新北市政府 107 年度自行研究報告

研究主題:

中文：

兒茶素在泌尿上皮癌細胞治療上扮演的角色

英文：

The role of (-)-epigallocatechin-3-gallate (EGCG) in treatment of urothelial carcinoma

研究機關：新北市立聯合醫院

研究人員：許富順醫師

研究期程：107.01.01-107.12.31

新北市政府衛生局編印

印製:107 年 12 月

新北市政府 107 年度自行研究計畫表

填 表 人：許富順
填表日期：107.12.07
聯絡電話：0978695055

計　畫　名　稱	兒茶素在泌尿上皮癌細胞治療上扮演的角色		
研究機關及人員	計畫主持人：新北市立聯合醫院 泌尿科 許富順醫師	期　　程	自議會 107 年預算審議通過後執行至 107 年 12 月 31 日
目　　的	1. 釐清不同濃度的 EGCG 在泌尿上皮癌的細胞毒殺作用及作用機轉之差異。確認低濃度 EGCG 是否引發腫瘤抗藥性? 2. 找尋低濃度 EGCG 產生抗藥性的作用機轉及檢測是否引起其他 tumor progress? 3. 以異體移植的方式，在動物的模式上，確認不同濃度、不同給藥方式 (包含口服和注射)的 EGCG 對腫瘤生長的作用為何?		
方　　法	*In vitro*: 針對不同的尿路上皮癌細胞株施以不同劑量之 EGCG 研究其細胞毒殺作用及抗藥性產生，並用 MTT assay 測量 cell viability， western blotting 研究不同濃度 EGCG 分子機轉以及高濃度 EGCG 逆轉化療藥物抗藥性的效應。 *In vivo*: 在裸鼠動物模式上進行腫瘤細胞株異體移植，待腫瘤生長至目標大小，使用不同給藥途徑後施以不同濃度之 EGCG 以研究對腫瘤生長之影響，每週測量腫瘤大小兩次，並在給藥達四週後犧牲並切除腫瘤保存。		
經　　費	800,000 元		
備　　註	實際研究人員：許富順、郭冠麟、楊劭芊、潘君儀		

備註：

一、研究機關及人員：包括研究機關、實際研究人員及參與工作人員。

二、方法：如研究方法之訂定、問題之發掘、研究設計、資料之蒐集與分析、解決方案之研擬、研究報告之提出。

三、已提報之自行研究計畫因故撤銷辦理者，應敘明原因行文通知。

四、自行研究報告內容應力求與所屬局處業務相關。

新北市政府 107 年度自行研究成果摘要表

計　畫　名　稱	兒茶素在泌尿上皮癌細胞治療上扮演的角色
期　　程	107 年 1 月 1 日至 107 年 12 月 31 日
經　　費	800,000 元
緣起與目的	1.釐清不同濃度的 EGCG 在泌尿上皮癌的細胞毒殺作用及作用機轉之差異。確認低濃度 EGCG 是否引發腫瘤抗藥性? 2.找尋低濃度 EGCG 產生抗藥性的作用機轉及檢測是否引起其他 tumor progress? 3.以異體移植的方式，在動物的模式上，確認不同濃度、不同給藥方式 (包含口服和注射)的 EGCG 對腫瘤生長的作用為何?
方法與過程	*In vitro*: 針對不同的尿路上皮癌細胞株施以不同劑量之 EGCG 研究其細胞毒殺作用及抗藥性產生，並用 MTT assay 測量 cell viability， western blotting 研究不同濃度 EGCG 分子機轉以及高濃度 EGCG 逆轉化療藥物抗藥性的效應。 *In vivo*: 在裸鼠動物模式上進行腫瘤細胞株異體移植，待腫瘤生長至目標大小，使用不同給藥途徑後施以不同濃度之 EGCG 以研究對腫瘤生長之影響，每週測量腫瘤大小兩次，並在給藥達四週後犧牲並切除腫瘤保存。
研究發現及建議	本研究結果發現，口服兒茶素 EGCG（類比日常飲用量）無法抑制裸鼠泌尿上皮癌腫瘤的生長。高濃度 EGCG 雖然可抑制腫瘤的生長，不過毒性亦不可忽視。
備　　註	

新北市政府 107 年度自行研究計畫執行情形季報表

填表日期：2018/12/06

計畫名稱	研究機關及人員	期程		執行情形概述
		起	訖	
兒茶素在泌尿上皮癌細胞治療上扮演的角色	泌尿科 許富順醫師	107.01.01	107.03.31	研究團隊的召集與訓練、實驗細節的擬定、細胞株與動物實驗的準備
兒茶素在泌尿上皮癌細胞治療上扮演的角色	許富順醫師	107.04.01	107.06.30	細胞實驗與動物實驗
兒茶素在泌尿上皮癌細胞治療上扮演的角色	許富順醫師	107.07.01	107.09.30	相關實驗的結果統計分析，討論與除錯，確認實驗結果的正確性、組織切片
兒茶素在泌尿上皮癌細胞治療上扮演的角色	許富順醫師	107.10.01	107.12.31	研究報告的撰寫、參加國際會議的發表，完成期末報告

壹、 摘要(研究目的、方法、重要發現、主要建議及政策意涵)

研究目的

Urothelial carcinoma (UC) is the most common cancer of urinary tract. Patients with metastatic UC are usually treated with systemic chemotherapy. There still existed 30% to 50% of advanced UC cases that are not responsive to cisplatin-based chemotherapy; the prognosis for patients with metastatic UC remains poor. (-)-epigallocatechin -3-gallate (EGCG) is the most abundant polyphenolic compound from green tea and have various bioactivity and could induce cytotoxicity to UC. The study showed that after 2 hours of intensive consumption of green tea, the concentration of EGCG in human plasma can only reach up to 0.6-1.8 μM. These concentrations in plasma are much lower than the concentrations used in studies and given through intraperitoneal injection. However, the anticancer effects of different concentrations of EGCG on UC have not been thoroughly explored.

研究方法

Urothelial carcinomas cell lines NTUB1 and T24 were treated with different concentrations of EGCG for different durations, with or without chemotherapy reagents: gemcitabine and doxorubicin were then analyzed by cell viability assay and western blot. Antibodies include: phospho-c-Raf (Ser259), phospho-Akt (Ser473), phospho-c-Raf (Ser259), c-PARP and c-caspase 3 were used to detect AKT, ERK and apoptosis related pathways in western blot. Six- to eight-weeks old mice were given low-dose EGCG by P.O.(1000mg/L in water), low-dose EGCG (5mg/kg/daily), high-dose EGCG (200mg/kg/daily), and normal

saline by intraperitoneal injection (I.P.), two weeks after xenograft with BFTC-905 UC cell line.

重要發現

Our data showed that high dose of EGCG (50 or 80 μM) can enhance the cytotoxicity of these chemotherapeutic drugs *in vitro*. The underlying mechanism of high-dose EGCG is associated with Akt-ERK pathway. On the other hand, low-dose EGCG (1.5 μM) did not reduce tumor cell viability and failed to activate apoptosis pathway. Our *in vivo* study showed low-dose EGCG (5 mg/kg/daily) that corresponds to concentration in plasma after tea consumption did not repress UC tumor growth in mice. Skepticism has been raised regarding safety of high-dose EGCG consumption due to the potential hepatotoxicity that may occur in human and rodents. Clinicians often face a dilemma of toxicity and effectiveness when using chemotherapeutic drugs as high dose EGCG (200 mg/kg/daily) is required to suppress cancer cells.

主要建議及政策意涵

Our *in vivo* mouse study with daily EGCG oral administration had shown no significant difference on either suppressing or promoting UC tumor growth in mice. Although some findings in our research are in agreement with other studies where EGCG could enhance chemotherapy drugs effect and potently reverse chemoresistance, the toxicity for high dosage and mechanisms for low dosage of EGCG should be fully evaluated.

貳、 主旨及背景說明(與現行業務關聯性)

背景：

抗癌藥物需嚴格精算藥物劑量，過高劑量因毒性太強產生嚴重副作用，劑量過低則無法毒殺腫瘤且容易導致抗藥性、甚至多重抗藥性的產生。尿路上皮癌(urothelial carcinomas; UC)是泌尿系統常見的惡性腫瘤，發生率近年來逐年上升。治療轉移性泌尿上皮細胞癌是以順鉑（cisplatin）為主的化學治療處方，一般治療反應率可達 40%至 70%，但多數之病人最後仍因腫瘤抗藥性而死亡，因此如何改善臨床上治療效果及降低抗藥性發生是最重要之課題。

兒茶素((-)-epigallocatechin-3-gallate ; EGCG)是綠茶萃取物中含量最多的多酚類成分，占所有水溶性綠茶萃取物的 16.5%。從 1983 年開始，EGCG 便被發現具有預防癌症的活性。有研究指出，每天喝十杯綠茶可以降低肺癌的發生率。研究指出 EGCG 會使得癌細胞週期停滯，進而造成細胞的凋亡。將 EGCG 與抗癌藥物併用，可以強化人類乳癌細胞株的抑制效果。此外，動物實驗模型，證實人類的鼻咽癌、肺癌、攝護腺癌、肝癌、胃癌等細胞株在投以 EGCG 後會增強抗癌效果。EGCG 也被證實具有毒殺尿路上皮癌的效果並被認定有在臨床上使用的潛力。唯研究顯示大量攝取綠茶後 2 小時，EGCG 在人體血漿中濃度僅可達 0.6-1.8 μM，此血漿中濃度遠小於研究中使用皮下注射給予的 EGCG 劑量，然而目前對於不同濃度之 EGCG 對泌尿上皮癌細胞作用的相關研究仍顯不足。

目的：

1. 釐清不同濃度的 EGCG 在泌尿上皮癌的細胞毒殺作用及作用機轉之差異。
2. 找尋低濃度 EGCG 產生抗藥性的作用機轉。
3. 以異體移植的方式，在動物的模式上，確認不同濃度、不同給藥方式 (包含口服和注射)的 EGCG 對腫瘤生長的作用為何?

透過以上的研究，能進一步確認 EGCG 毒殺腫瘤的濃度，並在臨床上能避免經食用後在體內的 EGCG 促進腫瘤抗藥性的產生，以為後續之臨床應用建立治療泌尿上皮細胞癌之新策略。本研究顯示，高濃度 EGCG 能對泌尿上皮癌細胞株造成顯著的細胞凋亡作用，引發 Caspases 與 PARP 的活化以及提高化療藥物如

doxorubicin 及 gemcitabine 的細胞毒殺作用。EGCG 抑制尿路上皮癌細胞存活率的結果與其他研究團隊相同，80-100 μM 之 EGCG 作用 24 小時可達 IC50。然而，*in vitro* 我們在觀察到在低濃度 0.25-10 μM EGCG(攝取綠茶後血漿中濃度)之下卻會促進尿路上皮癌細胞增生。我們也證實 80 μM EGCG 會抑制 AKT pathway；相反的，1.5 μM EGCG 卻會活化 AKT pathway。這些結果暗示 EGCG 能在高濃度下有抗癌效果，但低濃度之下則可能會誘發抗藥性。

參、 相關研究、文獻之檢討

尿路上皮癌在台灣及全球發生率逐年上升

Bladder urothelial carcinoma (UC) is the fifth most common cancer in western society, with a global incidence of over 356,000 new cases per year and a prevalence estimated at 2.7milion cases [1]. In the US, it is the fifth most common malignancies accounting for over 70,000 new cases a year and an estimated annual spend of $3.5 billion on treatment [2]. The global burden of bladder UC increase significantly in recent years as a result of population aging together with the progression of the tobacco epidemic and increasing exposure to occupational carcinogens in developing countries [3]. The incidence rate of UC in Taiwan also has been increasing, with an estimation of over 2000 new cases per year[4].

尿路上皮癌的治療面臨的難題: 化學抗藥性的發生──如何找尋新的治療藥物或處方，克服化學抗藥性是重要的課題

There still existed 30% to 50% of advanced UC that are not responsive to chemotherapy. Recent developments in immunotherapy, including PD-1 and PD-L1 inhibitors, have shown promising results, demonstrating its effectiveness in 15-29% of patients with metastatic UC following recurrence after platinum-containing chemotherapy [5]. Nevertheless, despite some new regimens were developed, the prognosis for patients with metastatic UC remains poor [6, 7]. This is due to chemoresistance that developed in many patients resulting treatment failure and mortality. Another limiting factor associated with standard regimen was the substantial toxicity [6-10]. Treatment-related mortality occurred in 2% to 4% of patients, especially in the elderly [6, 8]. Research in

finding novel compounds to enhance the treatment efficacy or reduce the toxicity of current therapeutic regimens is imperative. These novel compounds could also be incorporated into current chemotherapeutic regimen to achieve effective treatment.

EGCG是兒茶素(tea catechin)的主要成分，是一種茶多酚(tea polyphenol)，具有調控多項生理功能

Green tea is one of the most common beverages consumed worldwide. It is widely accepted that many of the physiological-function effects of green tea are mediated through polyphenols. Among various constituents of green tea, (-)-epigallocatechin-3-gallate (EGCG), the main and most significant catechin, accounts for nearly 15-65% of water extractable fraction from green tea leaves, i.e., 100–300 mg in a brewed cup of green tea [11].

EGCG has shown numerous health modulating effects through various pathways, as an antioxidant, anti-inflammatory compound, showing gene expression activities through growth factor-mediated and ubiquitin/proteasome degradation pathways [12, 13]. Metabolic studies have shown that administration of EGCG supplement yielded a maximum EGCG concentration of 4.4 μM in human plasma. Such concentration would be enough to exert antioxidative activity in the blood stream.

EGCG已被報告有抑制人類多種腫瘤生長的效果

Many cohort studies focusing on the relationships between tea consumption and the risk for several of cancers have showed an inverse association between tea consumption and cancer risk, **including bladder cancer**[14]. EGCG showed a wide range of bioactivities such as anti-viral, anti-oxidant and anti-

tumor activities studied both *in vitro* and *in vivo* [12]; in affinity column studies, EGCG also showed that it can bind to various proteins in cell lysates [15]. Further studies on EGCG activities in vitro showed that EGCG alone can have a wide range of bioactivities including inhibition of protein kinase, DNA methyltransferase and telomerase activity as well as induction of cell cycle-arrested and protein degradation [16-18].

Studies have also reported the tumorigenesis inhibitory effect of EGCG *in vivo*. In a rat colon cancer model, the administration of EGCG could reduce aberrant crypt foci (ACF) formation by 71%; in another mouse model, EGCG could inhibit the development of small intestinal tumors [19]. For bladder urothelial carcinoma cancer and prostate cancer models, EGCG could inhibit tumor growth; moreover, in combination with other constituents from green tea, EGCG could repress insulin-like growth factor 1(IGF1) level and further had influences on it downstream molecules, including PI3K, Akt and ERK [14, 19]. Another research in rat bladder tumor model also showed that ECGC is able to reduce implantation and growth of tumor cells compared to mitomycin C [20, 21]. Combination of EGCG and anticancer compounds enhances anticancer effect in human breast cancer cell lines [22, 23], as well as in xenograft mouse *in vivo* models: human cancer cell lines from head and neck, lung, breast, prostate, liver and stomach [24, 25]. The inhibitory activities of EGCG against tumorigenesis have been conducted in different animal models and EGCG could be a promising anti-cancer drug for human cancer treatment.

EGCG的代謝: 攝取後僅有相當低濃度的EGCG能進到血液循環中

The absorption of tea catechins is difficult to be measured precisely. Only about 1.7% of consumed EGCG are found in plasma, urine, and feces of a person who drinks tea. Various measuring methods, including HPLC, showed that an increase of EGCG concentration in human plasma in 1-2 hours after tea consumption. But the bioavailability of tea catechins is relatively low—only 0.2–2% of the consumed amount of EGCG gets into plasma of healthy humans [26]. When green tea is consumed in high doses, the overall level of EGCG in human plasma is about 0.6-1.8 μM [27-29]. One large review summarized pharmacogenetic parameters of tea metabolites in human body after consumption. After oral consumption of 100 to 1600 mg of pure epigallocatechin gallate, 0.26 to 6.35 μM of epigallocatechin gallate was detected in plasma in 2-3 hours; some conjugated compounds of epigallocatechin gallate in the amount of 0.28 to 7.40 μM were also found in 1.9 to 4.6 hours [30]. **These data showed that plasma concentrations of EGCG after oral administration are much lower than those used in studies *in vitro* and *in vivo* by intraperitoneal injection (I.P.).**

The above observation has also been found in clinical practices. A phase II randomized trial of green tea catechins and lycopene in men at increased risk of prostate cancer were carried out to compare two groups patients: first group of patients received daily gel capsule of 15 mg lycopene while the second group of patients received 600 mg/d EGCG capsule. However, no difference has been found in PSA levels between two groups after 6 months. The dose of green tea could have also been increased above 600 mg/L but was comparable with other prostate cancer prevention studies [31].

Dietary catechin	(mg)	Source	Metabolites in plasma	C_{max} (μM)	T_{max}(h)	AUC(μM·h)	$T_{1/2}$(h)
EGCG	600	Pure EGCG	Free EGCG	0.37	3.0	2.0	2.7
EGCG	800	Pure EGCG	Free EGCG	0.96	4.0	3.1	1.9
EGCG	50	Pure EGCG	Free EGCG	0.26	1.3	0.9	1.8
EGCG	100	Pure EGCG	Free EGCG	0.41	1.9	2.9	3.3
EGCG	200	Pure EGCG	Free EGCG	0.68	1.3	2.5	2.3
EGCG	400	Pure EGCG	Free EGCG	1.24	1.4	5.1	2.9
EGCG	800	Pure EGCG	Free EGCG	2.10	1.8	7.6	3.6
EGCG	1600	Pure EGCG	Free EGCG	6.35	1.4	21.2	4.9

Bioavailability and metabolism of tea catechins in human subjects. (Chi-Tang Ho, and Fereidoon Shahid, 2008.)

在研究結果中，高濃度EGCG會抑制*in vitro*細胞存活率及*in vivo*腫瘤生長；低濃度的EGCG雖然在*in vitro*會促進尿路上皮癌細胞增生，*in vivo*則對腫瘤生長不存在顯著差異

The results of our study showed that an appropriate dosage (>20 μM) of EGCG significantly inhibited cell viability of two UC cell lines (NTUB1 and T24) in a dependent manner that is consistent with the findings of previous reports [14]. Interestingly, we found that low dosage of EGCG (<10 μM) can enhance cell viability, at a concentration close to the level of EGCG in plasma after assimilation [30]. Although this phenomenon is also found in previous studies related to anti-cancer drug discovery [32-35], little has been reported about the adverse effect, may due to the very little chance under insufficient dose in clinical use. **In this study, our results indicated that low-dose EGCG may promote UC cell viability *in vitro* and have no significant effect either to repress or promote UC tumor size in *vivo*.** EGCG is a component of green tea and some healthy products which could be consumed by healthy people or cancer patients. **Therefore, we aim to understand the effect of insufficient dose of EGCG on UC cell proliferation and the underlying mechanism.**

肆、　研究方法(包含研究內容、範圍、對象、限制與過程)

研究方法與材料：

UC cell lines

Three UC cell lines, **NTUB1, T24 and BFTC-905**, were used in this study. NTUB1 cells were derived at National Taiwan University Hospital from the surgical specimen of a 70-year-old female patient with high grade transitional cell carcinoma and was proved to be tumorigenic in nude mice [36, 37]. The T24 cells were derived from a highly malignant grade III human urinary bladder carcinoma. NTUB1 cells were kindly provided from Dr. Yeong-Shiau Pu (Department of Urology, National Taiwan University Hospital, Taipei, Taiwan). T24 human UC cell line was obtained from the American Type Culture Collection (Manassas, VA). The other human urinary bladder UC cell line, BFTC-905, was derived from a 51-year-old Taiwanese female patient diagnosed with grade-III papillary UC in 1990. Cells were maintained at 37℃ in RPMI-1640 medium (for NTUB1 and T24 cells), or Dulbecco's Modified Eagle Medium (for BFTC-905 cells) supplemented with 10% or 15% fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin (Invitrogen, Carlsbad, CA) [38].

Reagents and chemicals

EGCG pure compound was obtained from Enzo Life Sciences, Inc. (NY, USA), purity ≥ 98% (HPLC) and **EGCG capsule** for P.O. was obtained from YUNGSHIN PHARM IND. CO (Taiwan), purity ≥ 90%. Chemotherapeutic agents used in this study were all from clinical medications: gemcitabine from Gemzar (Lilly, Fegersheim, France); doxorubicin from Adriblastina Rapid

Dissolution Pfizer (New York, NY); and paclitaxel from Sinphar (Taiwan). All other chemicals were obtained from Sigma-Aldrich or Merck Millipore (Billerica, MA, USA).

Antibodies

- AKT pathway: Phospho-c-Raf (Ser259), Phospho-Akt (Ser473), Phospho-c-Raf (Ser259)
- ERK pathway: Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204)
- Apoptosis related antibodies: cleaved-PARP, cleaved- caspase 3

Measurement of Cell Viability

Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyl tetrazolium (MTT, Sigma-Aldrich) assay. In brief, cells were seeded with culture medium in 96-well microplate (4500 cells/well) and incubated at 37℃ for 24 hours before drug treatments. After being treated for the indicated times, the cells were incubated with medium containing 0.4 mg/ml MTT at 37℃ for 4 hours. The reduced MTT crystals were dissolved in DMSO and measured using BioTek ELISA reader (Winooski, VT, USA) at a wavelength of 570 nm.

Western Blotting

After the various treatments, cells were washed with ice-cold PBS and lysed with cell lysis buffer (Cell Signaling Technology) on ice for 15 minutes followed by centrifugation at 14000 rpm for 15 minutes at 4℃. The clear supernatants were harvested and protein concentrations were determined by BCA protein assay (Thermo Scientific Pierce, Rockford, IL, USA). Equal quantities of each sample were resolved in SDS-PAGE and then transferred to a PVDF

membrane (Millipore, Billerica, MA, USA). The membranes were blocked with 5% BSA in TBST for at least 1 hour followed by incubation with the respective primary antibodies at 4℃ overnight. The membranes were then washed with TBST for 10 minutes three times, and incubated at room temperature for 1 hour with the applicable horseradish peroxidase (HRP)-conjugated secondary antibodies (Genetex, Irvine, CA, USA). After washing twice with TBST, the antibody-bound membranes were visualized by enhanced chemiluminescence Western blotting detection reagents (Millipore, Billerica, MA, USA). In addition, the Image J (NIH, USA) software was used to quantify the relative expression level of target proteins, followed by normalization based on each internal control.

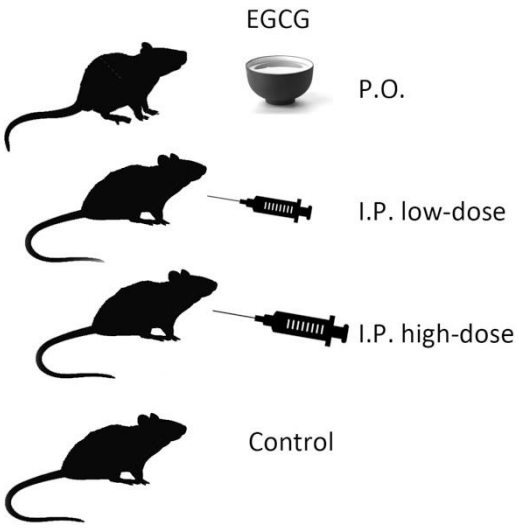
Immunohistochemistry

In order to determine ERK expression in different stage of UC, a bladder carcinoma tissue microarray (BL806, US Biomax, Rockville, MD, USA) was applied for Immunohistochemistry (IHC) staining. This tissue array contains 37 cases of transitional cell carcinoma, 2 squamous cell carcinoma, 1 adenocarcinoma, 40 adjacent normal tissue, single core per case. Tissue sections were deparaffinized in xylene and rehydrated through descending grades of alcohol. The endogenous peroxidase activity was quenched by treating the tissues with 3% H_2O_2 in methanol for 10 min and rinsed in PBS for 5 min. The nonspecific binding was blocked with 1:1000 dilutions of FBS in PBS and incubated at room temperature for 30 hours. After washing with PBS, slides were incubated over night at 4 °C with 1:500 dilution of anti-p-ERK primary antibodies. Next day, slides were washed twice by PBST and incubated for 30 min at RT and were conjugated with HRP (Polymer-HRP IHC Detection Systems, Biogenex). After washing in PBST, the sections were incubated for 10

min with 3', 3' -diaminobenzidine tetrahydrochloride (DAB) (Polymer-HRP IHC Detection Systems) The tissue sections were then briefly rinsed in water, counterstained with hematoxylin and then mounted for visualization.

In vivo Xenograft Experiments

A total of 10^6 BFTC-905 cells were suspended in 200 μl of serum-free medium and mixed with an equal volume of Matrigel (BD Biosciences, Bedford, MA), followed by subcutaneous injection into the dorsal flanks of 6- to 8-week-old nude mice obtained from National Laboratory Animal Center (NLAC, Taipei, Taiwan). After tumor is grown to a size of about 150-300 mm^3, the mice were randomly assigned to the following four groups: control, EGCG-low (P.O.), EGCG-low (I.P.) and EGCG-high (I.P.). EGCG-low (I.P.) and EGCG-high (I.P.) treated group received 5 or 200 mg EGCG/kg in normal saline respectively, once a day for four weeks, while EGCG-low (P.O.) group received 1000mg EGCG/L in drinking water that prepared in fresh every day. Paclitaxel was given at 2mg/kg I.P., three times a week. Control group received DMSO in normal saline alone. Tumor volume was measured with calipers twice a week, and calculated as: [$V= LD \times (SD)^2 / 2$], where V is the tumor volume, LD is the longest tumor diameter and SD is the shortest tumor diameter. The study that involve animal experiment has been approved by the National Taiwan University College of Medicine and College of Public Health Institutional Animal Care and Use Committee (IACUC) (No. 20130239 and 20170557).



Statistical analysis

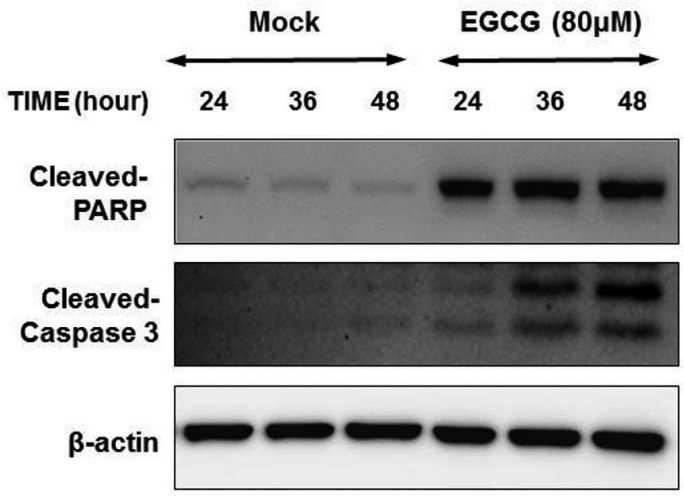
Statistical analyses were performed by GraphPad Prism® 7 software. Data were presented as means ± SD or SEM then analyzed by one-way ANOVA. Significant differences of each two group were compared by Bonferroni post hoc test and defined as *p* value < 0.05. （IHC score: intensity x percentage of cells with positive staining under at least 10 HPF）。

伍、研究發現

EGCG induced apoptosis in human UC cell line

To confirm whether apoptosis could be induced by EGCG in bladder cancer cell line, NTUB1 lysates were collected and western blot analysis was performed to measure the relative protein expression level. NTUB1 was first treated with ECGC at a high concentration of 80μM, then the cells were lysed and harvested after 24, 36 and 48 hours of treatment. The endogenous protein level was analyzed by western blot. High EGCG treatment resulted in a robust increase of cleaved-PARP and cleaved-caspase 3 in NTUB1 cells (Fig. 1).

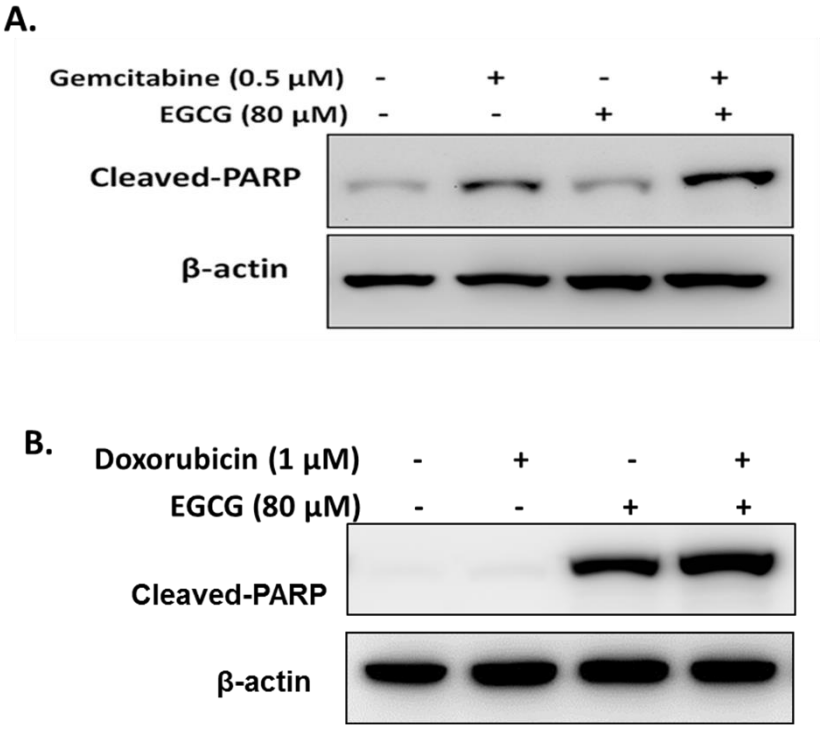
Figure. 1



Combination of EGCG with gemcitabine or doxorubicine results in an increased cleaved-PARP

To further analyze whether EGCG could enhance apoptosis effect of chemotherapy drugs treatment in UC, two chemotherapy drugs: gemcitabine and doxorubicine, were used in combination with EGCG on NTUB1 cell line. Cells were treated with following drugs, alone or combined, at a concentration of 80 μM EGCG, 0.5 μM gemcitabineand or 1 μM doxorubicine respectively. Western blotting was performed to detect endogenous level of cleaved-PARP. Results showed that cleaved-PARP strongly increased when (Fig. 2A) gemcitabime or (Fig. 2B) doxoribincine was used in combination with EGCG, compared with using the chemotherapy drug alone.

Figure.2

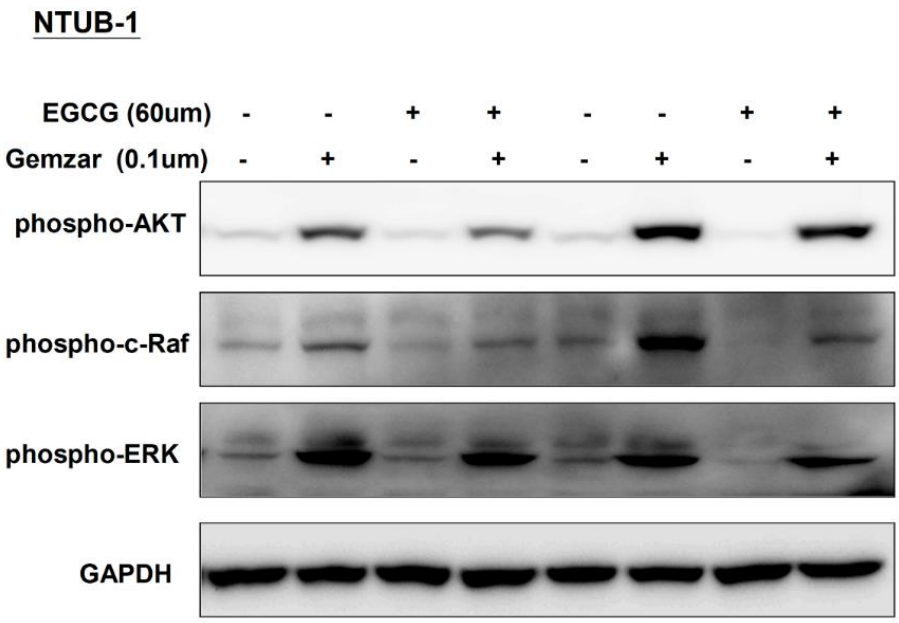


The mechanism for enhancing the cytotoxicity of chemotherapeutic agents may be mediated through Akt-ERK pathway.

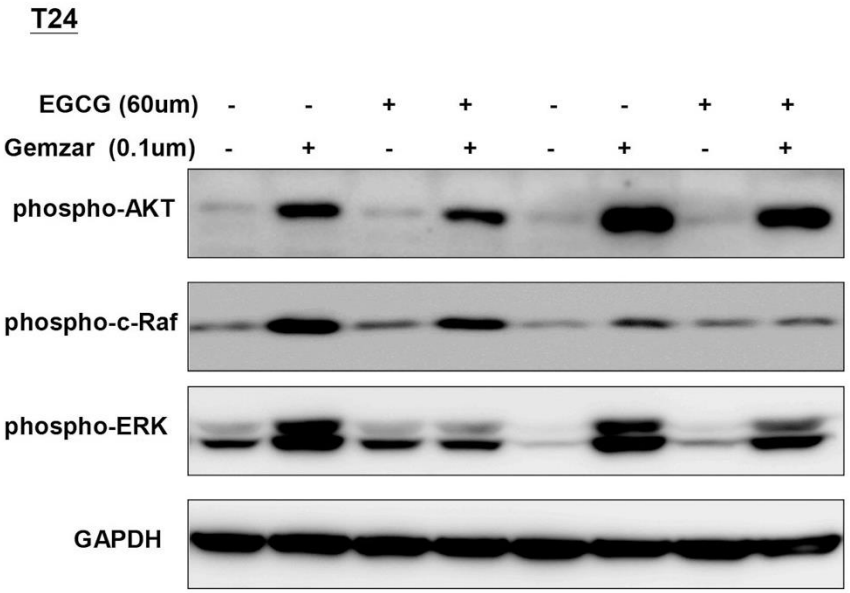
After it is confirmed that there is an increased apoptosis protein level when chemotherapeutic agents were used with EGCG, the underlining mechanism of the synergistic cytotoxic effect is then investigated. The activation of Akt-ERK pathway after gemcitabine treatment were suppressed by EGCG, which could explain the synergistic cytotoxic effect in combination with chemotherapeutic agents and EGCG (Fig. 3A, 3B). Consistent with the IHC results in bladder carcinoma tissue microarray, higher expression level of p-ERK also presented in tumor tissue of high-grade UC specimens compared to the one of low-grade specimens (Fig. 3C).

Figure. 3

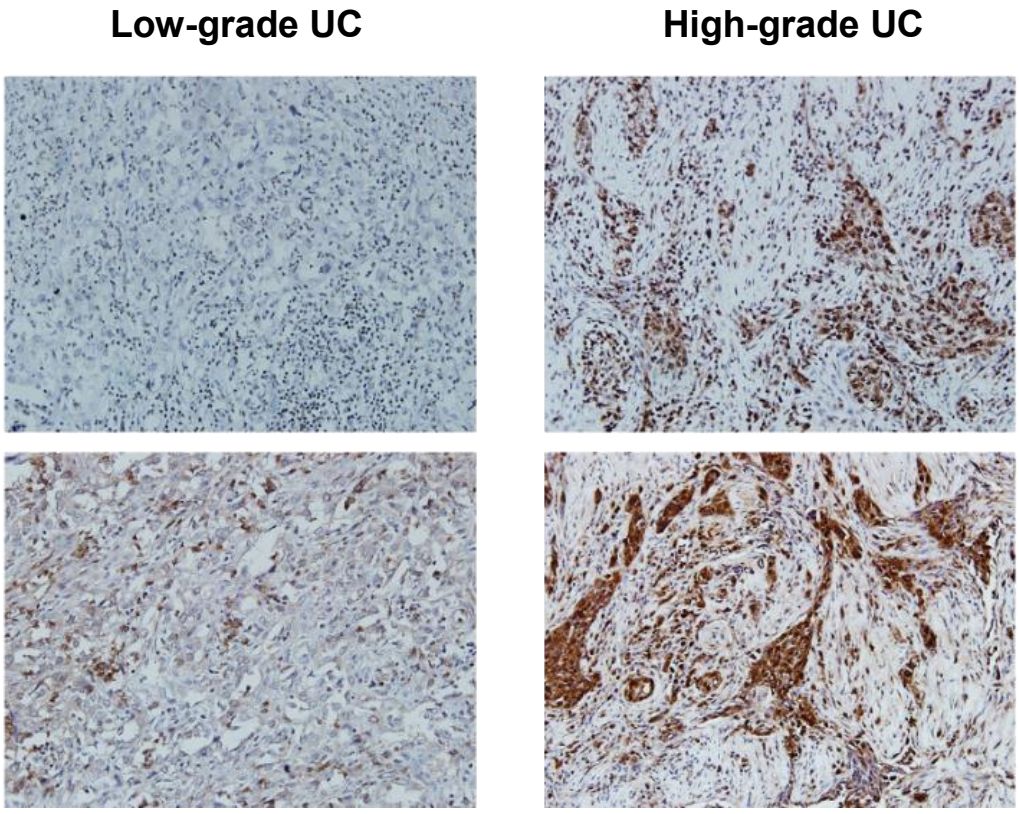
A.



B



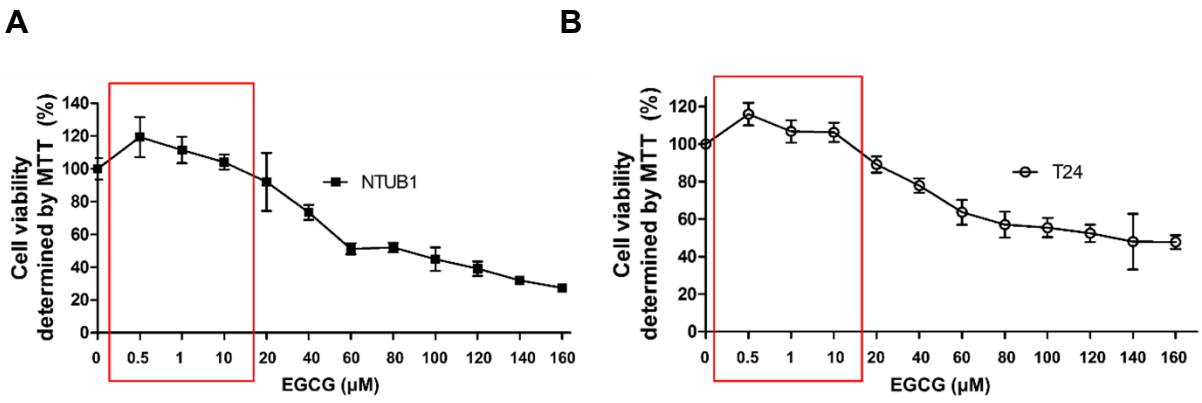
C. IHC: Phospho-ERK1/2



UC cell viability was inhibited by 20 to 160μM of EGCG, but enhanced with EGCG at the concentration below 10 μM.

So far, our results indicated a possible mechanism for induce synergistic cytotoxicity effect of using chemotherapeutic agents in conjunction with high concentration EGCG; however, apoptosis effect with different EGCG concentration — for example, low concentration of EGCG corresponding to the concentration in plasma after tea consumption — is still unclear. To further investigate the apoptosis when treated with different EGCG, we used cell viability assay (MTT) (n=6) to understand the effects of EGCG on the viability of human UC cell line by treating NTUB1 and T24 cells with various concentrations (0-160 μM) of EGCG and for 24 hours, respectively. As shown in figure 4, higher dose of EGCG (20-160 μM) have shown to inhibit UC cell viability in a dose-dependent manner, whereas lower dose EGCG (<10 μM) have shown to enhance cell viability, indicating low concentration of EGCG may fail to induce sufficient cytotoxicity.

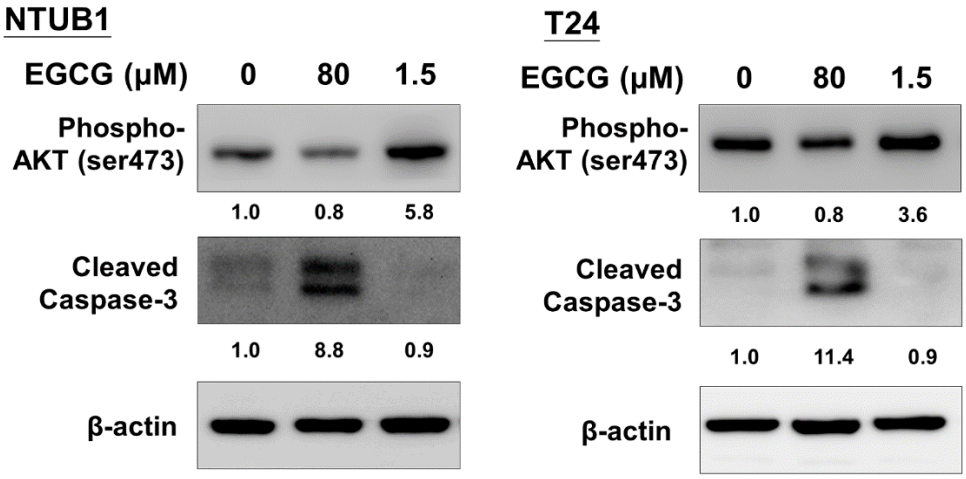
Figure. 4



AKT phosphorylation was suppressed under high-dose EGCG treatment while being conversely activated with low dose EGCG

At 80 μM concentration, EGCG partially inhibited AKT phosphorylation and largely enhanced caspase3 cleavage in both bladder cancer cell line NTUB1 and T24. However, low-dose EGCG significantly enhanced p-AKT and by 5.8- and 3.6-fold in NTUB1 and T24 respectively (Fig. 5). The results suggest that insufficient (low) dose of EGCG would not merely fail to trigger apoptosis, but also promote AKT-pathway for cell proliferation and drug resistance.

Figure. 5



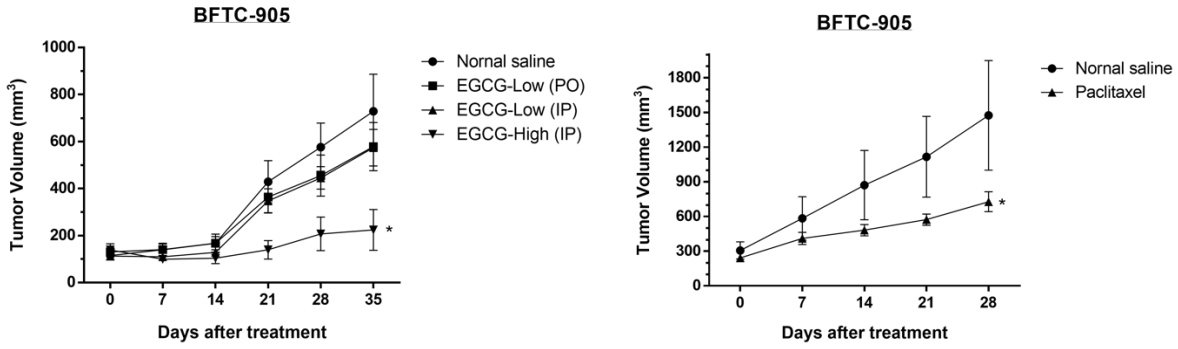
UC Xenograft mice presented no significant different of tumor size change in both P.O. and I.P. low-dose EGCG groups, yet revealed noticeable toxicity in high-dose EGCG group

To further investigate whether low-dose EGCG could potentially promote tumor growth *in vivo*, nude mice were grafted with BFTC-905 UC cell line xenografts following low- and high-dose EGCG treatment. Tumor volume presented as mean ± SEM, n= 6 or 10, $p < 0.05$ compare to control normal saline group. In Figure 6A, our results showed that there were no significant

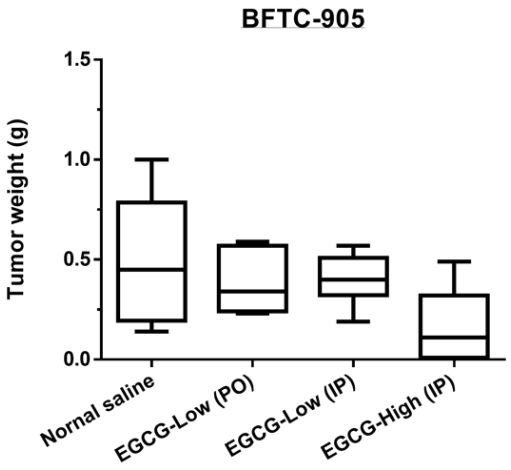
differences in tumor size between normal saline group and low-dose EGCG groups, suggesting low dose EGCG (1000 mg/L in water P.O. and 5 mg/kg/daily I.P.) would neither reduce nor promote tumor growth. High-dose EGCG groups (200 mg/kg/daily) served as a positive control for treatment and thus had shown a significant reduction in tumor size in the fifth week. However, 200mg/kg EGCG in the first week of treatment had caused a significant loss in body weight in mice (down to about 75%) and killed 2 of 5 mice in the group (survival rate reduced to 60% after 10 days) (Fig. 6E, 6F), suggesting a potential risk of toxicity for using such high dosage treatment to be effective. Furthermore, EGCG drug dosage in order to induce anti-tumor activity was far greater than a UC second-line chemotherapy drug, paclitaxel, in this case 2 mg/kg three times a week (Fig. 6B, 6D). Representative tumor samples excised from nude mice were shown as figure 6G. Although high concentration EGCG was able to induce anti-tumor cytotoxicity, the risk of toxicity would need to be carefully considered to determine if EGCG treatment would be a practical option compared to the existing chemotherapy drugs.

Figure. 6

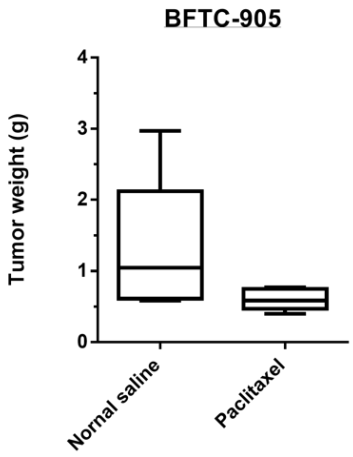
A **B**



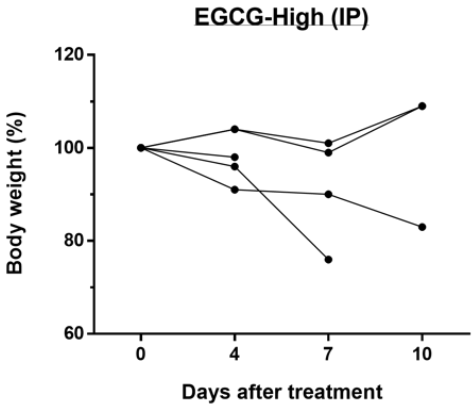
C


BFTC-905
Tumor weight (g)
1.5
1.0
0.5
0.0
Nornal saline
EGCG-Low (PO)
EGCG-Low (IP)
EGCG-High (IP)

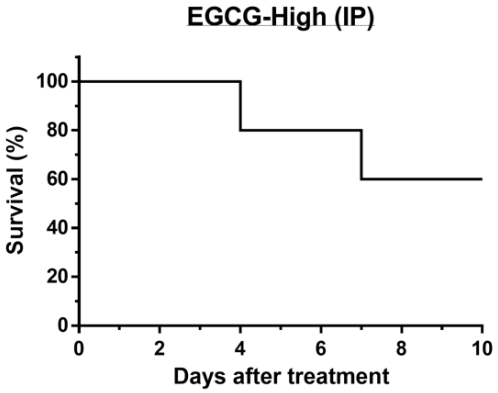
D


BFTC-905
Tumor weight (g)
4
3
2
1
0
Normal saline
Paclitaxel

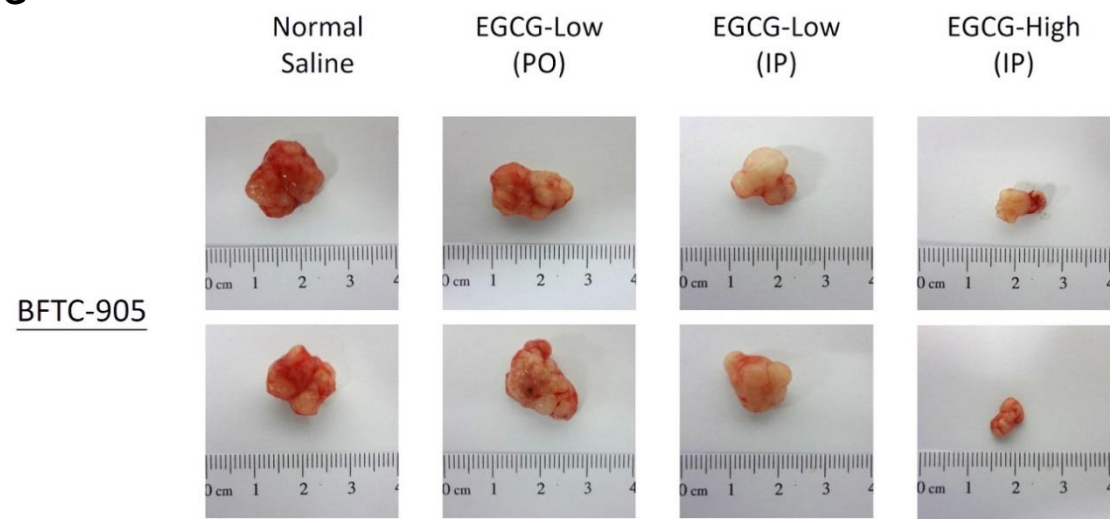
E


EGCG-High (IP)
Body weight (%)
120
100
80
60
0
4
7
10
Days after treatment

F


EGCG-High (IP)
Survival (%)
100
80
60
40
20
0
0
2
4
6
8
10
Days after treatment

G


Normal Saline
EGCG-Low (PO)
EGCG-Low (IP)
EGCG-High (IP)
BFTC-905

陸、結論與建議(分立即可行之建議及長期性建議)

EGCG, the main catechin of green tea, has been reported to elicit anti-tumor effect in different malignancies by intraperitoneal injection (IP) and intravenous injection (IV) in various studies. Conformably, our data showed that EGCG inhibited bladder cancer cell growth in cell-based model and in bladder cancer xenograft mice models by intraperitoneal injection (IP), indicating that EGCG could be an effective with anticancer drugs with exclusively sufficient dosage.

Due to many successful anti-cancer animal trials, oral medication of EGCG is widely considered to be effective as a supplement for cancer treatment. However, we also observed that the bladder tumor cells have not ceased growing with EGCG at a low-dose (<10 μM), at a concentration corresponding to the EGCG human plasma concentration (Cmax: 0.28-7.4 μM) after EGCG oral medication in adults. This raises the question of whether oral administration of green tea, even EGCG, is actually effective for treating bladder cancer.

Our *in vivo* study showed that low-dose EGCG (5 mg/kg/daily) corresponding to the EGCG concentration in plasma after green tea consumption did not repress UC tumor growth in mice. Although some research, such as 100 mg/kg/daily EGCG in bladder cancer SW780 cell mice [39], has showed high-dose EGCG treatment is safe and effective to suppress tumor growth, skepticism has been raised regarding safety of EGCG consumption due to the potential hepatotoxicity that may occur in human and rodents [40-42]. Our results also suggested a toxicity concern *in vivo* and an inefficient remedy of EGCG compare to extant chemo drugs. Another research also indicated a cytotoxicity of LD50 values that range from 78 to 84 μM in mice [2, 43], which

is the concentration corresponding to the high-dose EGCG in our study that have shown to induce strong apoptosis. To suppress cancer cells with such high dose of EGCG (200 mg/kg/daily), clinicians will need to face a dilemma of toxicity and effectiveness. For short-term research, mice serum collected from our EGCG-receiving mice should be further investigated. For long-term objective, although our research have similar findings to other studies where EGCG can enhance chemotherapy drugs with antitumor effect and can potently reverse chemoresistance [44, 45], the risk of toxicity and mechanisms of EGCG should be further evaluated to understand the viability of using EGCG for cancer treatment.

柒、參考文獻

1. Siegel, R., D. Naishadham, and A. Jemal, *Cancer statistics, 2012.* CA: a cancer journal for clinicians, 2012. **62**(1): p. 10-29.
2. Ploeg, M., K.K. Aben, and L.A. Kiemeney, *The present and future burden of urinary bladder cancer in the world.* World journal of urology, 2009. **27**(3): p. 289-93.
3. Latini, D.M., et al., *Bladder cancer detection, treatment and outcomes: opportunities and challenges.* Urology, 2010. **75**(2): p. 334-9.
4. http://tcr.cph.ntu.edu.tw/main.php?Page=A5, T.C.R.W.
5. Hsu, F.S., C.H. Su, and K.H. Huang, *A Comprehensive Review of US FDA-Approved Immune Checkpoint Inhibitors in Urothelial Carcinoma.* J Immunol Res, 2017. **2017**: p. 6940546.
6. Hussain, S.A. and N.D. James, *The systemic treatment of advanced and metastatic bladder cancer.* The Lancet Oncology, 2003. **4**(8): p. 489-497.
7. Harker, W., et al., *Cisplatin, methotrexate, and vinblastine (CMV): an effective chemotherapy regimen for metastatic transitional cell carcinoma of the urinary tract. A Northern California Oncology Group study.* Journal of Clinical Oncology, 1985. **3**(11): p. 1463-1470.
8. Roth, B.J.a.B., D. F., *Advanced bladder cancer: the need to identify new agents in the post-M-VAC (methotrexate, vinblastine, doxorubicin and cisplatin)* world. J Urol,, 1995. **153**.
9. von der Maase, H., et al., *Gemcitabine and Cisplatin Versus Methotrexate, Vinblastine, Doxorubicin, and Cisplatin in Advanced or Metastatic Bladder Cancer: Results of a Large, Randomized, Multinational, Multicenter, Phase III Study.* Journal of Clinical Oncology, 2000. **18**(17): p. 3068-3077.
10. Ueki, O., et al., *Methotrexate, vinblastine, doxorubicin, and cisplatin for advanced urothelial cancer.* Cancer Chemother Pharmacol, 1992. **30 Suppl**(0): p. S72-6.
11. Khan, N., et al., *Targeting multiple signaling pathways by green tea polyphenol (-)-epigallocatechin-3-gallate.* Cancer Res, 2006. **66**(5): p. 2500-5.
12. Tachibana, H., *Green tea polyphenol sensing.* Proceedings of the Japan Academy, Series B, 2011. **87**(3): p. 66-80.
13. Colomer, R., et al., *Natural Polyphenols and their Synthetic Analogs as*

Emerging Anticancer Agents. Curr Drug Targets, 2017. **18**(2): p. 147-159.

14. Qin, J., et al., *A component of green tea, (-)-epigallocatechin-3-gallate, promotes apoptosis in T24 human bladder cancer cells via modulation of the PI3K/Akt pathway and Bcl-2 family proteins.* Biochem Biophys Res Commun, 2007. **354**(4): p. 852-7.

15. Tachibana, H., *Molecular basis for cancer chemoprevention by green tea polyphenol EGCG.* Food Factors for Health Promotion, 2009. **61**: p. 156-169.

16. Shammas, M.A., et al., *Specific killing of multiple myeloma cells by (-)-epigallocatechin-3-gallate extracted from green tea: biologic activity and therapeutic implications.* Blood, 2006. **108**(8): p. 2804-2810.

17. Fang, M.Z., et al., *Tea polyphenol (-)-epigallocatechin-3-gallate inhibits DNA methyltransferase and reactivates methylation-silenced genes in cancer cell lines.* Cancer Res, 2003. **63**(22): p. 7563-70.

18. Meeran, S.M., et al., *A novel prodrug of epigallocatechin-3-gallate: differential epigenetic hTERT repression in human breast cancer cells.* Cancer Prev Res (Phila), 2011. **4**(8): p. 1243-54.

19. Yang, C.S., et al., *Cancer prevention by tea: animal studies, molecular mechanisms and human relevance.* Nat Rev Cancer, 2009. **9**(6): p. 429-39.

20. Jankun, J., R.W. Keck, and S.H. Selman, *Epigallocatechin-3-gallate prevents tumor cell implantation/growth in an experimental rat bladder tumor model.* Int J Oncol, 2014. **44**(1): p. 147-52.

21. Dettlaff, K., et al., *Formulation and characterization of EGCG for the treatment of superficial bladder cancer.* Int J Mol Med, 2017. **40**(2): p. 329-336.

22. Chisholm, K., B.J. Bray, and R.J. Rosengren, *Tamoxifen and epigallocatechin gallate are synergistically cytotoxic to MDA-MB-231 human breast cancer cells.* Anticancer Drugs, 2004. **15**(9): p. 889-97.

23. Sakata, M., et al., *Prevention of mammary carcinogenesis in C3H/OuJ mice by green tea and tamoxifen.* Asian Pac J Cancer Prev, 2011. **12**(2): p. 567-71.

24. Fujiki, H., et al., *Cancer Prevention with Green Tea and Its Principal Constituent, EGCG: from Early Investigations to Current Focus on Human Cancer Stem Cells.* Mol Cells, 2018. **41**(2): p. 73-82.

25. Fujiki, H., et al., *Synergistic enhancement of anticancer effects on numerous human cancer cell lines treated with the combination of EGCG, other green tea catechins, and anticancer compounds.* J Cancer Res Clin Oncol, 2015. **141**(9): p.

1511-22.

26. Wiseman, S., T. Mulder, and A. Rietveld, *Tea flavonoids: bioavailability in vivo and effects on cell signaling pathways in vitro.* Antioxid Redox Signal, 2001. **3**(6): p. 1009-21.

27. Shen, S.R. and S. Lay, *A discussion on "Tea and human health: biomedical functions of tea active components and current issues".* J Zhejiang Univ Sci B, 2015. **16**(9): p. 811-2.

28. Chen, Z.M. and Z. Lin, *Tea and human health: biomedical functions of tea active components and current issues.* J Zhejiang Univ Sci B, 2015. **16**(2): p. 87-102.

29. van het Hof, K.H., et al., *Consumption of green or black tea does not increase resistance of low-density lipoprotein to oxidation in humans.* Am J Clin Nutr, 1997. **66**(5): p. 1125-32.

30. Ting Sun , F.S., and Chi-Tang Ho, *Bioavailability and metabolism of tea catechins in human subjects.* . Tea and Tea Products. Chemistry and Health-Promoting Properties, ed. J.-K.L. Chi-Tang Ho , and Fereidoon Shahid. 2008.

31. Jacob, S.A., T.M. Khan, and L.H. Lee, *The Effect of Green Tea Consumption on Prostate Cancer Risk and Progression: A Systematic Review.* Nutr Cancer, 2017. **69**(3): p. 353-364.

32. Huang, K.H., et al., *Celecoxib-induced cytotoxic effect is potentiated by inhibition of autophagy in human urothelial carcinoma cells.* PLoS One, 2013. **8**(12): p. e82034.

33. Huanwen, W., et al., *Intrinsic chemoresistance to gemcitabine is associated with constitutive and laminin-induced phosphorylation of FAK in pancreatic cancer cell lines.* Mol Cancer, 2009. **8**: p. 125.

34. Kanno, T., et al., *1-[2-(2-Methoxyphenylamino)ethylamino]-3-(naphthalene-1-yloxy)propan-2-ol as a potential anticancer drug.* Pharmacology, 2013. **91**(5-6): p. 339-45.

35. Guo, J.R., et al., *Effects of karanjin on cell cycle arrest and apoptosis in human A549, HepG2 and HL-60 cancer cells.* Biol Res, 2015. **48**: p. 40.

36. Hour, T.C., et al., *Characterization of molecular events in a series of bladder urothelial carcinoma cell lines with progressive resistance to arsenic trioxide.* Anticancer Drugs, 2004. **15**(8): p. 779-85.

37. Yu, H.J., et al., *Characterization of a newly established human bladder carcinoma cell line, NTUB1.* J Formos Med Assoc, 1992. **91**(6): p. 608-13.

38. Huang, K.H., et al., *Down-regulation of glucose-regulated protein (GRP) 78 potentiates cytotoxic effect of celecoxib in human urothelial carcinoma cells.* PLoS One, 2012. **7**(3): p. e33615.

39. Luo, K.W., et al., *EGCG inhibited bladder cancer T24 and 5637 cell proliferation and migration via PI3K/AKT pathway.* Oncotarget, 2018. **9**(15): p. 12261-12272.

40. Luo, K.W., et al., *EGCG inhibited bladder cancer SW780 cell proliferation and migration both in vitro and in vivo via down-regulation of NF-kappaB and MMP-9.* J Nutr Biochem, 2017. **41**: p. 56-64.

41. Ramachandran, B., et al., *Repeated dose studies with pure Epigallocatechin-3-gallate demonstrated dose and route dependant hepatotoxicity with associated dyslipidemia.* Toxicol Rep, 2016. **3**: p. 336-345.

42. Li, F., et al., *Perspectives on the recent developments with green tea polyphenols in drug discovery.* Expert Opin Drug Discov, 2018. **13**(7): p. 643-660.

43. Chan, P.C., et al., *Fourteen-week toxicity study of green tea extract in rats and mice.* Toxicol Pathol, 2010. **38**(7): p. 1070-84.

44. Meng, Q., C.N. Velalar, and R. Ruan, *Effects of epigallocatechin-3-gallate on mitochondrial integrity and antioxidative enzyme activity in the aging process of human fibroblast.* Free Radic Biol Med, 2008. **44**(6): p. 1032-41.

45. Zhang, Y., et al., *Green tea polyphenol EGCG reverse cisplatin resistance of A549/DDP cell line through candidate genes demethylation.* Biomed Pharmacother, 2015. **69**: p. 285-90.