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子計畫五：皮膚癌及肺癌細胞 Hedgehog 訊息傳遞以及趨化 激素受體表現之關連性(1/3)

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一、 中英文摘要

趨化激素及其受體在調節細胞增殖， 凋亡， 以及血管新生方面扮演了重要的角色， 因而可能增進腫瘤增生及轉移。 在九十二年度的國科會計劃(92-2314-B-039-024)中， 我們探討趨化激素及其受體在基底細胞癌腫瘤生長病程中所可能扮演的角色。 我們發現趨化激素受體 CXCR4 於培養基底細胞癌細胞中有高度之表現。 為了進一步確定 CXCR4 之表現是否在基底細胞癌腫瘤增生過程中扮演重要之角色， 我們利用 retroviral transduction 技術將 CXCR4 cDNA 基因轉入基底細胞癌細胞， 而得到過度表現 CXCR4 之基底細胞癌細胞株 (CXCR4-BCC)。 進一步研究發現 CXCR4 之表現可增進細胞增生， 抵抗細胞凋亡， 以及促進血管新生。 這些結果顯示 CXCR4 之表達對基底細胞癌之腫瘤增生可能扮演了重要的角色， 然而， 為何癌細胞於演化過程中會發展出 CXCR4 表現之特性， 仍有待探索。

“Hedgehog 訊息傳導路徑” 在脊椎動物胚胎發育過程中扮演了重要的角色。 近來研究也發現此一訊息傳導路徑之不正常活化對於某些特定腫瘤之發生扮演了重要的角色。 例如此路徑中的兩個關鍵基因 PTCH 或是 SMO 的錯亂， 不論是 PTCH 的去活化或是 SMO 的活化， 都可能導致易生皮膚基底細胞癌或神經管胚細之 Gorlin 氏症候群， 或是單發性之基底細胞癌。 然而， 究竟 hedgehog 訊息傳導路徑如何藉由下遊之標的分子影響腫瘤發生仍然充滿疑問。

於此我們假設 hedgehog 訊息傳導路徑與趨化激素受體 CXCR4 之表現在基底細胞癌腫瘤發生中有密切之相關。 此假設乃是基於以下過去研究所揭露之事實： (1) CXCR4 之表達對基底細胞癌之腫瘤增生可能扮演了重要的角色， (2) hedgehog 訊息傳導路徑訊息傳導路徑之不正常活化對於皮膚基底細胞癌之發生扮演了重要的角色， (3) 腫瘤抑制基因 von Hippel-Lindau (VHL) 之不正常去活化可能導致腎藏癌(kidney clear cell carcinoma, RCC) CXCR4 之表達以及腫瘤發生， (4) 除了基底細胞癌之外， 小細胞肺癌 (small cell lung carcinoma, SCLC) 之腫瘤發生也同時受到 hedgehog 訊息傳導路徑以及 CXCR4 表達之調

控。 我們因此提出此計畫以驗證以上之假設， 此外， 我們也將嘗試探索 Hedgehog 訊息傳導路徑藉由何種機轉影響腫瘤發生。 我們將嘗試利用現有的 BCC-1/KMC 基底細胞癌細胞株： 首先我們將檢驗此細胞株中 hedgehog 訊息傳導路徑是否呈現不正常之活化， 並進一步確認此傳導路徑之活化是屬於 PTCH 的不正常去活化或是 SMO 的不正常活化。 接著再據此進一步改造 BCC-1/KMC 基底細胞癌細胞株之基因。 若是屬於 PTCH 的去活化， 我們將利用 retroviral gene transfer 技術將正常之 PTCH 基因轉入； 若是屬於 SMO 的不正常活化， 則利用 RNA 干擾 (RNA interference) 技術阻止 SMO 之表達。 如此將 hedgehog 訊息傳導路徑不正常之活化加以導正之後， 再進行一系列體外以及體內實驗比較基因改造前後之 BCC-1/KMC 基底細胞癌細胞株， 以求証是否 hedgehog 訊息傳導路徑之不正常之活化確實能影響 CXCR4 之表現以及基底細胞癌細之腫瘤新生。 類似之研究模式亦將同時應用於探索小細胞肺癌中 hedgehog signaling 與 CXCR4 表達之關係。

我們預期此計畫將會大幅提升我們對於 hedgehog 訊息傳導路徑如何影響皮膚癌及肺癌腫瘤新生的了解； 更重要者， 以 hedgehog 訊息傳導路徑構成分子為標的之新治療策略將可因此得進一步的發展。

Chemokines and their receptors play important roles in the regulation of cell proliferation, apoptosis, and angiogenesis, which may enhance tumor growth or metastasis. In our previous studies, we investigated the possible involvements of chemokine receptors in the pathogenesis of cutaneous basal cell carcinoma (BCC), the most common human cancer. We found high expression of chemokine receptor CXCR4 in a human BCC cell line and a subset of tissue samples from BCC lesions. Furthermore, by retroviral gene transduction, we have found expression of CXCR4 enhance BCC tumorigenesis by the up-regulation of cell proliferation, migration, apoptosis-resistance and angiogenesis. The results of our previous study clearly indicate that CXCR4 may play a critical role in BCC tumorigenesis, but the question remains about how an evolving tumor cell is

re-engineered to express CXCR4.

The hedgehog signaling pathway plays an essential role in embryonic development and patterning of diverse vertebrate structures. The role of the hedgehog pathway in tumorigenesis was based on the finding that the gene aberration of either one of two key members of hedgehog signaling (either inactivating mutations in the PTCH gene or the activating mutations in SMO gene) is responsible for the inherited disorder known as Gorlin syndrome, which is predisposed to develop BCC, medulloblastoma and other tumors, as well as sporadic BCC. However, it is still unclear, by what mechanisms, hedgehog signaling may lead to the tumorigenesis of BCC.

Herein we propose to test our hypothesis that hedgehog signaling pathway and CXCR4/CXCL12 axis may be closely related in the BCC tumorigenesis. This hypothesis is based on the following facts: (1) CXCR4 expression enhances BCC tumorigenesis, (2) Hedgehog signaling pathway activation (either inactivation of tumor-suppressor PTCH gene or activation of tumor-enhancer SMO) may lead to BCC tumorigenesis, (3) Inactivation of tumor-suppressor VHL gene mediates CXCR4 expression and tumorigenesis of RCC cells, (4) small cell lung carcinoma (SCLC) is another example whose tumorigenesis is controlled by hedgehog pathway and CXCR4 expression. Furthermore, we seek to address the mechanisms utilized by hedgehog signaling to enhance BCC tumorigenesis.

In order to test our hypothesis, herein we propose to take advantage of the human BCC cell line, BCC-1/KMC. We will firstly examine whether BCC-1/KMC cells possess an active hedgehog signaling pathway. We will try to delineate the nature of hedgehog activation, which may either attribute to the inactivation of PTCH or activation of SMO genes. Depending on the nature of hedgehog signaling in BCC-1/KMC cells, gene modification will be employed either using gene transfer to enhance the PTCH gene or gene knockdown to silence the SMO gene, in order to inactivate the inherent hedgehog signaling in BCC cells. The hedgehog-gene-modified BCC cells will then be subject to a series of *in vitro* and *in vivo* test to determine whether the inactivation of hedgehog

signaling will eventually affect the expression of CXCR4, and the tumorigenesis of BCC. This model will also be used to explore whether hedgehog signaling pathway and CXCR4 expression may also cooperate in the tumorigenesis of SCLC.

We envision this work may contribute greatly to the further understanding of how hedgehog signaling pathway may be involved in the evolution of skin and lung cancer cells, and new therapeutic strategies targeting certain component genes in hedgehog pathway may thus be developed.

二、緣由與目的

The goal of this proposal is to determine whether chemokine receptor CXCR4 expression in cutaneous basal cell carcinoma (BCC) may be related to hedgehog signaling pathway, well known to play a pivotal role in BCC tumorigenesis. Herein we will **firstly** demonstrate the results of our previous study (not published yet till Feb, 2004), revealing that CXCR4 is highly expressed by BCC and its expression enhances BCC tumorigenesis by several mechanisms. **Secondly**, we will give a background introduction for the role of hedgehog signaling pathway in BCC tumorigenesis. **Lastly**, we will explain why we hypothesize that CXCR4 expression by BCC may be related to hedgehog signaling.

Chemokines and their receptors are well known for their ability to regulate the directional migration of leukocytes, and thus broadly involved in normal developmental processes like organogenesis and host homeostasis like immune surveillance, as well as pathological conditions like infection or inflammation (reviewed by {Rossi, 2000 #39} and {Baggiolini, 2001 #40}). Furthermore, emerging evidences also show that chemokines/chemokine receptors may play important roles in several essential aspects of tumorigenesis, including tumor growth, angiogenesis, invasion and metastasis (review by {Vicari, 2002 #41}).

CXC chemokine receptor-4 (CXCR4) is one of those chemokines having been implicated in tumor progression recently. Müller et al {Muller, 2001 #34} demonstrated that CXCR4 was highly expressed in breast cancer cells and chemokine CXCL12 (the only cognate ligand for

CXCR4, also known as stromal-derived factor-1/SDF-1) had peak levels of expression in organs known as common metastatic sites for breast cancer. Moreover, *in vivo* neutralization of CXCR4 by mAb significantly impaired metastasis of breast cancers to regional lymph nodes and lung, indicating CXCR4's involvement in selective metastases of breast cancer to certain organs. In an attempt to further clarify CXCR4's role in cancer metastasis, Murakami et al {Murakami, 2002 #42} demonstrated that CXCR4's expression *in vivo* selectively enhances the metastatic potential of melanoma cells to lung, but not to other organs; and further showed that CXCR4 might act by enhancing tumor adhesion to endothelial cells and tumor growth under stress. Moreover, CXCR4 were also found expressed in other types of tumors and may be involved in tumor progression {Staller, 2003 #48; Taichman, 2002 #35; Koshiba, 2000 #50}.

Cutaneous basal cell carcinoma (BCC) is the most common human cancer and its incidence is continuously increasing {Miller, 1994 #51; Gloster, 1996 #53}. Typical lesions of BCC feature local skin invasion and angiogenesis, whereas the metastasis is rare. Although several factors may account for the pathogenesis of BCC, including ultraviolet light exposure {Gailani, 1996 #52} and mutations in hedgehog genes as aforementioned {Unden, 1997 #54; Oro, 1997 #56}, the molecular mechanisms for BCC tumor progression are still limited.

We hence hypothesized that certain chemokine receptor(s) may be involved in BCC tumor progression. In our previous work (supported by NSC **91-2314-B-037-299** and NSC **92-2314-B-039-024**), we have tried to address whether CXCR4 may play a critical role in BCC tumorigenesis and obtained **some interesting results as follows:**

CXCR4 is expressed by BCC cells. To address whether chemokine receptors may play a role in the tumorigenesis of BCC, we extensively examined the expression levels of CC and CXC chemokine receptors (CCR1–CCR10 and CXCR1–CXCR6) in a human BCC cell line (BCC-1/KMC){Chiang, 1994 #7} using real-time quantitative PCR (qPCR). We found CXCR4 was predominantly expressed by BCC-1/KMC cells compared with other chemokine receptors

(~72-fold higher than CCR10, the second highest expressed). We next determined the relative expression levels of CXCR4 in BCC-1/KMC, other skin cancer cell lines (melanoma and squamous cell carcinoma) and normal cells from human skin (keratinocytes, melanocytes and fibroblasts), and observed similarly highly expressed CXCR4 in BCC-1/KMC in comparison with other kinds of normal and transformed cells from skin (~1000- to 3000-fold higher expression than other cells) The expression of CXCR4 in BCC-1/KMC cells was further confirmed at protein level by immunoblotting.

We next determined whether CXCR4 is expressed in BCC tissues. BCC samples were subject to immunohistochemical studies. Anti-CXCR4 mAb staining was found positive in a subset of BCC samples. In situ hybridization studies using CXCR4 anti-sense oligonucleotide probes further showed positive staining of CXCR4 mRNA in BCC tumor cells.

Functional Transduction of BCC Cells with CXCR4. To determine whether CXCR4 alone may play important roles in BCC tumorigenesis, we used retroviral vector (pLNCX2, from Clontech, Palo Alto) to transfer CXCR4 gene into BCC-1/KMC cells, which were then subject to magnet-bead selection to enhance the CXCR4 expression as described {Murakami, 2002 #42}. Magnet-bead positive selection was further used to increase CXCR4 expression, as ~91% CXCR4-BCC showed clear staining with anti-CXCR4 mAb by flow cytometry. Measured by qPCR, CXCR4 expression was significantly enhanced in CXCR4-BCC compared with parental BCC-1/KMC (~37-fold) and pLNCX2-transduced BCC cells (pLNCX2-BCC, serving as vector-control) (~14-fold). Calcium flux assay was further used to confirm that CXCR4 receptors were functional in CXCR4-BCC.

CXCL12/CXCR4 Enhance CXCR4-BCC Proliferation by CXCR4 in vitro. To address whether expression of CXCR4 may enhance BCC tumor growth, pLNCX2-BCC and CXCR4-BCC cells were cultured with normal serum (10% FCS) or low serum (0.5% FCS) in the presence or absence of CXCL12. CXCR4-BCC and pLNCX2-BCC cells showed

similar proliferation rates in normal serum, either with or without CXCL12. By contrast, CXCL12 treatments resulted in significant increase of CXCR4-BCC cell proliferation compared with PBS treatments (24-hr, ~1.2-fold $p=0.027$; 48-hr, ~2.2-fold, $p=0.004$). The enhanced growth with the treatments of CXCL12 could be neutralized by anti-CXCR4 (300 ug/ml) mAb, but not by the isotype control mouse IgG2b. CXCL12 did not significantly enhance the proliferation of pLNCX2-BCC cells in low serum ($p=0.4$, data not shown). Thus, CXCL12 may enhance BCC tumor growth by CXCR4 expression under stressful condition like low serum.

CXCL12/CXCR4 Interactions Protect CXCR4-BCC from Apoptosis by UVB Irradiation. To determine whether CXCR4 signaling may provide apoptosis resistance for BCC cells, UVB irradiation was used to induce apoptosis in pLNCX2-BCC and CXCR4-BCC cells. With CXCL12 pre-treatment, there was ~38% ($p=0.04$) decrease of apoptosis in CXCR4-BCC cells (using caspase-3 as marker). This protective effect could be negated by co-treatment with CXCR4-blocking T22 peptide but not control peptide ALA. The presence of CXCL12 and T22 did not significantly affect the UVB-induced apoptosis in pLNCX2-BCC cells. Thus, CXCL12/CXCR4 pathway might provide BCC cells with significant resistance to apoptosis.

Hedgehog Pathway Plays a Critical Role in the Tumorigenesis of Basal Cell Carcinoma (BCC)

The hedgehog signaling pathway plays an essential role in embryonic development. It is well known to be involved in the patterning of diverse vertebrate structures, including neural tube, lungs,

skin, axial skeleton, teeth, hair and limbs {Bellusci, 1997 #82; Hardcastle, 1998 #83; Marigo, 1996 #84; Riddle, 1993 #85; St-Jacques, 1998 #86}. This pathway has recently also been shown to play a role in haematopoiesis {Bhardwaj, 2001 #87}. The importance of the hedgehog pathway is somewhat reflected by its high degree of conservation through evolution, and our knowledge about its signaling in vertebrates is largely based on studies in *Drosophila* {McMahon, 2000 #88}. The role of the hedgehog pathway in tumorigenesis was

based on the finding that the gene aberration of two key members of hedgehog signaling, either inactivating mutations in the PTCH gene or the activating mutations in SMO gene, is responsible for the inherited disorder known as Gorlin syndrome (or naevoid basal cell carcinoma syndrome, NBCCS), which is predisposed to develop BCC, medulloblastoma and other tumors, as well as sporadic BCC {Taipale, 2001 #96; Wiley, 2001 #7; Wetmore, 2003 #99}. Several other tumor types have also been shown to have mutations in key members of the hedgehog signaling pathway {Wetmore, 2003 #99; Watkins, 2003 #94}. However, it is still unclear, by what mechanisms, hedgehog signaling may lead to the tumorigenesis of BCC. These discoveries have highlighted the potential role of genes in hedgehog pathway, already known to be important in normal development, in controlling cell growth and differentiation.

Our Hypothesis: CXCR4 and Hedgehog Signaling Pathway May be Related in BCC Pathogenesis.

A model for CXCR4 activation in tumorigenesis: inactivation of tumor-suppressor von Hippel-Lindau (VHL) in clear cell renal cell carcinoma: The results of our previous study clearly indicate that CXCR4 may play a critical role in BCC tumorigenesis, but the question remains about how an evolving tumor cell is re-engineered express CXCR4. Given that it has already been established that hedgehog signaling pathway play a pivotal role in the tumorigenesis of BCC, as aforementioned, we wonder whether CXCR4 and hedgehog signaling pathway may be related in some way in the pathogenesis of BCC. This hypothesis is largely based on Staller et al's recent report (published by Nature, ref. {Staller, 2003 #15}) which demonstrated that CXCR4 is a novel target gene for von Hippel-Lindau (VHL) tumor-suppressor gene. As inactivation of PTCH gene in hedgehog pathway may lead to pathogenesis of hereditary and sporadic BCC as mentioned above, inactivation of the

VHL tumor suppressor gene is linked to the development of several different types of human tumors, including hereditary and sporadic clear cell carcinoma of kidney (RCC) {Kaelin, 2002

#89}. The best characterized function of VHL protein (pVHL) is as a recognition subunit of an E3 ubiquitin protein ligase complex that targets the α -subunit of the DNA-binding transcription factor hypoxia-inducible factor (HIF) {Semenza, 2002 #91} for ubiquitin-mediated degradation in the presence of oxygen {Pugh, 2003 #90}. Tumor-derived pVHL mutants are defective in this regard and manifest constitutive activation of HIF target genes {Iliopoulos, 1996 #92}. Indeed, HIF activation is an early event in the evolution of neoplastic kidney lesions in VHL disease {Mandriota, 2002 #93}. In their paper, Staller et al demonstrated that pVHL negatively regulate CXCR4 expression by its capacity to target HIF for degradation under normal oxygen condition. Importantly, their analysis of RCC that manifests mutation of the VHL gene revealed an association of strong CXCR4 expression with poor tumor-specific survival {Staller, 2003 #15}. These results indicate VHL inactivation acquired by tumor cells early in tumorigenesis leads to CXCR4 activation during tumor cell evolution, which may result in a selective survival advantage and metastasis.

Similar role for VHL axis and hedgehog signaling pathway in tumorigenesis: It is clear that VHL gene and PTCH gene in hedgehog signaling pathway are common in several ways. They are both tumor-suppressor gene, and their inactivation mutations strongly associate with the tumorigenesis of RCC and BCC respectively. Given that Staller et al have found that VHL's inactivation may turn on CXCR4 expression by RCC, and our previous results demonstrating CXCR4 expression is important for BCC tumorigenesis, it will be reasonable to hypothesize that the mutation of certain genes in hedgehog signaling pathway may regulate the expression of CXCR4 by BCC.

Small-cell lung carcinoma (SCLC): another tumor model that may be governed by hedgehog signaling pathway and CXCR4 expression: Recent reports have shown that BCC is not the only type of tumor whose pathogenesis is regulated by both hedgehog signaling pathway and CXCR4 expression {Watkins, 2003 #94; Burger, 2003 #70}. Watkins et al, in their recent paper published by Nature {Watkins, 2003 #94}, demonstrated that hedgehog signaling plays an critical role in a

subsets of SCLC, a highly aggressive and frequently lethal human tumor with neuroendocrine features. These tumors maintain their malignant phenotype in vitro and in vivo through ligand-dependent hedgehog pathway activation.

This pattern of hedgehog signaling also similarly governs normal differentiation of pulmonary neuroendocrine precursor cells and repair of airway epithelium subject to acute airway injury {Watkins, 2003 #94}. On the other hand, Burger et al {Burger, 2003 #70} recently also reported tumor cells from SCLC patients express high levels of CXCR4. Furthermore, they found CXCR4 expression mediates migration, integrin activation and adhesion to stromal cells of SCLC cells, indicating that CXCR4 expression may enhance SCLC tumor progression. These reports clearly demonstrate that, like BCC, SCLC tumorigenesis is under the regulation of both hedgehog signaling pathway and CXCR4 signaling axis.

In summary, herein we propose to test our hypothesis that hedgehog signaling pathway and CXCR4/CXCL12 axis may be closely related in the BCC and SCLC tumorigenesis. This hypothesis is based on the following facts: (1) CXCR4 expression enhances BCC tumorigenesis; (2) Aberrant hedgehog signaling pathway activation (inactivation of tumor-suppressor PTCH gene or activation of SMO gene) leads to BCC tumorigenesis, (3) Inactivation of tumor-suppressor VHL gene mediates CXCR4 expression and tumorigenesis of RCC cells, (4) SCLC is another example whose tumorigenesis is controlled by hedgehog pathway and CXCR4 expression. This work may contribute greatly to the further understanding of how hedgehog signaling pathway may be involved in the evolution of skin and lung cancer cells, and new therapeutic strategies targeting certain component genes in hedgehog pathway may thus be developed.

三. 結果與討論

Detection of Gene Aberration in Hedgehog Signaling Axis

In order to determine whether there may be gene aberration of Hedgehog signaling axis in BCC

cell line. Using DNA sequencing, thorough examination of all exons of SMO and PTCH genes was performed, which revealed no gene mutation in all the exons examined. Nonetheless, PTCH exon 20 did showed some polymorphism according to previously report.

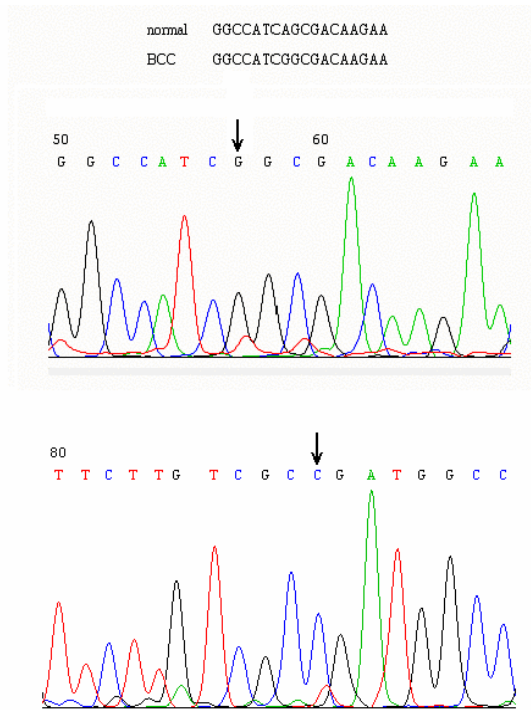


Fig.1 DNA sequencing revealed polymorphism upon PTCH gene exon 20

Gene Modification of CXCR4 using RNA interference

In order to determine whether modification of CXCR4 gene expression may affect the expression of Hedgehog genes, we employed RNA interference technique, using retroviral vector encompassing siRNA, and successfully knock down the CXCR4 expression (6.28-fold) by BCC cells, as shown in Fig.

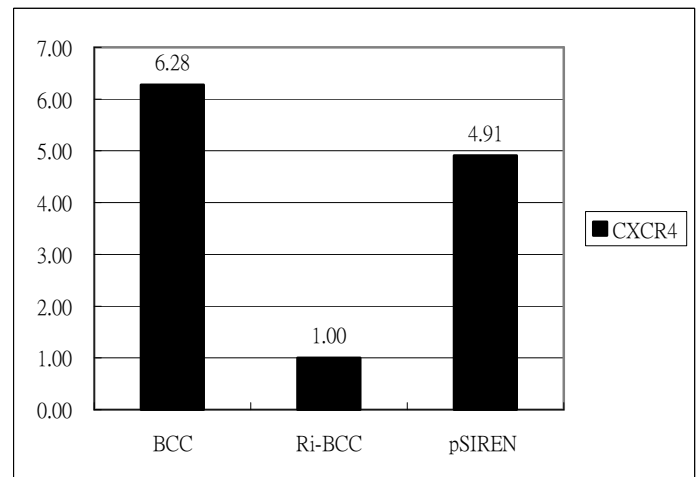


Fig. 2 CXCR4 gene knock-down in BCC cell line by RNA interference (Ri-BCC, BCC transduced with retroviral vector encompassing si-RNA targeting CXCR4 gene; pSIREN, mock-transduced cells)

Activation of CXCR4 and Hedgehog Gene Expression

In order to determine whether signaling of CXCL12/CXCR4 is related to the up-regulation of Hedgehog genes, herein we use exogenous CXCL12 to stimulate various BCC cell lines with different degree of CXCR4 expression. As shown in Fig 3 A-D, wild type BCC cell line, BCC with CXCR4 interference (Ri-BCC), and mock-transduced BCC were stimulated by CXCL12 (ligand for CXCR4) or not. After 24- or 48-h, essential genes in Hedgehog signaling pathway, including PTC, SMO, GLI, and SHH were tested for their expression by various BCC lines. Although there was mild increase of expression level of PTC in wild-type BCC line after the triggering of CXCR4/CXCL12 axis, the alteration does not appear to be significant. However, we detected a high expression of SHH by lung cancer cells, although its expression seemed not affected significantly by the addition of CXCL12

Discussion

According to the results we collected thus far, it appears that Hedgehog signaling genes showed

little change in terms of expression at RNA level. In the next approach, we will seek to determine whether Hedgehog signaling will alter the expression of CXCR4. Moreover, we will try to compare and determine whether there is difference between BCC and lung cancer cells in terms of signaling of CXCR4/CXCL12 and Hedgehog axes.

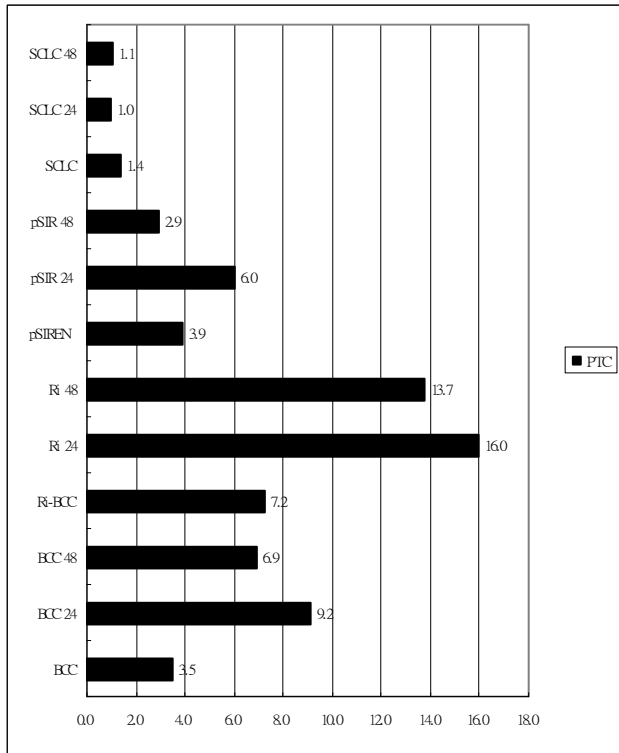


Fig. 3A PTC expression by various cell lines

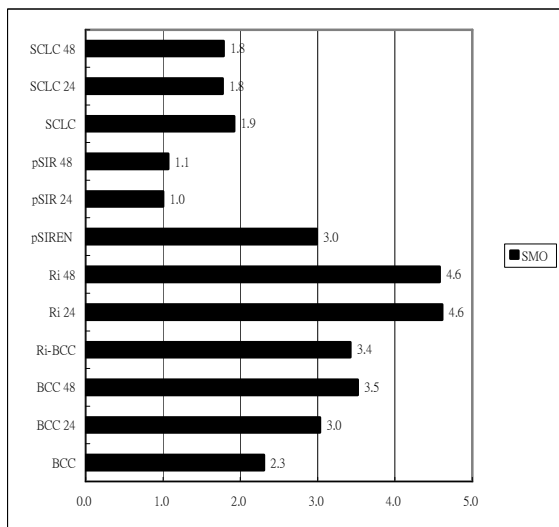


Fig. 3B SMO expression by various cell lines

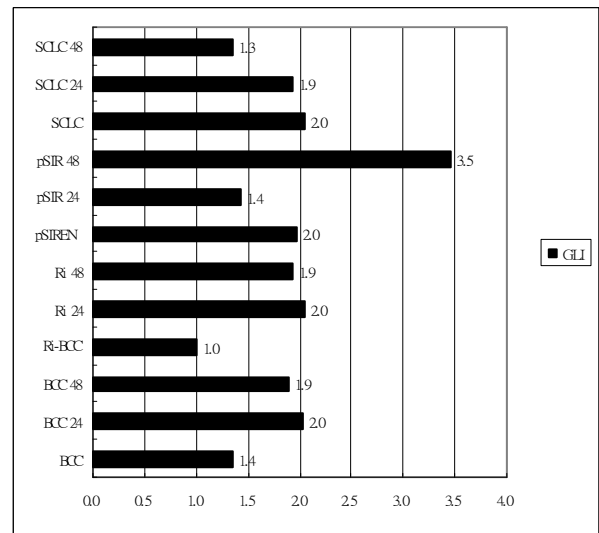


Fig. 3C
Gli expression by various cell lines

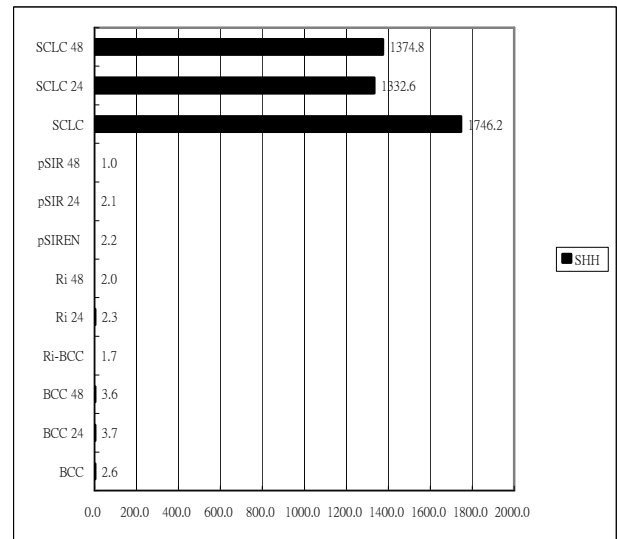


Fig. 3D
Shh expression by various cell lines

四. 参考文献

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