

中 文 摘 要 ： Slit2蛋白在腫瘤的發展、血管新生、免疫反應上扮演重要的角色。我們發現Slit2在exon15的外顯子位置有兩個剪接變異型的表現: Slit2-WT(具有exon15)及Slit2-E15 (沒有exon15)。肺癌組織的Slit2表現遠低於周圍正常組織，有趣的是肺癌病人之正常肺組織表現高度的Slit2-WT，而Slit2-E15的表現非常低或不表現。若將Slit2-E15過度表現於CL1-5肺癌細胞時，可以有效的抑制癌細胞的生長及轉移，但過度表現Slit2-WT則只能抑制細胞的轉移但對於生長沒有抑制的能力。除此之外，我們發現肺癌細胞的條件培養液會增加臍帶內皮細胞(HUVECs)的通透性及降低管柱的完整性，並使得雞胚胎表面的血管直徑變細。但是如果條件培養含有Slit2-E15時，則可以抑制肺癌細胞之條件配養液所誘導的HUVECs通透性及管柱完整性，並使得雞胚胎表面的血管直徑變正常。而條件培養含有Slit2-WT時只能抑制癌細胞之條件配養液所誘導的HUVECs通透性，但對於管柱的完整性及新生血管直徑則沒有影響。我們也發現環境中葡萄糖的濃度、FGF會增加Slit2-WT的表現，而gallic acid則會降低Slit2-WT的表現。除此之外，當動物以LPS誘導肺發炎及以尾靜脈注射肺癌細胞產生肺轉移時，Slit2-WT的表現也會下降。由我們所建構的Slit2-splicing reporter constructs發現，在短暫轉染的情況下，Slit2-E15是主要表現的剪接變異型，而Slit2-WT則表現非常低。綜合我們的研究，在沒有調控的情形下主要表現的是Slit2-E15剪接變異型，而 Exon 15則需要調控因子的幫助才能保留下來產生Slit2-WT。由於肺癌病人的肺組織之Slit2-E15表現非常低，若能找到抑制Slit2-WT剪接變異型的因子，而提高Slit2-E15的表現型，將有助於讓腫瘤微環境中的Slit2-E15的表現量增加，進而抑制肺癌細胞的生長，並讓腫瘤所誘導的新生血管正常化，以增加化學治療及放射治療的療效。

中 文 關 鍵 詞 ： Slit2、剪接變異型、HUVECs、內皮細胞通透性、體外管柱形成、血管新生、腫瘤微環境

英 文 摘 要 ： Slit2 plays an important role in tumor development, angiogenesis, and immune response. We have identified alternative splicing at exon 15 of Slit2, Slit2-WT (presence of exon 15) and Slit2-E15 (absence of exon 15). The expression of Slit2 is highly reduced in lung tumor when compared with their normal lung counterpart. However, Slit2-WT is the predominant splicing form with little or undetectable Slit2-E15 form in lung cancer specimen. Ectopic expression of Slit2-E15 inhibited growth and invasion of CL1-5 lung cancer cells while Slit2-WT repressed growth capability only. In addition, the conditioned medium (CM) of lung cancer cell enhanced permeability of HUVECs and inhibited tube formation of HUVECs as well as reduced diameter of peripheral blood vessels in Chicken chorioallantoic membrane assay (CMA). CM containing Slit2-E15 inhibited CM-induced permeability and restored tubes quality and diameter of blood vessels whereas CM containing Slit2-WT only reduced CM-induced

HUVECs permeability but had no effect on tubes and vessels quality. We also observed that higher glucose level and fibroblast growth factor increased the expression of Slit2-WT while gallic acid reduced the expression of Slit2-WT. Besides, both lung inflammation induced by LPS and lung metastasis via tail vein injection of CL1-5 decreased the expression of Slit2-WT. In order to monitor Slit2 splicing, we constructed Slit2-reporter constructs. Interestingly, Slit2-E15 is the predominant form expressed in the transient transfected H661 cells, suggesting that Slit2-E15 is the bona fide splicing form while inclusion of exon 15 in Slit2-WT requires additional regulation. Since Slit2-E15 form is greatly repressed while Slit2-WT is highly expressed in tumor microenvironment, it would be an attractive therapeutic strategy if the Slit2-Exon 15 splicing event can be shifted from Slit2-WT to Slit2-E15, which would in turn inhibit growth, invasion of tumor cells as well as normalize blood vessels for enhancing efficacy of chemotherapy and radiotherapy.

英文關鍵詞：Slit2, HUVECs, endothelial permeability, tube formation, angiogenesis, tumor microenvironment.

附件一

科技部補助專題研究計畫成果報告

（□期中進度報告/■期末報告）

Gallic acid 對腫瘤微環境中 Slit2-Exon15 剪接變異型表現的影響

計畫類別：■個別型計畫　□整合型計畫
計畫編號：MOST 102－2320－B－040－010－MY2
執行期間：2013 年 08 月 01 日至 2013 年 07 月 31 日

執行機構及系所：中山醫學大學 醫學研究所

計畫主持人：蔡菁華
共同主持人：
計畫參與人員：孫仕容、廖振毅、王秀羽、陳威廷、吳祥維、呂凱迪

本計畫除繳交成果報告外，另含下列出國報告，共 ___ 份：
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□出席國際學術會議心得報告

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中　華　民　國 104 年 09 月 15 日

中文摘要

Slit2 蛋白在腫瘤的發展、血管新生、免疫反應上扮演重要的角色。我們發現 Slit2 在 exon15 的外顯子位置有兩個剪接變異型的表現: Slit2-WT(具有 exon15)及 Slit2-ΔE15 (沒有 exon15)。肺癌組織的 Slit2 表現遠低於周圍正常組織，有趣的是肺癌病人之正常肺組織表現高度的 Slit2-WT，而 Slit2-ΔE15 的表現非常低或不表現。若將 Slit2-ΔE15 過度表現於 CL1-5 肺癌細胞時，可以有效的抑制癌細胞的生長及轉移，但過度表現 Slit2-WT 則只能抑制細胞的轉移但對於生長沒有抑制的能力。除此之外，我們發現肺癌細胞的條件培養液會增加臍帶內皮細胞(HUVECs)的通透性及降低管柱的完整性，並使得雞胚胎表面的血管直徑變細。但是如果條件培養含有 Slit2-ΔE15 時，則可以抑制肺癌細胞之條件配養液所誘導的 HUVECs 通透性及管柱完整性，並使得雞胚胎表面的血管直徑變正常。而條件培養含有 Slit2-WT 時只能抑制癌細胞之條件配養液所誘導的 HUVECs 通透性，但對於管柱的完整性及新生血管直徑則沒有影響。我們也發現環境中葡萄糖的濃度、FGF 會增加 Slit2-WT 的表現，而 gallic acid 則會降低 Slit2-WT 的表現。除此之外，當動物以 LPS 誘導肺發炎及以尾靜脈注射肺癌細胞產生肺轉移時，Slit2-WT 的表現也會下降。由我們所建構的 Slit2-splicing reporter constructs 發現，在短暫轉染的情況下，Slit2-ΔE15 是主要表現的剪接變異型，而 Slit2-WT 則表現非常低。綜合我們的研究，在沒有調控的情形下主要表現的是 Slit2-ΔE15 剪接變異型，而 Exon 15 則需要調控因子的幫助才能保留下來產生 Slit2-WT。由於肺癌病人的肺組織之 Slit2-ΔE15 表現非常低，若能找到抑制 Slit2-WT 剪接變異型的因子，而提高 Slit2-ΔE15 的表現型，將有助於讓腫瘤微環境中的 Slit2-ΔE15 的表現量增加，進而抑制肺癌細胞的生長，並讓腫瘤所誘導的新生血管正常化，以增加化學治療及放射治療的療效。

關鍵詞: Slit2、剪接變異型、HUVECs、內皮細胞通透性、體外管柱形成、血管新生、腫瘤微環境

Abstract

Slit2 plays an important role in tumor development, angiogenesis, and immune response. We have identified alternative splicing at exon 15 of Slit2, Slit2-WT (presence of exon 15) and Slit2-ΔE15 (absence of exon 15). The expression of Slit2 is highly reduced in lung tumor when compared with their normal lung counterpart. However, Slit2-WT is the predominant splicing form with little or undetectable Slit2-ΔE15 form in lung cancer specimen. Ectopic expression of Slit2-ΔE15 inhibited growth and invasion of CL1-5 lung cancer cells while Slit2-WT repressed growth capability only. In addition, the conditioned medium (CM) of lung cancer cell enhanced permeability of HUVECs and inhibited tube formation of HUVECs as well as reduced diameter of peripheral blood vessels in Chicken chorioallantoic membrane assay (CMA). CM containing Slit2-ΔE15 inhibited CM-induced permeability and restored tubes quality and diameter of blood vessels whereas CM containing Slit2-WT only reduced CM-induced HUVECs permeability but had no effect on tubes and vessels quality. We also observed that higher glucose level and fibroblast growth factor increased the expression of Slit2-WT while gallic acid reduced the expression of Slit2-WT. Besides, both lung inflammation induced by LPS and lung metastasis via tail vein injection of CL1-5 decreased the expression of Slit2-WT. In order to monitor Slit2 splicing, we constructed Slit2-reporter constructs. Interestingly, Slit2-ΔE15 is the predominant form expressed in the transient transfected H661 cells, suggesting that Slit2-ΔE15 is the bona fide splicing form while inclusion of exon 15 in Slit2-WT requires additional regulation. Since Slit2-ΔE15 form is greatly repressed while Slit2-WT is highly expressed in tumor microenvironment, it would be an attractive therapeutic strategy if the Slit2-Exon 15 splicing event can be shifted from Slit2-WT to Slit2-ΔE15, which would in turn inhibit growth, invasion of tumor cells as well as normalize blood vessels for enhancing efficacy of chemotherapy and radiotherapy.

Keywords: Slit2, HUVECs, endothelial permeability, tube formation, angiogenesis, tumor microenvironment.

前言：

Lung cancer is the most common cause of cancer death worldwide. In the process of screening genes involved in lung tumorigenesis, slit2 was identified by suppressive subtractive hybridization. Slit is a secreted glycoprotein that was first identified in the midline of glia cells in drosophila brain. It acts as a repellent cue for axon migration in both vertebrate and invertebrate via Robo receptors. Recently, Slit2 has been shown to function as a tumor suppressor gene. We have identified two alternative splicing forms of Slit2, Slit2-WT and Slit2-ΔE15. Slit2-ΔE15 inhibits both growth and invasion of lung cancer cells while Slit2-WT possesses invasion inhibitory capability only. Slit2-WT is expressed predominantly in normal lung counterpart of lung cancer specimen, while Slit2-ΔE15 is expressed at higher ratio in non-tumor lung specimen, normal murine lung tissue and transformed normal bronchial epithelial cells. These observations strongly suggested that alternative splicing of Slit2 may be occurred during tumorigenesis. Interestingly, we found that gallic acid enhanced expression of Slit2-ΔE15 splicing form, suggesting that some natural compounds may protect tumorigenesis, in part by modulating Slit2 alternative splicing.

研究目的：

Increasing evidences demonstrate that alternative splicing regulates many aspects in cancer development. We identified alternative splicing forms of Slit2. Slit2-ΔE15 (absence of exon 15) inhibits both growth and invasion of lung cancer cells while Slit2-WT possessed invasion inhibition activity only. Our observation suggested that expression of Slit2 is switched from high ratio of Slit2-ΔE15 toward high ratio of Slit2-WT in the tumor microenvironment thus enabling cancer cells to evade growth suppression. To prove the hypothesis, splicing reporter constructs were constructed to monitor alternative splicing of Slit2. These constructs not only allow us to study mechanism of Slit2 alternative splicing but also be used to screen nature compounds for restoring Slit2-ΔE15 expression in lung. Restoration of Slit2-ΔE15 expression would be able to block growth, invasion of cancer cells, as well as normalization of blood vessel. This study aims to construct splicing reporter construct that allow us to identify factors in the tumor microenvironment and environmental factors that modulate Slit2-Exon 15 splicing. If imbalance expression of Slit2-WT and Slit2-ΔE15 splicing variants is a significant pathologic factor in lung cancer microenvironment, restoring Slit2-ΔE15 expression via natural compounds may be an attractive strategy to inhibit tumor progression by normalizing tumor microenvironment.

文獻探討：

Slit is a secreted glycoprotein that was first identified in the midline of glia cells in drosophila brain. It acts as a repellent cue for axon migration in both vertebrate and invertebrate via Robo receptors (Brose et al., 1999; Kidd et al., 1999; Li et al., 1999). In vertebrates, three Slit homologs were identified with similar structure organization containing four leucine rich repeats (LRRs) followed by seven to nine EGF repeats and a laminin G domain, ending with a C-terminal cyctein-rich knot (Brose et al., 1999; Rothberg and Artavanis-Tsakonas, 1992; Rothberg et al., 1990) (Figure 1). Four Robo receptors have been identified in mammal, which serve to transduce Slit2 signal. All of these receptors exhibit similar ectdodomain structure, consisting of five immunoglobulin-like (IG) domains followed by three fibronetin type 3 (FN3) repeats, except for Robo4 which contains only two IG domains and two FN3 repeats (Sabatier et al., 2004; Sundaresan et al., 1998). The intracellular domains of Robo receptors are mostly unstructured and poorly conserved. However, there are two to four cytoplasmic conserved motifs (CC0-3) that may be associated with a variety of

adaptor proteins (Dickson and Gilestro, 2006). Slit2/Robo1 signaling requires the second LRR (LRR2) domain to interact with the first and second IG domain of Robo1 (Howitt et al., 2004; Liu et al., 2004; Morlot et al., 2007).

In addition to the role in axon guidance and neuronal migration (Wu et al., 1999), Slit2 also inhibits leukocyte chemotaxis (Wu et al., 2001) and plays roles in angiogenesis (Wang et al., 2003). Interestingly, promoter hypermethylation of slit2 gene or deletion of chromosome 4 (location of Slit2) has been found in many cancers, including lung, breast, liver, colorectal, cervical cancers, and glioma, as well as leukemia, implicating that Slit2 is involved in the neoplastic process (Dallol et al., 2002; Dallol et al., 2003a; Dallol et al., 2003b; Dunwell et al., 2009; Singh et al., 2007). Evidence from previous studies has demonstrated that Slit2 has the ability to repress growth, migration and invasion of tumor cells (Dallol et al., 2002; Kim et al., 2008; Mertsch et al., 2008; Werbowetski-Ogilvie et al., 2006; Yiin et al., 2009). Recently, down regulation of Slit2 has been shown to associate with poor prognosis in lung cancer through deregulation of β-catenin and E-cadherin (Tseng et al., 2010). Although Slit2/Robo1 is widely accepted as a suppressing signal of invasion, this signal plays a role in inducing brain metastasis of breast cancer cells (Schmid et al., 2007). Moreover, Slit2/Robo1 has also been shown to enhance angiogenesis and to support tumor growth in melanoma (Wang et al., 2003). Thus, the role of Slit2/Robo1 signaling in tumor progression or metastasis may dependent on the origin of the cancer. Moreover, Slit2 also modulates immune responses. Slit2 has been shown to inhibit migration of neutrophil, lymphocytes, and dendritic cells in inflammatory responses indicating that it may play an important role in anti-inflammation (Altay et al., 2007; Guan et al., 2003; Tole et al., 2009; Wu et al., 2001).

Although Slit2 functions as a tumor suppressor in most of the cases, relative few studies pursued the tumor suppression role of Slit2 from tumor microenvironment point of view. Tumor microenvironment composes of tumor associated fibroblasts, endothelium, and various infiltrated immune cells. Secretary factors secreted by cells in the microenvironment play critical role in regulating tumor growth. Breast tumors secret CXCL1 to recruit endothelial cells to tumor microenvironment and recruited endothelial cells in turn secrete S100A8/9 to enhance tumor growth (Acharyya et al., 2012). It has been shown that EphA2 represses Slit2 expression in endothelium and expression of EphA2 is negatively correlated with Slit2 level in human ductal carcinoma. High level of Slit2 suppresses angiocrine factors-induced tumor proliferation and motility (Brantley-Sieders et al., 2011). Fibroblasts are the major cell type in tumor microenvironment, which also express Slit2. It has been shown that fibroblasts have different effect on the growth of breast cancer cells. Co-culture fibroblasts with breast cancer cells either enhances or represses growth of cancer cells dependent on Robo1 expression in cancer cells. Activation of Slit2/Robo1 pathway sufficient to repress fibroblast-induced cancer growth (Chang et al., 2012).

In addition to antitumor role, Slit/Robo signaling plays both anti- and pro-angiogenic roles in angiogenesis. However, how Slit2/Robo affects angiogenesis is still unclear. Robo4 is expressed in all endothelial cells, while Robo1 is expressed quite diversely among different endothelial cells [32,33]. Slit2 binds to Robo1 monomer or Robo1/Robo4 heterodimer promotes endothelial mobility [33], while Slit2/Robo4 signaling counteracts VEGF-induced angiogenesis and maintains vascular stabilization [20], [25],[26]. A recent genetic study has clearly shown that Slit2 is involved in Robo4–mediated down-regulation of VEGF angiogenesis in breast [39]. Although Slit2 has been shown to stabilize blood vessels through Robo4 receptor [25,26], it is not known whether various Slit2 Exon 15 splicing forms differentially affect vessel

stability.

研究方法：

Constructing Slit2-alternative splicing reporter constructs & analysis of the alternative splicing forms:
Slit2 genomic DNA from exon 14 to exon 16 was PCR amplified and cloned into T/A vector for generating insertional mutations at the beginning and the end of the exon 15. The mutated Slit2 genomic DNA was subcloned into pcDNA which has been inserted with F-luciferase or R-luciferase as splicing reporters. Each of the final reporter construct was then transfected into H661 lung cancer cell transiently or for selection of stable clones.

Animal studies:
Balb/c nude mice were injected with CL1-5 lung cancer cells (1x 10^6 cells/100 μl PBS) or PBS via tail vein injection twice. The second injection of lung cancer was performed one month after the first injection. Mice were sacrificed 107 days after first injection. Lung and Brain tissues were removed for RNA extraction, cDNA synthesis and Slit2 splicing form analysis. For Lipopolysaccharides (LPS) study, Mice were treated with 10 μg LPS/20 μl PBS/day via transnasal instilling for three days and were sacrificed at the fourth day. Lung tissue was removed for RNA extraction, cDNA synthesis and Slit2 splicing form analysis.

Matrigel tube formation assay:
HUVECs were pretreated with/without conditioned medium (CM) (with 10% FBS) for 24 h. Ibidi u-slide plates (Applied Biophysics, NY, USA) were coated with 10 μl Matrigel (10 mg/ml) and incubated at 37℃ for 1 hr. The pretreated HUVECs were suspended in fresh CM (with 10% FBS and ECGS) and plated onto a layer of Matrigel at a density of 5.3 x 10^3 cells/well. The plates were then incubated at 37℃ for an additional 6 hr and capillary-like tube formation was observed under microscope. For Slit2 depletion, 0.25 μg/ml of anti-IgG (A9044, Sigma, St Louis, USA) and anti-Myc (05-419, Millipore, CA, USA) were used to incubate CM/Slit2-ΔE15 at 4℃ for 16 hr followed by protein G precipitation. After centrifugation, the CM was used for tube formation assay. For blocking Robo receptors, 0.25 μg/ml of anti-IgG, anti-Robo1 (ab7279, abcam, UK) and/or anti-Robo4 (ab10547, abcam, UK) were incubated with HUVECs at 37℃ for 16 hr followed by CM treatment for 24h prior to tube formation assay. For RNAi, 50 pmol of si-Robo1 (5'-GCAGACACGUGGCCUAAUATT-3') , 100 pmol of si-Robo4 (5'-GCUUCUGGCUGUGCGAAUUTT-3') and si-NC (5'-UUCUCCGAACGUGUCACGUTT-3') were transfected into HUVECs and incubated at 37℃ for 16 hr, respectively. Cells were then treated with CM of Slit2-ΔE15 for 24 hr prior to tube formation assay.

Chicken chorioallantoic membrane assay:
Fertilized chicken eggs were transferred to an egg incubator maintained at 37℃ and 50% humidity for 8 days. To separate the chicken chorioallantoic membrane (CAM) from the shell membrane, two small holes were drilled into the shell, one at the blunt end of the egg where the air sac is located and the other at 90° halfway down the length of the egg. Gentle suction was applied at the hole located at the blunt end of the egg to create a false air sac directly over the CAM. Then, a 1 cm^2 window was removed from the eggshell immediately over the second hole. Since Slit2 is a 200 KDa protein, holes were made in the CAM to facilitate penetration of Slit2. CM of CL1-5/VC, CL1-5/Slit2-WT or CL1-5/Slit2-ΔE15 or RPMI (control group) was placed on the CAM and the embryos were incubated for an additional 48 hr. The neovascular zones under the disks were

photographed and the ratios of the diameters of the peripheral blood vessels close to the CAM relative to those far below the CAM were determined by Wimasis software (Wimasis GmbH, Munich, Germany).

Permeability assay:

Coating of 1.0 μm Transwell membrane (PIHT 30R 48; Millipore, MA, USA) was carried out with 0.1% gelatin at 37 ^{o}C for 1h. Aspirated gelatin solution from the membrane and 4 x 10^4 HUVECs in 200 μl of M199 with 10% FBS were added to the upper chamber. The same medium was added to the lower chamber until the surface of the medium was the same as in the upper chamber, with incubation at 37°C, 5% CO_2 for 4 days until cells formed a tight monolayer. The insert of the Transwell membrane (upper chamber) was moved to a new well and the cells were treated with/without CM (1% FBS), followed by incubation at 37°C, 5% CO_2 for 16h. The next day, the medium in the lower chamber was replaced with new CM with 100 pg/ml of VEGF165 for 3.5h. Then, the medium in the upper chamber was replaced with 100 μl of new medium containing 10 μg/ml of HRP followed by incubation for 1h. The lower chamber medium was moved to a new tube and mixed well. Then, 10 μl of the medium were diluted to 50-fold and treated with TMB for 15 min. The reaction was terminated with 50 μl of 2N H_2SO_4. The permeability of HRP in HUVECs was then determined at 450 nm absorbance. Standard curve of HRP was established with 1, 2, 4, 8, 16 and 32 μl of 100 ng/ml HRP in a volume of 50 μl. For anti-Robo4 treatment, HUVECs were treated with 0.25 μg/ml of anti-IgG or anti-Robo4 after tight monolayer was formed, followed by CM for 24 hr prior to permeability assay.

Statistical analysis:

All HUVEC assays were carried out in at least three independent experiments with three repeats for each experiment. Values were presented as mean ± SD. Statistical analysis was performed using the SPSS statistical software program (version 13, SPSS, Inc.). One-way analysis of variance (ANOVA) was used to analyze the significance among groups followed by Scheffé test for comparisons between two groups. $P < 0.05$ was considered statistically significant.

結果:

Generation of Slit2 reporter constructs.

We have successfully generated Slit2 reporter constructs. Slit2/F-Luc is used to monitor Slit2-WT form and Slit2/R-Luc is for monitoring Slit2-ΔE15 form (Figure 1). After transient transfection, these two genomic constructs were expressed and spliced. However, only the Slit2-ΔE15 form was expressed. We suspected that one base insertion at the beginning or the end of exon 15 may affect the retention of Exon 15. Therefore, we reconstructed a wildtype

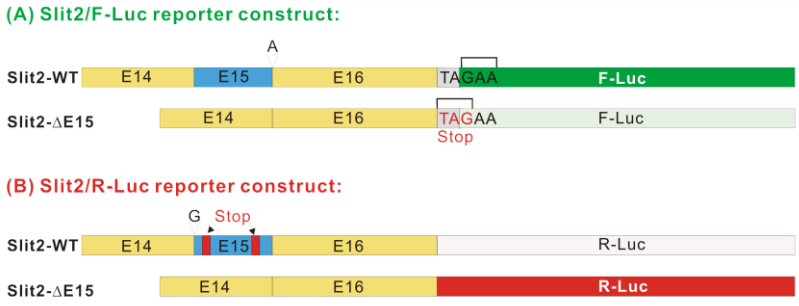

Figure 1. Slit2 splicing reporter constructs. (A) SLit2/F-Luc is used to detect Slit2-WT form by assay F-luc activity. (B) Slit2/R-Luc is used to detect Slit2-DE15 by assay R-Luc activity.

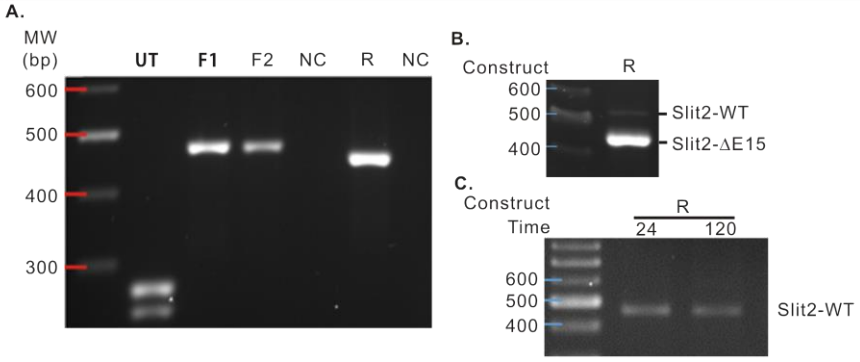

Figure 2. The expression of Slit2-splicing forms in splicing reporter construct. (A) Only Slit2-ΔE15 form was expressed in both Slit2/F-luc and Slit2/R-luc constructs. (B) Slit2-WT was able expressed in wild-type Exon15. (C) Slit2-WT was detected by Exon 15 specific primer.

Exon 15 in the reporter construct. Finally, the WT splicing form was expressed but the level was much lower than Δ15 splicing form in transient transfection (**Figure 2**). One possibility is that chromatin structure may play an important role in Slit2-Exon 15 splicing. These results suggested that Slit2-ΔE15 splicing form is a default splicing process. Thus, it is very important to identify the mechanism involved in high level of Slit2-WT splicing form expression in lung cancer patients.

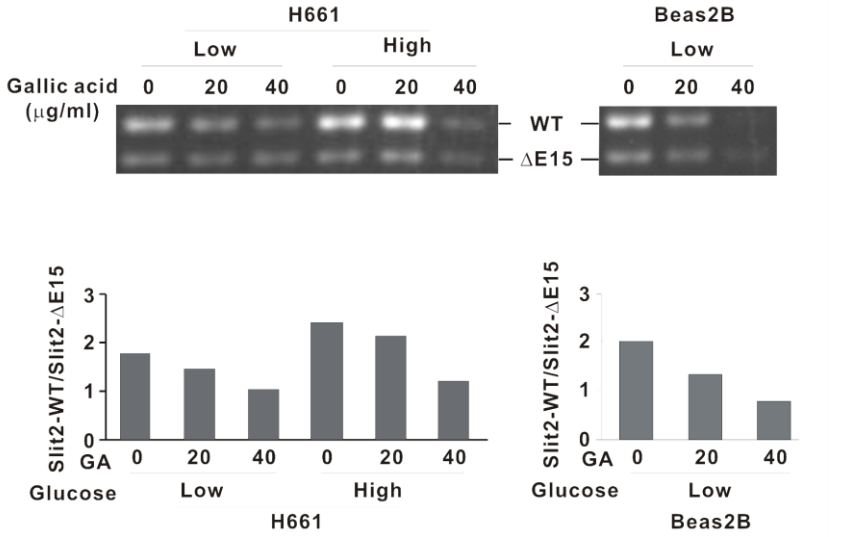

Figure 3. The effect of glucose and GA in the expression of Slit2 splicing forms. H661 cells were incubated with General (Low, 11.1 mM) or high glucose (36.1mM) RPMI and treated with different concentration of gallic acid. Beas2B cells were only treated with GA in Low glucose medium.

The Effect of Glucose, GA, FGF and $NiCl_2$ on Slit2-Exon 15 splicing.

It has been reported that Slit-Robo signaling is required for pancreatic beta-cell survival and function of beta-cell to secret insulin (PNAS, 2013, 110:16480). We thus investigated whether glucose concentration affect the expression patterns of Slit2. We found that incubation H661 in high glucose medium enhanced the expression of Slit2-WT when compared with low glucose medium (Figure 3). When these cells were treated with gallic acid (GA), the Slit2-WT was preferentially repressed (Figure 3). Similar effect of GA was observed in Beas 2B cells (Figure 3). In $NiCl_2$ treatment, Slit2-WT expression was reduced in H661 but was enhanced in Beas 2B cells (Figure 4). Whereas, FGF greatly induced Slit2-WT expression in MRC5 and lower extent in Beas2B cells (Figure 5).

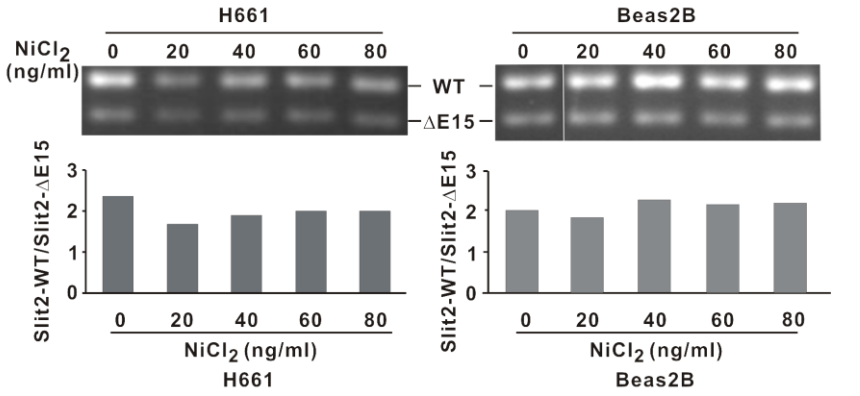

Figure 4. NiCl2 treatment in H661 and Beas2B cells. NiCl2 reduced the expression of Slit2-WT splicing form while enhanced the expression of Slit2-WT form in Beas2B.

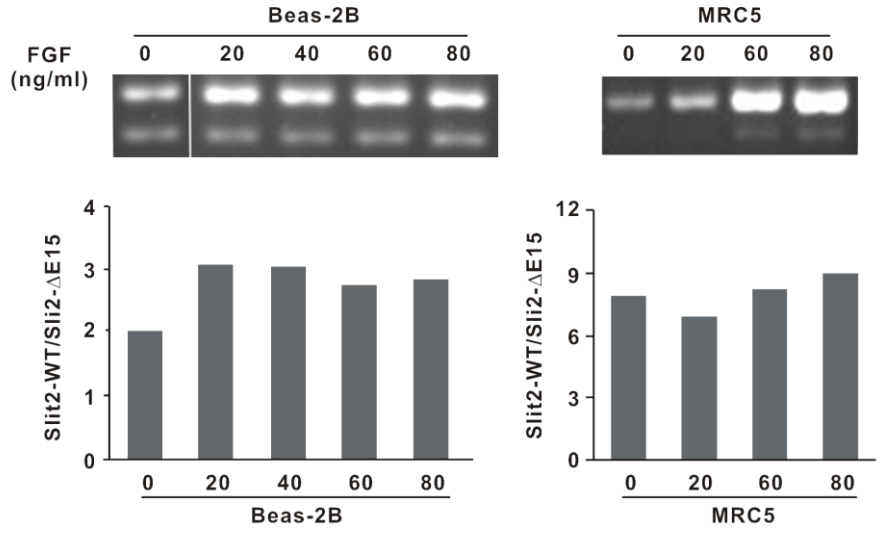

Figure 5. The effect of FGF on the expression of Slit2 splicing forms. FGF enhanced Slit2-WT expression in both Beas-2B and MRC5 cells.

The Effect of LPS induced lung inflammation on Slit2-Exon15 splicing.

To examine whether inflammation would alter splicing of Exon 15 in Slit2, we inject LPS (10 μg/100 μl) nasally to wildtype mice for three consecutive days and sacrificed the mice at the fourth day

for analysis. Interestingly, LPS greatly induced overall Slit2 expression as well as Slit2-ΔE15 splicing form expression (**Figure 6**). This result suggested that Slit2-ΔE15 may be differentially up-regulated by lung inflammation.

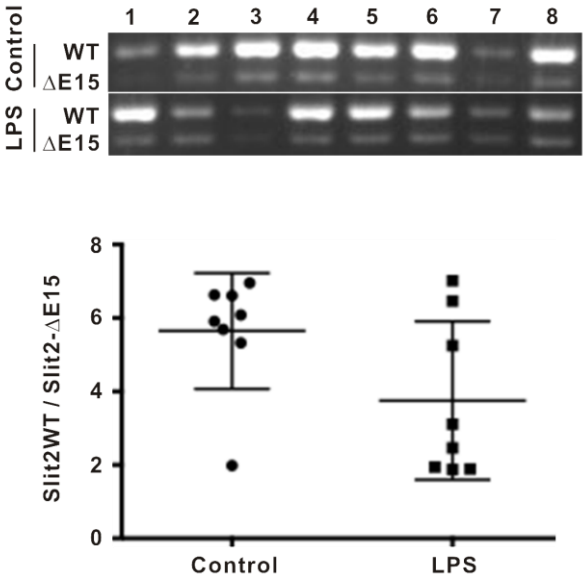

Figure 6. Slit2 splicing patterns in LPS induced lung and brain. LPS repressed the expression of Slit2-WT.

The Effect of lung metastasis on Slit2-Exon15 splicing.

We have tried intratracheal injection of Lewis lung cancer cells to C57BL/6 mice to examined whether growth of tumor in chest would affect Slit2-Exon15 splicing pattern or not. However, up to five weeks, none of the mice developed obvious lung tumor and no significant difference in the expression of Slit2 splicing form between PBS and Lewis lung cancer cells injected group. Perhaps, C57BL/6 mice have effective clearance of the cancer cells. So, we repeated the experiment with tail vein injection of human CL1-5 lung cancer cells into Balb/c nude mice. The result showed that Slit2-WT splicing form was greatly repressed in mice with CL1-5 induced lung metastasis (Figure 7).

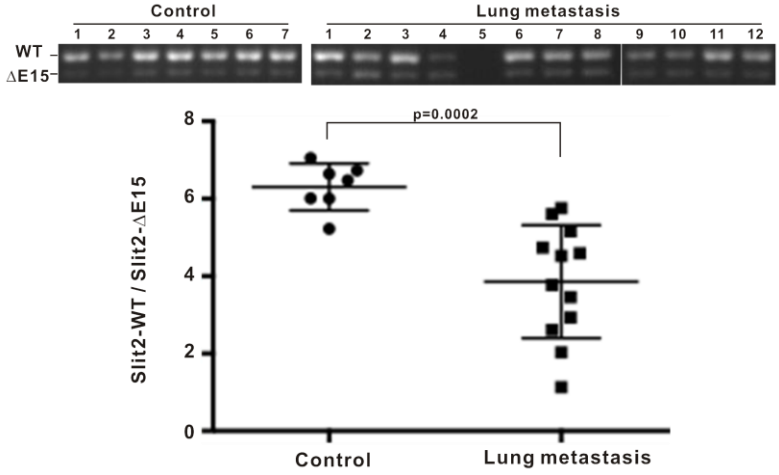

Figure 7. Slit2 splicing pattern in CL1-5 induced-lung metastasis. Tail vein injection of CL1-5 lung cancer induced lung metastasis. The expression of Slit2-WT was greatly reduced in metastasized lung thus significantly reduced SLit2-WT/Slit2-ΔE15 ratio.

The effects of Slit2 splicing forms on permeability of HUVECs.

Slit2-Robo4 signaling promotes vascular stability and impedes pathological angiogenesis by inhibiting endothelial hyper-permeability (Jones, 2008; 2009). It has not been observed if Slit2-Exon15 splicing forms have different effects on the permeability of HUVECs. Monolayered HUVECs were incubated with conditioned medium (CM) containing different Slit2 splicing forms for 16h with permeability induced by $VEGF_{165}$ for 3.5h. The permeability was determined by the diffusion of HRP from upper chamber to lower chamber on Transwell membrane. The results showed that CM of both CL1-5/Slit2-WT and CL1-5/Slit2-ΔE15 reduce cell permeability of HUVECs when compared with CM of CL1-5/VC. However, the reduction in cell permeability was more significant with Slit2-ΔE15 (Figure 8).

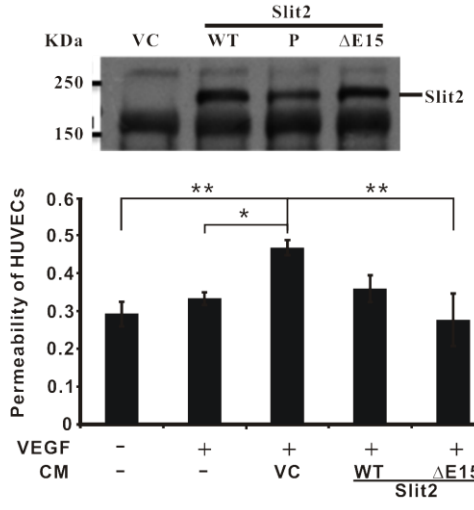

Figure 8. The effect of Slit2 splicing forms on permeability in HUVECs.

The effects of Slit2-WT and Slit2-ΔE15 on tube formation by HUVECs and angiogenesis on chicken chorioallantoic membrane (CAM) assay.

Next, we examined whether Slit2 splicing forms differentially affect tube formation by HUVECs, HUVECs were pretreated with/without CM from CL1-5/Slit2-WT, CL1-5/Slit-ΔE15 or CL1-5/VC prior to assay. Without CM, HUVECs formed high quality tubes within 6h. Although the speed of tube formation of HUVECs treated with VC CM was similar to that of untreated cells, the tubes were thinner and highly disconnected following treatment with CM collected from CL1-5/VC (Figure 9A & B). Similar to VC CM, CL1-5/Slit2-WT CM negatively affected tube formation by HUVECs. Interestingly, when pretreated with CL1-5/Slit2-ΔE15 CM, HUVECs formed thicker tubes when compared with untreated cells (Figure 9A & B). These results suggested that Slit2-ΔE15 enhances the quality of tube formation by HUVECs while Slit2-WT does not. Next, we used chicken embryos to examine the effect of CM containing various Slit2 Exon15 splicing forms on angiogenesis via CAM assay. Similar to the results of tube formation assay, CM of CL1-5/VC greatly reduced the diameter of peripheral vessels when compared with the control group (RPMI medium). CM of CL1-5/Slit2-ΔE15 restored the diameter of peripheral vessels while CM of CL1-5/Slit2-WT did not (Figure 9C & D). These results suggested that the function of Slit2-ΔE15 in angiogenesis is conserved between humans and chickens. To demonstrate that Slit2-ΔE15, not Slit2-ΔE15-induced content in CM, is responsible for restoring tube quality, Slit2-ΔE15 was depleted in CM prior to tube formation assay. Slit2-ΔE15-depleted CM resulted in poor quality tubes in HUVECs when compared with anti-IgG treated CM (Figure 9E), indicating that Slit2-ΔE15 directly enhances tube quality.

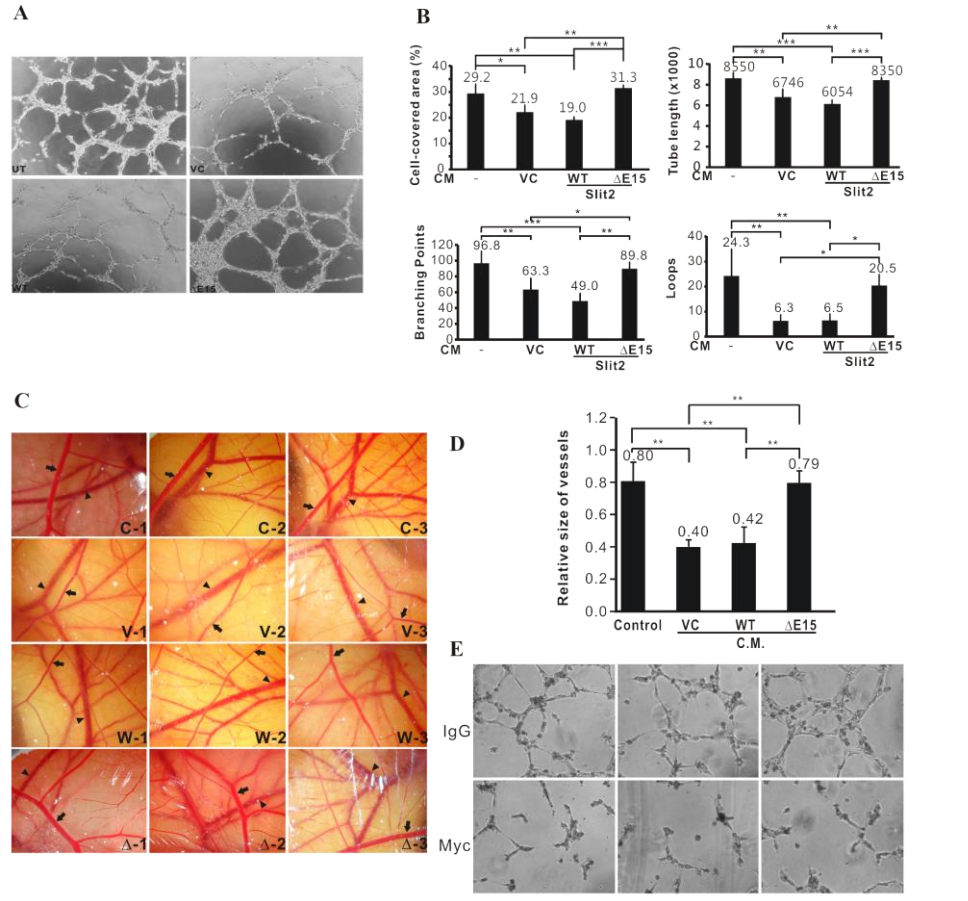

Figure 9. The effect of Slit2 splicing form on tube formation and vessel size in CAM assay. A. HUVECs were treated with CM with/without Slit2-WT or Slit2-ΔE15 and were subjected to tube formation analysis. B. Tube quality was determined by tube cover area, tube length, branch point and loops. C & D. CAM assay showed that CM of VC and Slit2-WT reduced the diameter of peripheral blood vessel (arrow) while CM of Slit-ΔE15 restore the diameter of blood vessel. Arrow head refers to deep vessels.

Robo4 participated in Slit2 mediated permeability inhibition however neither Robo1 nor Robo4 is involved in Slit2-ΔE15-mediated tube formation.

It has been shown that Slit2/Robo4 pathway inhibits VEGF-induced permeability. It is important to determine whether Robo4 is also involved in Slit2-ΔE15-mediated reduction in permeability. When the function of Robo4 was blocked by anti-Robo4, the permeability of HUVECs increased following treatment with CM of CL1-5/Slit2-WT or CM of CL1-5/Slit2-ΔE15 in comparison with IgG control (Figure 10A & B). Neutralization of Robo1 did not increase permeability of HUVECs. Similar results were obtained when the expression of Robo4 was interfered with by si-Robo4 (Figure 10C & D), suggesting that Robo4 but not Robo1 is required for Slit2-ΔE15-mediated inhibition of permeability. To explore the roles of Robo1 and Robo4 in Slit2-ΔE15-mediated tube formation, we neutralized Robo1 or Robo4 in HUVECs, followed by treatment with CM of CL1-5/Slit2-ΔE15. In comparison with anti-IgG treatment, blocking of Robo1 or Robo4 alone did not reduce tube formation of HUVECs treated with CM of Slit2-ΔE15. It is possible that Robo1 and Robo4 compensate for each other in Slit2-ΔE15-mediated tube formation or that these antibodies are unable to block the function of Robo receptor in angiogenesis. Double blocking with anti-Robo1 and anti-Robo4 did not affect Slit2-ΔE15-mediated tube formation (Figure 11A). Similar results were obtained in cells with si-Robo1 or si-Robo4 single knockdown and si-Robo1/si-Robo4 double knockdown (Figure 11B). These results are important because they point to the possibility of yet to be elucidated Slit2 signaling pathway(s) in angiogenesis.

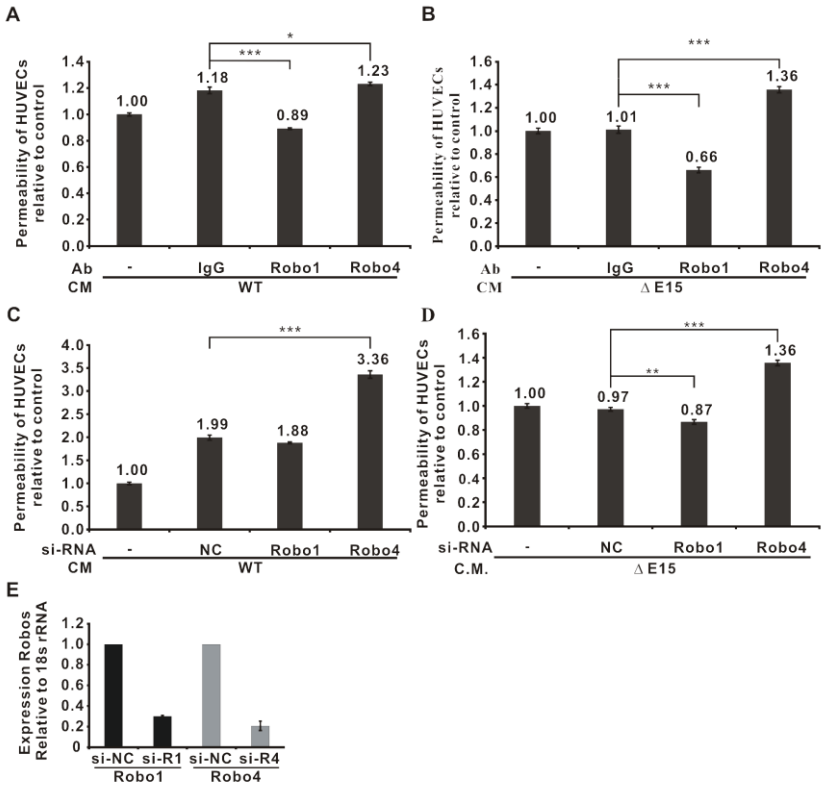

Figure 10. The role of Robo1 and Robo4 in Slit2-splicing form mediated permeability inhibition in HUVECs. (A & B) Antibody neutralization assay revealed that Robo4 but not Robo1 is involved in both Slit2-WT and Slit2-ΔE15 mediated permeability inhibition in HUVECs. (C & D) Similar results were obtained when Robos were knockdown by siRNAs. (E) The effectiveness of RNAi.

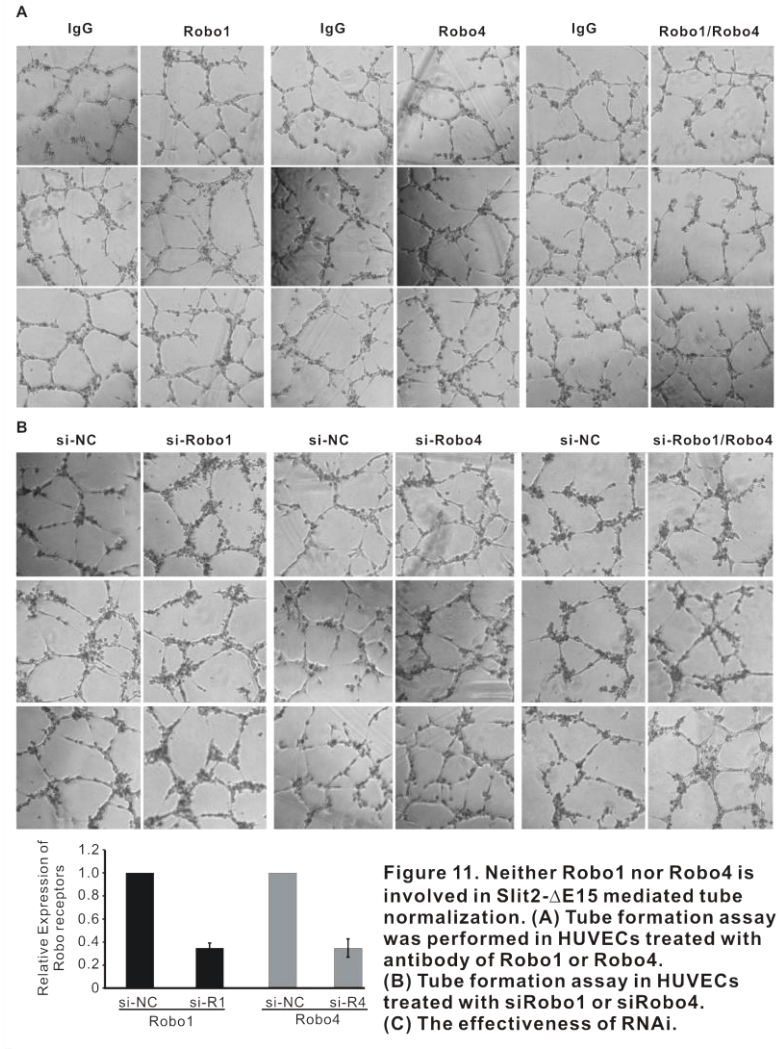

Figure 11. Neither Robo1 nor Robo4 is involved in Slit2-ΔE15 mediated tube normalization. (A) Tube formation assay was performed in HUVECs treated with antibody of Robo1 or Robo4. (B) Tube formation assay in HUVECs treated with siRobo1 or siRobo4. (C) The effectiveness of RNAi.

結論、討論與建議:

Exon15 of Slit2 contains 8 amino acids which can be alternative spliced into Slit2-WT and Slit2-ΔE15. We have observed that Slit2-WT is the predominant form expressed in tumor microenvironment in lung cancer, while Slit2-ΔE15 can be expressed at higher level in pneumothorax patients. Thus, this study aims to identify whether Slit2-Exon15 splicing can be regulated by process of tumorigenesis, inflammation and other factors. Our results showed that the expression of Slit2 was down-regulated when acute inflammation in lung was induced by LPS. In addition, the ratio of Slit2-WT relative to Slit2-ΔE15 was decreased. On the other hand, when lung metastasis was induced by injection of CL1-5 cells in Balb/c nude mice, the ratio of Slit2-WT relative Slit2-ΔE15 was also decreased. These results revealed that LPS and lung metastasis induced lung inflammation and is correlated with lower expression of Slit2 and higher Slit2-WT/Slit2-E15 ratio. It has been reported that Slit2 methylation is positively correlated with inflammation status in inflammatory bowel disease (World J gastronenterol, 2014, 20:10591). Slit2 also function as anti-inflammatory factor in human placenta (Am J reprod Immunol, 2015, 73:66-78). This effect may due to the role of Slit2 in preventing chemoattraction of leukocyte and anti-platelet function (Pediatr Nephrol, 2015, 30:561). Thus, induction of Slit2 expression could effectively inhibit inflammation.

Our results suggested that high glucose enhances overall Slit2 expression with higher level of Slit2-WT splicing form. Gallic acid reduced the overall expression level of Slit2 preferentially inhibition of Slit2-WT. FGF enhances overall Slit2 expression preferentially the Slit2-WT form. Thus, different factors may regulate the overall expression of Slit2 however it appears that the Slit2-WT form is the predominant form to be regulated. Our splicing reporter constructs suggested that Slit2-ΔE15 is a predominant splicing form in transient transfected condition. It is possible that Slit2-WT form splicing may be regulated at chromatin level during transcription. It has been demonstrated that histone modification status may recruit different adaptor proteins that could attract different splicing factors to regulate splicing event (Cell, 2011, 144:16). Thus, it is possible that Exon 15 is a weak exon to be included in mRNA and requires splicing regulatory protein(s) to activate the inclusion splicing. Binding of splicing regulatory protein(s) to Slit2 mRNA may be regulated by the expression level of the regulatory protein(s) or the epigenetic modification of the chromatin.

Since Slit2-WT is highly expressed in non-tumor lung of lung cancer patients, and since Slit2 has been demonstrated to reduce VEGF-induced permeability in HUVECs, we wondered how different Slit2 splicing forms affect angiogenesis. It is known that blood vessels are abnormal around tumor with characteristic of increasing permeability and abnormal structures (Science, 2005, 307:58; Physiol Rev, 2011, 91:1071). We also observed that CM of lung cancer cells inhibited tube formation in HUVECs and reduced diameter of blood vessels in CAM assay. CM collected from Slit2-ΔE15, but not Slit2-WT, overexpressing cells also restored tube quality and diameter of blood vessels. Although both Slit2-WT and Slit2-ΔE15 inhibited lung cancer CM-induced permeability, Slit2-ΔE15 has better effect. Thus, high level of expression of Slit2-WT in lung cancer not only lost the ability to inhibit growth of tumor cells but also lost the ability to maintain the integrity of blood vessel.

It is interesting to note that there are distinct Slit2-Exon 15 splicing expression patterns between brain and lung in mice. As shown in figure 12 the ratio of Slit2-WT relative Slit2-ΔE15 is lower in brain than in lung. Higher level of Slit2-ΔE15 level in brain may be important to maintain endothelial integrity and that may play an important role in blood-brain barrier. Thus, the balance between Slit2-WT and Slit2-ΔE15 splicing forms may be regulated in normal physiological and pathological conditions.

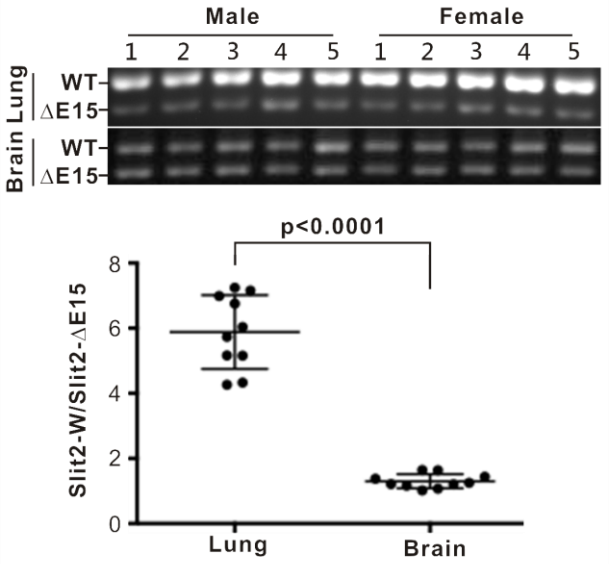

Figure 12. Slit2-Exon 15 splicing patterns in brain & lung. Lung expressed higher level of Slit2-WT splicing form while the expression of Slit2-WT and Slit2-ΔE15 forms are equivalent in brain.

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附件二

科技部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（簡要敘述成果是否有嚴重損及公共利益之發現）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），如已有嚴重損及公共利益之發現，請簡述可能損及之相關程度（以 500 字為限）

Slit2 蛋白在腫瘤的發展、血管新生、免疫反應上扮演重要的角色。我們發現 Slit2 在 exon15 的外顯子位置上會產生兩個剪接變異型-Slit2-WT(含有 exon15)及 Slit2-ΔE15 (沒有 exon15)。肺癌組織的 Slit2 表現遠低於周圍正常組織，有趣的是肺癌病人之正常肺組織表現高度的 Slit2-WT，而 Slit2-ΔE15 的表現非常低或不表現。我們發現 Slit2-ΔE15 具有抑制肺癌細胞的生長、轉移的能力而 Slit2-WT 則只能抑制肺癌細胞的轉移能力。除此之外，Slit2-ΔE15 可以抑制癌細胞所有導的 HUVECs 內皮細胞通透性及恢復血管的完整性，而 Slit2-WT 卻只能抑制內皮細胞的通透性，而無法恢復血管的完整性。近來的研究顯示，當腫瘤所誘導的新生血管較完整時，可以促進化學治療及放射治療的療效。因此，我們更進一步想要了解 Slit2-Exon15 剪接調控，我們發現在短暫轉染的細胞中 Slit2 的表現以 Slit2-ΔE15 型為主，暗示要產生 Slit2-WT 型需要額外的因子參與調控。我們發現環境中葡萄糖的濃度、纖維母細胞生長因子(FGF)可以促進 Slit2-WT 的表現，而 gallic acid 及發炎反應則會降低 Slit2-WT 的表現，目前我們仍繼續積極的尋找可以促進 Slit2-ΔE15 剪接變異性表現的因子。若能讓腫瘤微環境中 Slit2-WT 的表現轉化成 Slit2-ΔE15 的表現，不僅可以抑制肺癌細胞的生長、轉移並可以讓腫瘤附近不正常的血管正常化，改善腫瘤為環境中缺氧的狀況，降低缺氧不僅可以讓腫瘤較不會惡化更可以促進化療及放療的療效。除此之外，我們在 Slit2 之 1520 個胺基酸中，鑑定出 8 個胺基酸，此 8 個胺基酸本身即具有抑制 CL1-5 細胞的生長及轉移能力，此胺基酸已經在專利申請中。

附件三

科技部補助計畫衍生研發成果推廣資料表

日期：___年__月__日

<table>
<tr><td>科技部補助計畫</td><td colspan="3">計畫名稱：
計畫主持人：
計畫編號：　　領域：</td></tr>
<tr><td rowspan="2">研發成果名稱</td><td colspan="3">（中文）</td></tr>
<tr><td colspan="3">（英文）</td></tr>
<tr><td>成果歸屬機構</td><td></td><td>發明人
(創作人)</td><td></td></tr>
<tr><td rowspan="2">技術說明</td><td colspan="3">（中文）

（200-500 字）</td></tr>
<tr><td colspan="3">（英文）</td></tr>
<tr><td>產業別</td><td colspan="3"></td></tr>
<tr><td>技術/產品應用範圍</td><td colspan="3"></td></tr>
<tr><td>技術移轉可行性及預期效益</td><td colspan="3"></td></tr>
</table>

註：本項研發成果若尚未申請專利，請勿揭露可申請專利之主要內容。

附件四

科技部補助專題研究計畫執行國際合作與移地研究心得報告

日期：___年___月___日

計畫編號	MOST　　—　　—　　—　　—　　—		
計畫名稱			
出國人員 姓名		服務機構 及職稱	
出國時間	年　月　日至 年　月　日	出國地點	
出國研究 目的	□實驗 □田野調查 □採集樣本 □國際合作研究 □使用國外研究設施		

一、執行國際合作與移地研究過程

二、研究成果

三、建議

四、本次出國若屬國際合作研究，雙方合作性質係屬：(可複選)

□分工收集研究資料
□交換分析實驗或調查結果
□共同執行理論建立模式並驗証
□共同執行歸納與比較分析
□元件或產品分工研發
□其他 (請填寫) _____

五、其他

附件五

科技部補助專題研究計畫出席國際學術會議心得報告

日期：___年___月___日

計畫編號	MOST　　—　　—　　—　　—　　—		
計畫名稱			
出國人員 姓名		服務機構 及職稱	
會議時間	年　月　日至 年　月　日	會議地點	
會議名稱	(中文) (英文)		
發表題目	(中文) (英文)		

一、參加會議經過

二、與會心得

三、發表論文全文或摘要

四、建議

五、攜回資料名稱及內容

六、其他

附件六

科技部補助專題研究計畫國外學者來臺訪問成果報告

日期：___年___月___日

計畫編號	MOST　　—　　—　　—　　—　　—		
計畫名稱			
邀訪學者 姓名		服務機構 及職稱	
國籍		來臺時間	年　月　日至　年　月　日
來訪目的 (可複選)	□技術指導 □實驗設備設立 □計畫諮詢/顧問 □學術演講 □國際會議主講員 □其他		

一、訪問過程

二、對本項專題計畫產生之影響、貢獻或主要成果

三、建議

四、其他

科技部補助計畫衍生研發成果推廣資料表

日期:2015/10/26

科技部補助計畫	計畫名稱: Gallic acid 對腫瘤微環境中Slit2-Exon15剪接變異型表現的影響
	計畫主持人: 蔡菁華
	計畫編號: 102-2320-B-040-010-MY2　　學門領域: 保健營養

無研發成果推廣資料

102年度專題研究計畫研究成果彙整表

計畫主持人：蔡菁華	計畫編號：102-2320-B-040-010-MY2
計畫名稱：Gallic acid 對腫瘤微環境中Slit2-Exon15剪接變異型表現的影響	

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	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	1	1	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	3	3	100%	人次	
		博士生	3	2	100%		
		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	1	2	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	3	3	100%	人次	
		博士生	3	2	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。）		無					

	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	

科技部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）
   Slit2蛋白在腫瘤的發展、血管新生、免疫反應上扮演重要的角色。我們發現Slit2在exon15的外顯子位置上會產生兩個剪接變異型-Slit2-WT(含有exon15)及Slit2-E15（沒有exon15)。肺癌組織的Slit2表現遠低於周圍正常組織，有趣的是肺癌病人之正常肺組織表現高度的Slit2-WT，而Slit2-E15的表現非常低或不表現。我們發現Slit2-E15具有抑制肺癌細胞的生長、轉移的能力而Slit2-WT則只能抑制肺癌細胞的轉移能力。除此之外，Slit2-E15可以抑制癌細胞所有導的HUVECs內皮細胞通透性及恢復血管的完整性，而Slit2-WT卻只能抑制內皮細胞的通透性，而無法恢復血管的完整性。近來的研究顯示，當腫瘤所誘導的新生血管較完整時，可以促進化學治療及放射治療的療效。因此，我們更進一步想要了解Slit2-Exon15剪接調控，我們發現在短暫轉染的細胞中Slit2的表現以Slit2-E15型為主，暗示要產生Slit2-WT型需要額外的因子參與調控。我們發現環境中葡萄糖的濃度、纖維母細胞生長因子(FGF)可以促進Slit2-WT的表現，而gallic acid 及發炎反應則會降低Slit2-WT的表現，目前我們仍繼續積極的尋找可以促進Slit2-E15 剪接變異性表現的因子。若能讓腫瘤微環境中Slit2-WT的表現轉化成Slit2-E15的表現，不僅可以抑制肺癌細胞的生長、轉移並可以讓腫瘤附近不正常的血管正常化，改善腫瘤為環境中缺氧的狀況，降低缺氧不僅可以讓腫瘤較不會惡化更可以促進化療及放療的療效。除此之外，我們在Slit2之1520個胺基酸中，鑑定出8個胺基酸，此8個胺基酸本身即具有抑制CL1-5細胞的生長及轉移能力，此胺基酸已經在專利申請中。