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行政院國家科學委員會補助專題研究計畫成果報告

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計畫類別：■ 個別型計畫　　□整合型計畫

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執行期間：90 年 8 月 1 日至 90 年 7 月 31 日

計畫主持人： 黎 慶

共同主持人： 蔡宗欣等

計畫參與人員： 蔡宜鈞、鄧如琇

本成果報告包括以下應繳交之附件：

□赴國外出差或研習心得報告一份

□赴大陸地區出差或研習心得報告一份

■ 出席國際學術會議心得報告及發表之論文各一份

□國際合作研究計畫國外研究報告書一份

執行單位：中山醫學大學 醫學院 微免科

中 華 民 國 90 年 6 月 30 日

中文摘要

在先前的實驗裡，我們發現因為 *ras* 基因過度的表達使 T24 膀胱癌細胞株產生對 Genistein 的抵抗力，一但使用 Anitsense oligonucleotide 抑制 Ras 的表達，該細胞株的生長如同其他膀胱癌細胞株 TCCSUP、TSGH8301 一樣，就會被 Genistein 強烈的抑制。在這研究裡，我們欲探討 Genistein 是經由那一個與 Ras 有關的訊息傳遞途徑，達到抑制 T24 細胞生長的結果。經過使用許多磷酸脢抑制劑，如 PD98059、U0126（以上對抗 MEK/ERK）、LY294002（對抗 PI3K）、H7（對抗 PKC）及 Anti-*fos* anitsense oligonucleotide 處理 T24 細胞後，再定量它們的生長能力，我們發現只有 *ras* → *raf* → MEK/ERK → *c-fos* 的途徑可以傳遞 Genistein 抑制 T24 細胞生長的訊息。由於 Genistein 正在臨床上測試其抑制各種癌症的能力，而許多癌症包括膀胱癌的起因與被 *ras* 基因的變異與表達有關，所以本實驗結果可以做為預先評估 Genistein 治療膀胱癌之參考；假如病患膀胱癌證實有 *ras* 基因的變異，則 Genistein 的療效是很差的。

關鍵詞： *ras* 基因、T24 膀胱癌細胞株、Genistein、訊息傳遞途徑、抗癌療效

ABSTRACT

In the previous experiments, we have shown that the antisense oligonucleotide blocking the expression of the mutated *ras* gene in T24 bladder transitional carcinoma cells lead to the susceptibility of growth inhibition by genistein. In this research, we aimed to define the *Ras*-associated signal transduction pathway that mediated this inhibitory effect. By assaying the growth rates of T24 cells treated with a variety of protein kinase inhibitors, including PD98059 and U0126 that were for against of MEK/ERK; LY294002 that was for against PI3K; and H7 that was for against PKC, and anti-*fos* antisense oligonucleotide, we found that the inhibitory effect of genistein was mediated through the pathway of *ras* → *raf* → MEK/ERK → *c-fos* and no other pathway was involved. Since genistein is currently being evaluated as an anticancer drug in many clinical trials and the *ras* mutation with over expression has been associated with many cancers including bladder cancer, the result of this study provides a possible prognostic factor of the efficacy of the drug to treat patients with bladder transitional carcinomas with or without the *ras* expression abnormality.

Keywords: *ras*, T24 bladder transitional carcinoma cells, signal transduction, Genistein, anticancer drug

INTRODUCTION

Genistein, an isoflavone present in soya, has been shown to inhibit murine bladder tumorigenesis, *in vivo*, via the combined effects of reducing angiogenesis and proliferation and increasing apoptosis [1]. With multiple functions [2-6], genistein is currently being evaluated as a therapeutic drug for human bladder tumor. In the previous experiments, our laboratory showed that among eight bladder cell lines TCCSUP [7] and TSGH-8301 [8] cells were most sensitive to the growth inhibition by genistein, whereas T24 cell line [9] resisted to the inhibition, indicating the molecular mechanisms by which genistein exerts its effects were different in different cells. Subsequently, antisense oligonucleotide against the expression of Ras reversed the genistein-resistant phenotype of T24 cells, suggesting that the signal transduction pathway mediated through *ras* is important for the growth inhibitory effect of the drug. Since Ras controls many physiological processes such as growth and other functions [10-12], this study was therefore investigating which signal pathway was specifically mediating the resistance of T24 cells to growth inhibition by genistein. By using protein kinase inhibitors in conjunction with anti-*c-fos* antisense oligonucleotide, we determined, step-by-step, the components in the mechanism that were involved in the genistein inhibitory signaling.

METHODS AND RESULTS

Genistein, PD98059, LY294002, H7, and common chemicals and reagents were obtained mainly from Sigma (St. Louis, MO, USA), whereas U0126 were purchased from Promega (Madison, WI, USA). All reagents were used

according to the recommendations from the manufacturers, if applicable. When performing cell growth rate determinations, approximately 5,000 T24 tumor cells were incubated with various concentrations of protein kinase inhibitors (0 to 50 μM) or the anti-*c-fos* antisense or control oligonucleotide (0, 0.1, 0.5, or 5 μM) for 6-8 hours preceded to the treatment of genistein for 72 hours. The cultural cells, after removal of supernatants, were then subjected to a cell proliferation assay with reagents and protocols from CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Kit (Promega). The sequences of anti-*c-fos* antisense or control phosphorothioate oligodeoxynucleotides were 5'-GsAsAsGsCsCsCsGAGAsAsCsAsTsCsAsT -3' and AsTsGsAsTsGsTsTCTCsGsGsGsCsTsTsC-3' respectively; as an "s" indicates of a phosphorothioate moiety. Under these experimental procedures, we were able to determine whether or not growth of T24 cells could be inhibited by genistein in the presence of the particular protein kinase inhibitors or at the time Fos protein was down-regulated. By performing the experiments, the component protein kinases that were involved in genistein-mediated pathway were sequential identified.

CONCLUSION AND DISCUSSION

Cell proliferation assay showed that when T24 cells were treated with 50 μM genistein, the cell growth rate was approximately equal to untreated cells, while the rates dropped to 40 % of untreated cells when T24 cells were treated with both genistein and PD98059 (20 μM) or U0126 (10 μM)[Figure 1]. This and the previous results suggest that the genistein growth inhibitory effect was interfered by the presence of overexpressed Ras which transduced signal to the down stream protein kinases. Since the Ras mediated signal is lastly transudced to *c-fos* and therefore we tested the involvement of the onco-protein in the process. Figure 2 shows that only anti-*c-fos* oligonucleotide but not mutated *c-fos* oligonucleotide retarded the growth of T24 cells in the presence of genistein; it again indicates that the *ras* → *c-fos* pathway is actually mediating the genistein inhibitory effect. Taken together, overexpression of Ras in T24 cells renders the drug resistance phenotype. In order to assess the possible involvement of other signaling pathway in this process, inhibitors of PIK3 (LY294002) and PKC (H7) were also included in cell proliferation assays. The result showed that the treatment of cells with these inhibitors did not alter the cell growth rate (data not shown), suggesting that the pathways where these protein kinases resided have not thing to do with the genisten resistant phenotype of T24 cell line.

Since *ras* mutation and overexpression has been found in a variety of tumors and is account for 10-25 % of human maglinancies, including bladder tumor [13-15], the presence of high levels of oncogenic Ras will have the worst impact on the efficacy of anticancer therapy with genistein, the drug is currently evaluated in human trials; according to the result of this study. Therefore, testing of the *ras* mutation in bladder tumor is recommended before enrolling the patients in genistein-based anticancer chemotherapy.

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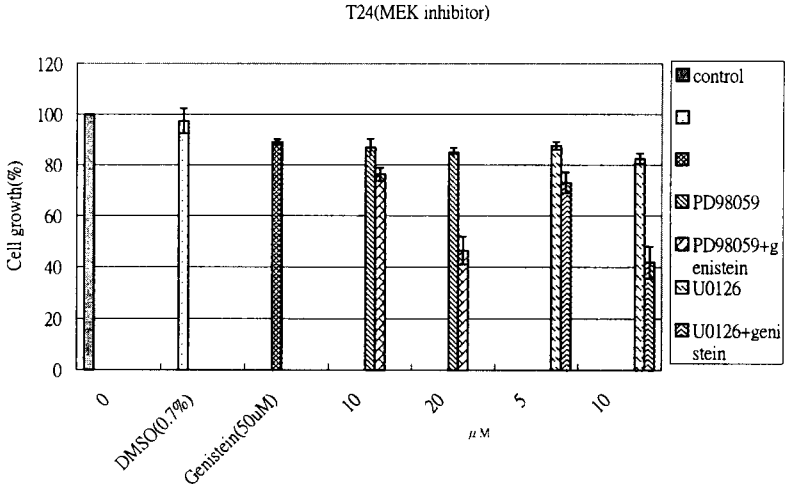

圖 1. T24 細胞株被 PD98059 及 U0126 處理後對的生長抑制狀況

T24 細胞株處理 MEK 抑制劑 PD98059 (0、10、20 μM)、U0126 (0、5、10 μM) 2 小時後，再以 genistein 50 μM 處理 72 小時之 cell proliferation assay。T24 細胞增生在 MEK 被抑制後，有明顯受到 genistein 影響；DMSO 是 PD98059 及 U0126 的 solvent。

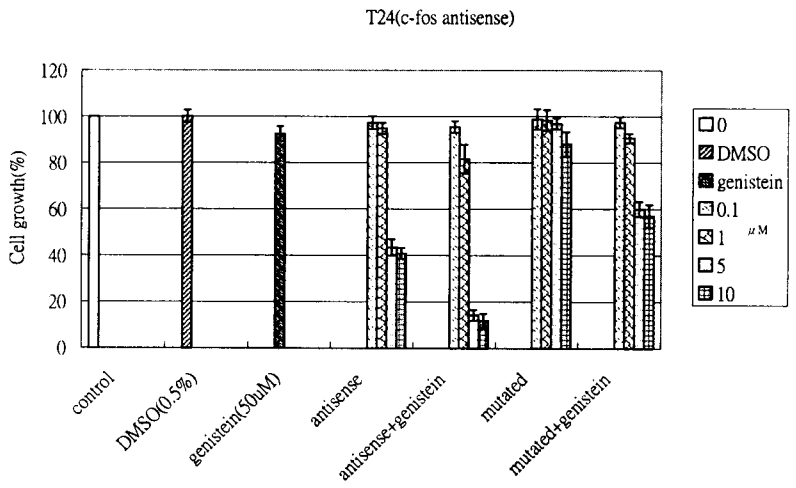

圖 4. T24 細胞內 c-fos 蛋白質受到抑制後對 Genistein 的抵抗性

以不同濃度的 *c-fos* antisense oligonucleotide 處理 T24 細胞 8 小時後，再以 Genistein 50 μM 處理 T24 細胞株 72 小時，看其生長的抑制情況，發現 T24 細胞的生長有明顯的下降。而 Mutated oligonucleotid 是 antisenseoligonucleotid 置換其中 4 個鹼基得到的序列 ，作為對照組。

附件一：出席國際學術會議心得報告

行政院國家科學委員會補助國內專家學者出席國際學術會議報告

91 年 2 月 26 日

報告人姓名	黎　慶	服務機構及職稱	中山醫學大學 醫學系 副教授
會議時間地點	91 年 2 月 9-12 日 法國 巴黎 巴斯德學院	本會核定補助文號	臺會合字第 0910000335 號
會議名稱	(中文)第六屆國際預測腫瘤學與癌症治療策略研討會 (英文)6th International Symposium on Predictive Oncology & Intervention Strategies		
發表論文題目	(中文)以微陣列方法探索膀胱癌細胞株T24對抑癌藥物Genistein抑制生長時之基因表達狀況及其分子機轉 (英文)Microarray profiling of gene expression patterns in bladder tumor cell line T24 responding to the growth inhibition mediated by anticancer drug Genistein		

報告內容應包括下列各項：

一、參加會議經過

本人於 2 月 7 日抵達巴黎，次日下午到會議會場的巴斯德學院報到。該學院雖已有 100 歷史，但仍然維護的很好，並有現代化且容量大的會議場地。正式會議由 9 日一直開到 12 日，每一天有一主題，並且可區分為大會的主題演講（與會學者共同參加）、同時進行的子題演講（與會學者選題目參加）、及全天壁報展示等學術活動，在此安排下，本會議一共發表了來自世界各國超過 550 篇的研究著作。除此外，由於與與會學者的直接交談及交換研究成果與心得的機會很多，所以參加本次研討會的收穫十足。

研討會大會還安排晚宴，讓我們品嚐世界聞名又昂貴的法國料理。另外也安排夜遊塞納河風光的活動，欣賞兩岸百年文化的建築及風光，令人目不暇給，流連忘返，十分印象深刻。

二、與會心得

「學如逆水行舟，不進則退」，這是我與會的心得。全世界有太多科學家在探討與癌症有關的研究題目，而且均有相當的成績，所以我們要立足在國際研究的舞台上，就要努力不懈的去做；但不只如此，還要加緊速度、求新求變，才能說跟上世界的潮流及腳步，也才有機會領先研究。要做到此，我必須老生常談的提出：1. 增加經費、2. 建立研究溝通合作管道、3. 隨進步增加適時儀器、4. 減少不必要公務以增加研究時間及思考、5. 國家各單位應整合以全力配合、及 6. 加重學校、研究單位、教師、學者的研究壓力及考核。也許以上幾點在國內已受到重視，或已正在實施，但很顯然，我們要努力的地方還很多。

除了得到上述的收穫外，也激發我未來研究方向的點子，在回國春假結束後與研究生的第一次見面，我就要求去訂購裸鼠，以測試因參加本研討會所得到想法的可行性。

三、考察參觀活動(無是項活動者省略)

本人無參訪任何學術單位的行程，但因就近而去看了巴斯德紀念博物館，知道這位偉大的微生物學家，如何分離礦物左旋及右旋的物理結構，及研發出包括狂犬病及天花等疫苗的經過（請了解，在那時代對細菌的明瞭尚未清楚，遑論天花病毒），我還看到在那時為研究微生物所研發出的各種儀器。

既然在巴黎，當然要走一趟歷史文化之旅，我自行搭地鐵到羅浮宮及凡爾賽宮去參觀法國的文藝作品。之後，我順道到奧地利維也納去參觀音樂文化聖地。

四、建議

如上面第二點已詳述；另外需要讓各地區及學校（不僅是台北市的中研院）有機會舉辦國際研討會，並持之以恆成為慣例，所以讓研究風氣如星火燎原的普植全國。

五、攜回資料名稱及內容

會議之摘要彙報及巴斯德學院介紹。

六、其他

無

附件三 出席國際學術會議發表之論文

Microarray profiling of gene expression patterns in bladder tumor cell line T24 responding to the growth inhibition mediated by anticancer drug Genistein

Biehuoy Shieh, Ph.D.[a] and Ching Li, Ph.D.[b*]

Departments of [a] Biochemistry and [b] Microbiology and Immunology
Chung Shan Medical University, Taichung 402, Taiwan, R.O.C.

***Presenting Author:** Ching Li, Ph.D., Department of Microbiology and Immunology, Chung Shan Medical University, No. 110, Sec. 1, Chien-Kuo N. Rd., Taichung 402, Taiwan, R.O.C.; 886-4-24730022 ext. 1630 (Office), 886-4-24727178 (Fax), e-mail: chingli@csmu.edu.tw

ABSTRACT

Aim: Unlike many other bladder tumor cell lines, T24 cells resisted the growth inhibition mediated by anticancer drug Genistein. The molecular mechanism of the drug resistance was therefore under investigation, and ultimately the approaches for clinical applications may be suggested.
Methods: cDNA-chip hybridization was used to profile gene expression patterns in cell lines susceptible or resistant to the growth inhibition mediated by Genistein. Antisense technology was then employed to investigate the roles of the potential genes identified by microarray techniques in the inhibition process.
Results: H-ras oncogene was identified and determined to play the central role in mediating the resistance of bladder tumor cell line T24 to the effect of Genistein.
Conclusion: Microarray technology is a reliable and powerful tool for gene expression profiling and potential gene identifying in cancer research. Furthermore, bladder tumor exhibiting H-ras overexpression indicates the probability of the failure in chemotherapy with Genistein.

INTRODUCTION

Transitional cell carcinoma (TCC) is the tumor that occurs mostly in urinary bladder, with an

exceptionally high mortality rate in blackfoot disease (BFD) endemic areas of Southern Taiwan [1,2]. The rates for TCC in the area could reach 30 times of that of the general population in Taiwan. In townships nearby the BFD prevalent areas, the incidence rate of bladder cancer is also elevated [3]. Therefore, bladder cancer in Taiwan is a severe disease, regardless of the association with BFD or not, that it is mandatory to find a complete medical procedures including detailed diagnostic and prognostic markers and anti-cancer treatments for the tumor.

Recently, an isoflavone genistein has been shown to inhibit murine bladder tumorigenesis, *in vivo*, via the combined effects of reducing angiogenesis and proliferation and increasing apoptosis [4]. This compound can be isolated from the fermentation broth of *Pseudomonas* sp. [5] and it is the predominant isoflavone existed at high quantity in soy [6]. Genistein has previously been reported to possess the inhibitory activities against the functions of estrogen receptor [7], growth factor-associated tyrosine kinases [8], and DNA topoisomerase II [9], as well as against the process of angiogenesis *in vitro* [10]. Furthermore, the compound is an anti-oxidant [11] but it stimulates the synthesis of sex hormone-binding globulin [7]. With multiple functions, genistein is currently being evaluated as a therapeutic drug for human bladder tumor. After testing eight bladder tumor cell lines available to us (data not shown), TCCSUP [12] and TSGH-8301 [13] cells showed most sensitive to the growth inhibition by the drug, whereas T24 cell line [14] resisted to the inhibition, indicating the molecular mechanisms by which genistein exerts its effects were different in different cells. This study was therefore investigating how T24 cells were differ to other bladder cell lines in terms of gene expression patterns that render the resistant phenotype to anticancer drug genistein.

MATERIALS AND METHODS

Molecular biology enzymes were mainly purchased from Stratagene (La Jolla, CA, USA) unless otherwise specified, whereas common chemicals and reagents were obtained mainly from Sigma (St. Louis, MO, USA). All reagents were used according to the recommendations from the manufacturers, if applicable. cDNA Microarray hybridization experiments were performed to detect the gene expression profiles in TCCSUP, TSGH-8301, and T24. The hybridization protocol and the cDNA-chip (Figure 1) used in here have been detailed described previously [15, http://w3.csmc.edu.tw/~chingli-Biochip]. Since the H-Ras protein was shown to be highly expressed in T24 cells but not in other cell lines in our study, we used anti-H-ras antisense or control phosphorothioate oligodeoxynucleotides (Antisense: 5'-CsCsAsCsAsCsCsCsGACsGsGsCsGsCsCsC-3'; Control: GsAsGsCsTsCsCsCAGsGsCsTsCsAsGsA-3'; whereas an "s" indicates of a phosphorothioate moiety) to target the synthesis of the protein, followed by assaying the proliferation rate of the cells responding to the treatment of 50 μM genistein (Sigma). Briefly, approximately 5,000 T24 tumor cells were incubated with various concentrationsof the antisense or control oligonucleotide (0, 0.1, 0.5, or 5 μM) for 6-8 hours preceded to the treatment of genistein for 72 hours. The cultural cells, after removal of supernatants, were then subjected to a cell proliferation assay with reagents and protocols from CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Kit (Promega; Madison, WI, USA). Under these experimental procedures, we were able to determine whether or not the expression of H-ras in T24 cells played a central role in drug resistance. cDNA microarrays were once again employed to investigate the gene expression profiles in T24 cells treated with anti-H-ras antisense or control phosphorothioate oligodeoxynucleotides in the presence or absence of genistein, as they are shown in Figure 2. By performing the experiments, the genes that are involved in the growth inhibition of the cancer cells mediated by genistein may be identified.

RESULTS AND DISCUSSION

H-ras Expression and Drug Resistance

Unlike TCCSUP and TSGH-8301 cell lines, T24 cell line resists to the growth inhibition mediated by genistein. In our previous experiments, cDNA-chip hybridizations produced gene expression patterns showing the ras gene was highly expressed in only T24 cells (data not shown), which is consistence with the previous report on the detection of overexpressed onco-protein H-Ras

in the cells [14]. In the same experiments, two inducible immediately early genes egr-1 and c-fos were detected to be constitutively expressed (Figure 3), which is presumably due to the coordinate regulation of gene expressions mediated by high level H-Ras in T24, but not TSGH-8301, cells as it was described in the previous report [16]. Therefore, we hypothesized that the expression of H-ras must be essential for the drug-resistant phenotype of the cells. Subsequently, we found out that, under the condition of H-ras expression knocked out (by treating with anti-H-ras antisense phosphorothioate oligodeoxynucleotide, Figure 4), T24 cells became susceptibility to growth inhibition mediated by genistein (Figure 5). We thus further investigated the gene expression differences in T24 cells treated with anti-H-ras or control oligonucleotides in the presence or the absence of 50 μM genistein to elucidate potential gene candidates that play roles in the molecular mechanism of growth inhibition. The schematic diagram of the entire experimental design is shown in Figure 2. According to the design, mRNA samples were isolated from T24 cells treated with anti-H-ras antisense oligonucleotide for 0, 8, or 12 hours. These mRNA samples were used in cDNA microarray hybridizations to obtain the gene expression patterns in T24 cells with low H-Ras level. The patterns were then compared to those derived from the hybridization of the identical cDNA-chips (the example shown in Figure 1) with mRNA samples isolated from the cells treated with anti-H-ras antisense oligonucleotide and 50 μM genistein for 0, 0.5, 1, 2, 4, or 12 hours. As the control, the hybridizations of the cDNA-chips with mRNA samples derived from T24 cells treated with the control oligonucleotide and 50 μM genistein for 0, 0.5, 1, 2, 4, or 12 hours were also performed and the deduced gene expression patterns were account for the alternations induced by nonspecific phosphorothioate oligonucleotide and genistein.

Profiling of Gene Expression Patterns

The cDNA microarray hybridizations designed in Figure 2 had performed more then four times for each mRNA samples isolated from T24 cells and the consistent gene expression profiles were obtained and listed in Table 1. By using egr-1 [15] as an example to illustrate the reliability of the microarray technology, the gene expression patterns for the immediately early gene detected by cDNA-chip hybridizations were compared to those by RT-PCR (reverse transcription-polymerase chain reaction), which is shown in Figure 6. The patterns derived from both methods were found to be consistent to each other. From Table 1 we further detect the expressions of 10 genes are upregulated whereas another 4 genes are downregulated when H-Ras expression is blocked by antisense oligonucleotide. On the other hand, 18 gene expressions were higher upon treating with control oligonucleotide and 50 μM genistein. These alternations in gene expression indicated the molecular adjustments within the cells responding to H-Ras synthesis inhibition, phosphorothioate oligonucleotide treatment, or genistein stimulation. The most importantly, the experiment with anti-H-ras antisense oligonucleotide and genistein revealed that there were 5 and another 10 genes whose gene expression levels were increased and decreased, respectively, and some of them had been detected in the previous cDNA-chip hybridization experiments (Table 1). Particularly, topoisomerase 2α gene was upregulated upon treating with anti-H-ras antisense oligonucleotide alone, but became downregulated if 50 μM genistein was added. The expressions of hdlc1, transcription factor II, fibronetin receptor β, and coagulation factor 3 were increased in control antisense oligonucleotide and genistein-treated cells, but were decreased in T24 cells when the olignucleotide was replaced by the one specific against H-Ras expression under the identical experimental conditions. Since differently expressed, these genes are the candidates for mediating the growth inhibition of bladder tumor cell line T24 by anticancer drug genistein and this warrant further investigations.

ACKNOWLEDGEMENTS

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