國立嘉義大學生命科學院

學生學術研究成果優良海報評選獲獎名單

時間:108 年 6 月 5 日

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Research and Development of the Extract *from Cirsium arisanense* Kitamura Roots as Hepatoprotective Agents

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Introduction

According to the statistics of the Department of Health, Executive Yuan Taiwan, liver disease is one of the leading causes of death in Taiwan. Liver disease is mainly resulting from hepatitis virus, followed by alcoholism and drug abuse. Therefore, finding therapy of liver disease is in need. While modern people advocate healthy eating, herbal medicines are becoming more and more popular, but most of them lack scientific evidences to prove their effects. Thus, how to develop and evaluate the value of herbal medicines for treating certain diseases is also a top priority. Compositae plants are the third biggest flowering plants in Taiwan. Many of the compositae plants have been frequently practiced as folk medicines locally, such as thistle plants, which have been claimed for hepatoprotective effects, like silymarin from milk thistle herb. Silymarin is first-line drugs used clinically on liver diseases, but due to its long-term efficacy leading to its poor clinical efficacy. A unique native plant in Taiwan: *Cirsium arisanense* Kitamura (CaK), which is traditionally documented as herbal medicine with hepatoprotective property by aboriginal culture. Science evidences to fulfill the wisdom of aboriginal ancestral practices were demonstrated in this study. This experiment would prove whether the root extract of CaK can effectively improve the efficacy of treatment of liver disease.

Cytotoxicity assay

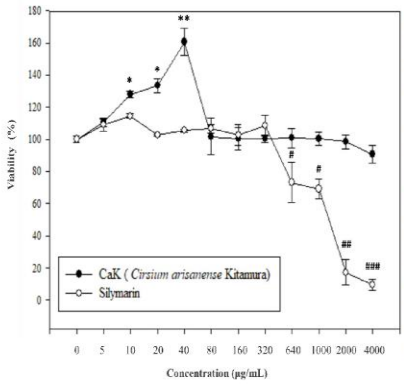

Figure 1. CaK was less cytotoxic than silymarin
The investigation on cytotoxicity of CaK and silymarin was performed on BNL CL.2 by MTT assay.
*,** P<0.05, 0.01 CaK V.S. 0 μg/mL, #,##,### P<0.05, 0.01, 0.001 silymarin V.S. 0 μg/mL.

The therapeutic effect on ethanol-induced injury of BNL CL.2

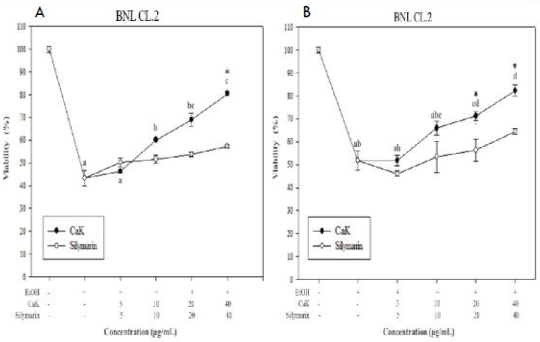

Figure 2. CaK dose-dependently protected and repaired BNL CL.2 cells from ethanol-induced injury.

A. BNL CL.2 cells were pre-incubated with different levels of CaK and silymarin (0-40 μg/mL) for 24 hours prior to the 10% ethanol-induced injury. Cells viability monitored by MTT assay. n=3,*P<0.05 V.S. 40 μg/mL silymarin, [abc] Bars of the same compound treatment with different superscript are significantly different (P<0.05)

B. BNL CL.2 cells were exposed to 10% ethanol-induced injury for 1 hour. After removal of ethanol, cells were incubated with CaK and silymarin (0-40 μg/mL) for 24 hours. The regenerative capacity from ethanol-induced injury was evaluated by the cell viability monitored with MTT assay. n=3, *P<0.05 V.S. 20, 40 μg/mL silymarin, [abc] Bars of the same compound treatment with different superscript are significantly different (P<0.05)

Cell regeneration ability

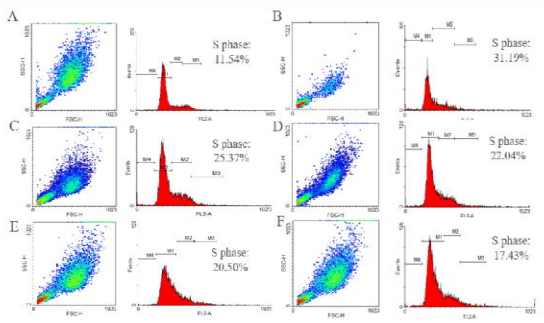

Figure 3. CaK dose-dependently normalized cell-integrity and accelerated BNL CL.2 cell cycle from ethanol-induced injury.
Except for the (A) negative control group, BNL CL.2 cells post ethanol-induced injury were treated with different levels of CaK using (B) 0, (C) 5, (D) 10, (E) 20, (F) 40 μg/mL for 24 hours.

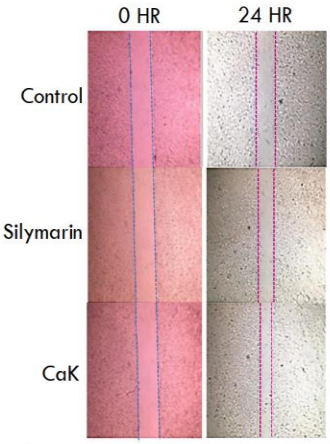

Figure 4. BNL CL.2 cells treated with CaK had averagely improved wound recovery than those treated with silymarin.
BNL CL.2 cells were incubated with 40 μg/mL CaK or silymarin for 24 hours after scratch injury. The scratch area and the gap recovery were quantitatively analyzed.

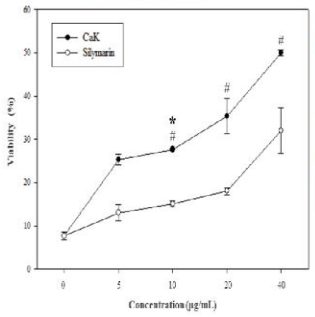

Figure 5. The effects of CaK on BNL CL.2 cells regeneration post scratch-induced injury.
BNL CL.2 cells were incubated with different levels of CaK or silymarin (0-40 μg/mL) for 24 hours after scratch injury. #P<0.05 V.S. 0 μg/mL, *P<0.05 V.S. 10 μg/mL silymarin.

CCl_4-intoxicated mouse liver-injury model

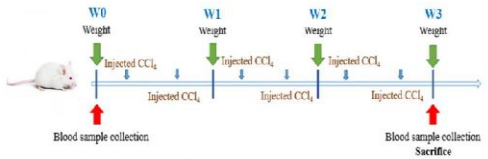

Figure 6. Flow chart of animal study
Mice were randomly assigned to normal control group and CCl_4-intoxicated treatment groups. The treatment groups included: dH_2O vehicle control, silymarin (200 mg/kg/BW, Surai PF *et al.*, 2015), two levels of CaK (100, 200 mg/kg/BW). With the exception of the normal control group, all treatments were orally administered every morning. For CCl_4 intoxication, mice were injected with 10 μl CCl_4 (80% v/v in olive oil) intraperitoneally twice a week for an entire 3-week period.

Table 1. CaK reduced liver inflammation and normalized serum biomarkers of CCl_4-induced liver injury in mice.
*P<0.05 vs. house keeping control (normal control group), **P<0.01 vs. house keeping control, ***P<0.001 vs. house keeping control, ****P<0.0001 vs. house keeping control.

Serum component	House keeping control	dH_2O vehicle	Silymarin	Low CaK	High CaK
GOT (IU/l)	121.20±38.47	2264.00±33.86****	1587.00±92.58****	486.00±42.94***	431.33±67.62**
GPT (IU/l)	24.36±4.94	1088.00±46.54****	974.00±66.38***	222.50±77.50**	234.40±40.32**
T-p (g/ml)	5.76±1.57	3.50±2.51*	5.00±1.11	4.00±0.92	5.10±0.23
Albumin (g/dl)	6.03±0.93	3.40±0.77*	6.50±0.51*	5.20±0.92	6.50±0.71
Globulin (g/dl)	0.47±0.10	0.90±0.01**	0.82±0.02*	0.45±0.02	0.67±0.12
Mean body weight (g)	30.65±0.95	23.50±0.92***	29.64±1.92	29.70±2.34	31.12±1.45
Relative liver weight	4.24±0.20	6.72±0.21***	5.65±0.22**	5.65±0.27**	5.90±0.15**

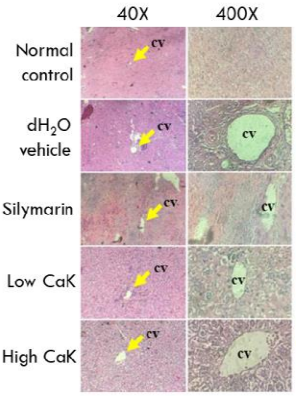

Figure 7. Steatosis and infiltrated Kupffer cells were clearly observed in the dH_2O vehicle-control groups , whereas steatosis as well inflammatory infiltration were less evident of the CaK and silymarin groups.
H&E staining; magnification x40,x400 of liver tissues. (CV: central vein)

Analysis and comparison of major active components

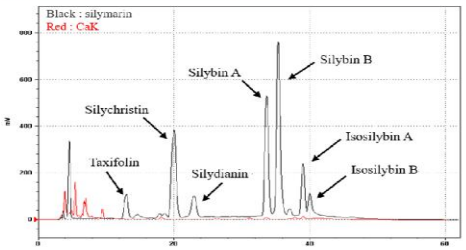

Figure 8. Major active components of CaK were distinguished from those in silymarin.

Discussion

CCl_4 generated radical metabolites during liver cell metabolism can cause inflammation, impact electron transport chain in mitochondria and induce membrane lipid peroxidation, and fatty degeneration of liver. To compare with silymarin, CaK didn't show beneficial effect in reducing liver steatosis (Fig.7); however it significantly lowered serum GOT and GPT levels (P<0.05) at 3-week blood sample (Table 1.); furthermore, relative liver weight was lower (P<0.05) as compared to that of dH_2O vehicle group indicated less liver inflammation. *In vitro* study of cell viability, flow cytometric cell integrity and cell cycle of ethanol-damaged BNL CL.2 cells (Fig 2. and Fig 3.) was in concert with the protective property of CaK in *in vivo* CCl_4 model which maybe resulted of accelerated hepatic regeneration. In scratch wound healing assay, the marginal cells could release growth factors and initiate cell replication to complete repairing action (Zahm. *et al.*,1997). When compared with the silymarin, it demonstrated that CaK had superior wound healing property than silymarin (Fig 4.) and dose-dependently improved wounded cells viability (Fig 5.), it might be speculated that CaK played the role of coenzymes assisting the cells to express higher level of growth factors and creating a superior healing environment. Moreover, based on the cell integrity and cell cycle analyzed by flow cytometry during ethanol-induced injury, CaK dose-dependently normalized cell-integrity as normal cells and induced transient cell cycle arrest in S phase that progressed to G2/M phase (Fig.3.). One argument of the hepatoprotective mechanisms of silymarin was to suppress the activation of NF-κB leading to inhibit the production of inflammatory TNF-α, affect the transcription of IL-6, and restrain the activation of STAT 3, in which it was speculated that liver repair and liver cell regeneration was hampered (Fig 9). CaK may be an alternative to silymarin in distinctive hepatoprotective mechanism by strengthening liver cell viability and improving liver cells regeneration. Additionally, the HPLC chromatogram showed major active components of silymarin were devoid in extracts of CaK (Fig.8). Silymarin and CaK belongs to same genesis in taxonomy, the active hepatoprotective components of CaK remains to be identified.

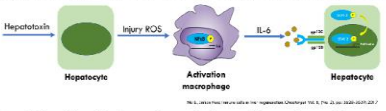

Figure 9. The relationship of macrophage activation, cytokine secretion, and liver regeneration.

Reference

1. Kuo-Lung Ku, Chu-Tsai Tsai, Wei Min Chang, Mei-Lin Shen, Chia-Tien Wu, Hui-Fen Liao Hepatoprotective Effect of Cirsium arisanense Kitamura in Tacrine-Treated Hepatoma Hep 3B Cells and C57BL Mice. The American Journal of Chinese Medicine, 355-368; 2008
2. Li Z, Lin H, Zhu Y, Wang M, Luo J. Disruption of cell cycle kinetics and cyclin-dependent kinase system by ethanol in cultured cerebellar granule progenitors. Elsevier, 14,132(1):47-58, 2001
3. Wang, C.P.; Zhou, L., Su, S. H.; Chen, Y.; Lu, Y.Y.; Wang, F.; Jia, H., Yang, Y.P. . Augmenter of liver regeneration promotes hepatocyte proliferation induced by Kupffer cells. World J Gastroenterol 12: 4859-4865; 2006

比較膀胱癌病患與非膀胱癌者*GSTM2*、*3*、*4*、*5*基因甲基化之程度

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Absctract

Due to the high recurrence rate of bladder cancer, it is urgent to find biomarkers as its prognostic indicators. It is known that glutathione *S*-transferase Mu1 (*GSTM1*) null genotype is a risk factor for bladder cancer. Our previous study found that *GSTM5* DNA is highly methylated in bladder cancer cells, and *GSTM5* gene expression could be increased by a DNA methyltransferase inhibitor. Here, we aim to analyze whether *GSTM1* null genotype and *GSTM2*、*3*、*4*、*5* DNA methylation level could be utilized as biomarkers for bladder cancer. We extracted the genomic DNA from urine of 50 normal people and the cancer tissues of 50 bladder cancer patients, and processed them with bisulfite conversion to obtain the *GSTM5* gene fragment. The coverage ranged from -209 bp to +198 bp, a total of 407 bp and containing 25 CpG sites. Comparing the results of *GSTM5* methylation in 100 subjects, it was found that the highest and lowest *GSTM5* methylation rates in cancer tissues of bladder cancer patients were 95.2% and 30%, with an average of 65.92%; The highest and lowest *GSTM5* methylation ratio of normal people were 95.6 % and 4.8%, with an average of 54.44%. After statistical calculation, the two groups did have statistical significance ($P < 0.05$), representing bladder cancer. The degree of *GSTM5* methylation in patient cancer tissues is higher than that in normal human. In addition, 10 bladder cancer patients had cancerous tissues and their adjacent non-cancerous tissues. Compared with their *GSTM5* methylation degree, 6 bladder tumor tissues were found to have higher *GSTM5* methylation degree than advanced noncancerous tissue. There was no difference between the bladder tumor tissue and the adjacent noncancerous tissue after statistical comparison of the mean values of the 10 patients. In the *GSTM1* gene test results, it was found that 60% or more of the gene deletion ratios were 64% and 62%, respectively, in bladder cancer patients or non-bladder cancer subjects, that is, the *GSTM1* gene in bladder cancer patients. Based on the above results, we propose to use urine samples to detect the methylation ratio of *GSTM5* gene, which can predict whether the risk of bladder cancer is high. Only 100 subjects are currently used. If the number of subjects can be increased, and the urine samples of bladder cancer patients can be tested at the same time, it may be possible to use the *GSTM5* gene methylation ratio test in the future. One of the screening programs and one of the post-treatment tracking programs for patients with bladder cancer.

Introduction

GST Mu methylation regulate cancer

- In Barrett's adenocarcinoma, promoter DNA hypermethylation regulate gene expression of several members of the Mu-class Glutathione-*S*-Transferases.

Gut. 2009; 58(1): p.5–15.

Downregulation of *GSTM5* by DNA CpG methylation

- In our previous studies found that *GSTM5* DNA is highly methylation in bladder cancer cells, and *GSTM5* gene expression could be increased by a DNA methyltransferase inhibitor.

PLoS One. 2016; 11(7): e0159102

Diagnosis of bladder cancer on specific gene methylation from voided urine

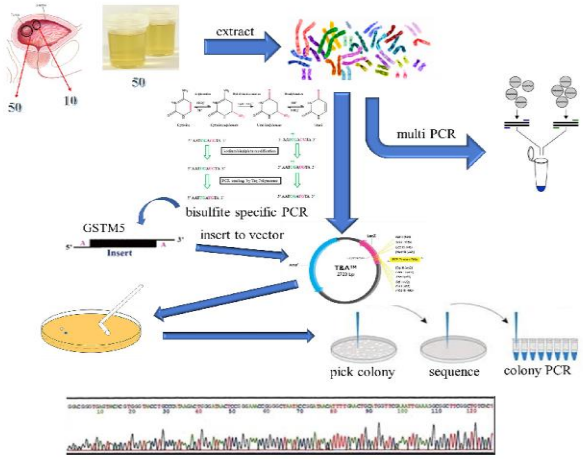
- Distinct methylation pattern in bladder cancer with frequent methylation of RARβ, DAPK, E-cadherin, and p16. Detection of gene methylation in routine voided urine using selected markers appeared to be more sensitive than conventional urine cytology.

Clinical Cancer Research. 2002;8(2):464–470.

Aim

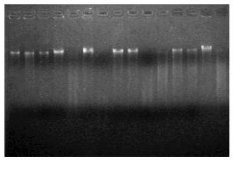
We aim to analyze whether *GSTM1* null genotype and *GSTM2*、*3*、*4*、*5* DNA methylation level could be utilized as biomarkers for bladder cancer.

Materials and Methods



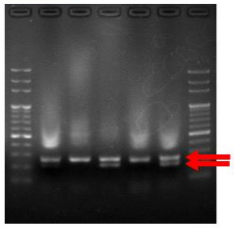
Result

Result 1. Make sure the sample of extracted gDNA.



Assessed gDNA quality by 1% agarose gel electrophoresis. If extracted gDNA well, could observe well gDNA bands with no obvious signs of fragmentation.

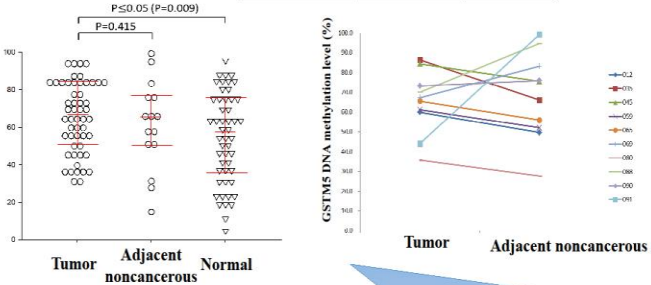
Result 2. Use multi PCR to detection *GSTM1* expression.



The β-globin was used as an internal control for gDNA extraction, *GSTM1* was target gene. Potential problems in a simple PCR include false negatives due to reaction failure or false positives due to contamination, so use multi PCR to prevent false negatives,

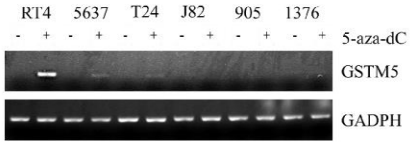
Result 3. Basic data of one hundred subjects and summary of *GSTM1*-null (-) and *GSTM5* DNA methylation level in human bladder tumor tissues, adjacent noncancerous tissues and health urine pellets.

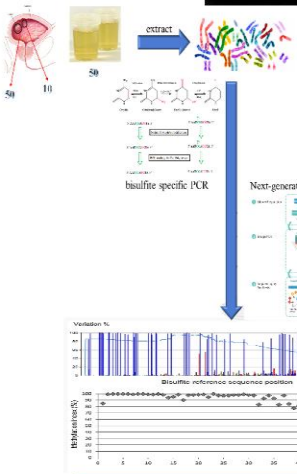
	Bladder cancer patient (n=50)	Normal person (n=50)
Sexuality (Male/Female)	32/18	11/39
Age (Min/Max)	28/92	21/81
Methylation level (Low/High)	30.8/95.2	4.8/95.6
GSTM1(+/-)	18/32	19/31



According to the *GSTM1* expression under PCR analysis, we found that about 64.5% people lacked *GSTM1* gene. Besides, it showed that the DNA CpG methylation level of *GSTM5* gene were higher in bladder cancer tissues than in adjacent noncancerous tissues and normal urine pellets.

Result 4. Effects of 5-aza-dC on the expression of *GSTM5* in human bladder cancer cell lines, and sequencing by NGS.





NGS v.s sanger sequencing

NGS can read thousands of sequences at the same time is more representative than sanger sequencing which only has 10 results. And sanger needs to undergo plasmid reproduction by bacteria. NGS can be directly sequenced without this step to reduce the incidence of errors.

	Read length (base pair)	Templates per run
Next-generation sequencing	600 bp	> 5000 reads
Sanger sequencing	700~800 bp	1 read

GSTM5 mRNA was increased by 5-aza-dC treatment in RT4 and 5637cells. Preparing sample by NGS, and compare with sanger sequencing.

Conclusion

- DNA methylation level of *GSTM5* has the potential to become a biomarker of bladder cancer.
- In the future, we hope to collect the urine and tumor of bladder cancer patients for *GSTM2-5* methylation analysis, together with normal person to observe the difference.

尋找提升*GSTM4*轉錄活性之轉錄因子與小分子化合物

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Abstract

Nowadays, due to tremendous changes in lifestyle, people often accumulate large amounts of toxins. Our body needs to metabolize these toxins by enzymes. Glutathione *S*-transferase (GST) is one of the important detoxifying enzymes that catalyze the conjugation of electrophilic substrates to glutathione. *GSTM* family is one of the *GST* gene superfamilies and can be divided into five subtypes *GSTM1-5*. Among them, polymorphisms of *GSTM1* gene is homozygous deletion (null genotype), which has been suggested to be associated with the loss of enzyme activity and resulted in the increased susceptibility to cancer. And there is increasing evidence demonstrating that oxidative injury due to electrophilic compounds is a major cause of DNA damage that correlates with cancer. However, about 50% of humans lack *GSTM1*, which belongs to a null mutation, but other *GSTM* family genes *GSTM2-5* don't have the null mutation. Therefore, if we increase the expression of other *GSTM* family genes, perhaps it can compensate for the absence of *GSTM1* and reduce the incidence of bladder cancer. GSTM4 is widely distributed in human tissues including the bladder and can be metabolized to the same substrate as GSTM1-3. Since *GSTM4* has the potential to compensate for the lack of antioxidant capacity caused by the deletion of the *GSTM1* gene, therefore, the purpose of this study is to analyze human *GSTM4* gene promoter transcriptional factors regulating the gene expression and looking for small molecule compounds that enhance its transcriptional activity to reducing the risk of bladder cancer by up-regulating *GSTM4* gene. We cloned the *GSTM4* gene promoter and six other different lengths of 5'-deletion *GSTM4* gene promoter and transfected into BFTC 905 bladder cancer cells using a reporter gene assay to observe its transcriptional activity. We found that at -5/ 93 human *GSTM4* gene promoter fragment has a slightly down-regulation of activity in cell experiments. This result means that there is an important transcription factor binding site that regulates the *GSTM4* transcription activity in -88~-5 bp. On the other hand, small molecule compounds, in which we found that three organic phytochemicals such as Resveratrol, Wogonin, and Baicalein can enhance the transcriptional activity of *GSTM4*, and it is expected to be a small molecule compound that can enhance the expression of *GSTM4* gene.

Introduction

◆ Glutathione *S*-transferase

Glutathione *S*-transferases (GST) are important detoxifying enzymes that catalyze the conjugation of electrophilic substrates to glutathione.

Trends in Pharmacological Sciences. 2012;33(12):656-68.

◆ Glutathione *S*-transferase Mu

Polymorphisms of *GSTM1* gene is homozygous deletion (null genotype), which has been suggested to be associated with the loss of enzyme activity, and resulted in the increased susceptibility to cancer.

Journal of Toxicology and Environmental Health. 2017;80(7-8):423-429.
Annual Review of Pharmacology and Toxicology. 2005;45:51-88.
Journal of Cancer Research and Therapeutics. 2016;12(2):712-5.
Oncogene. 2006;25:1639-48

◆ Glutathione *S*-transferase Mu 4

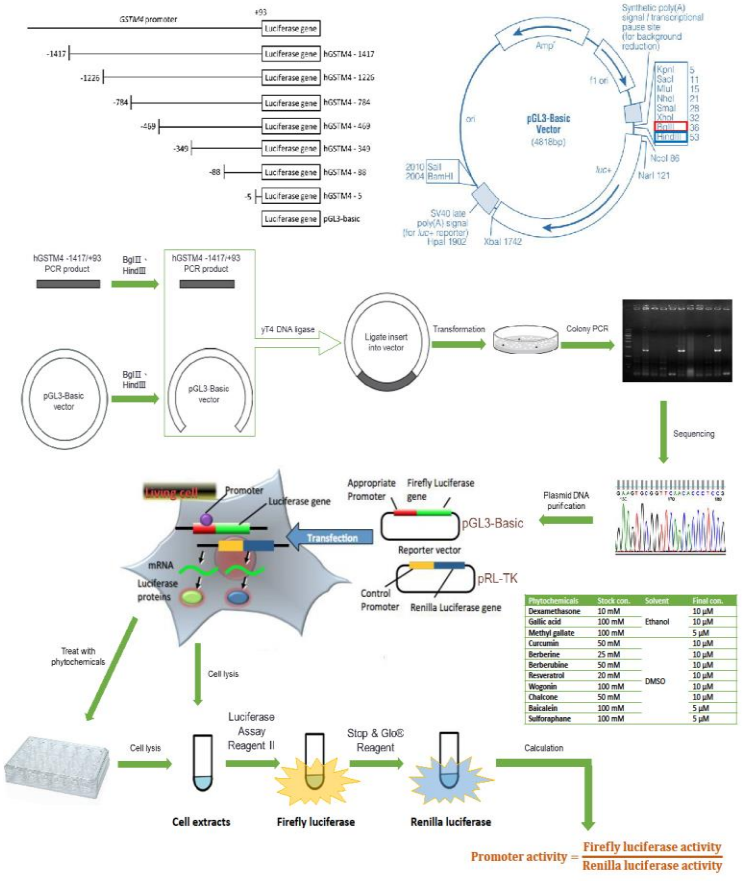
GSTM4 can metabolize the same substrate as GSTM1-3, but it is lower metabolic activity for 1-chloro-2, 4-dinitrobenzene (CDNB).

Archives of Biochemistry and Biophysics. 1994;311(2):487-495.

Aim

I would like to explore the mechanism of GSTM4 expression from the gene promoter. If we find a way to regulate GSTM4 expression, perhaps it will provide the function of protecting cells lacking *GSTM1* before cancer, and inhibit the growth of bladder cancer cells.

Materials and Methods



Phytochemicals	Stock con.	Solvent	Final con.
Dexamethasone	10 mM	Ethanol	10 μM
Gallic acid	100 mM		10 μM
Methyl gallate	100 mM		5 μM
Curcumin	50 mM	DMSO	10 μM
Berberine	25 mM		10 μM
Berberubine	50 mM		10 μM
Resveratrol	20 mM		10 μM
Wogonin	100 mM		10 μM
Chalcone	50 mM		10 μM
Baicalein	100 mM		5 μM
Sulforaphane	100 mM		5 μM

Results

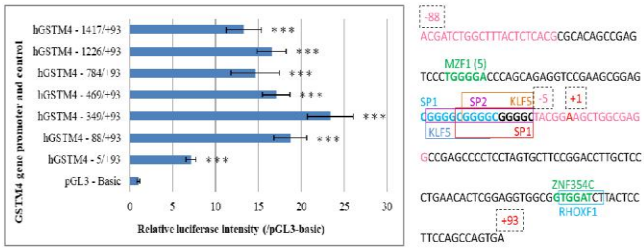


Fig. 1 Transcriptional activity analysis of 5' deletion human *GSTM4* gene promoter

The activity of the shortest -5/+93 *GSTM4* gene promoter fragment was more down-regulated. In addition, all the fragment activities were higher than pGL3-Basic and there were significant differences. P values, 0.05 were considered to be statistically significant. *p<0.05, **p<0.01, ***p<0.001.

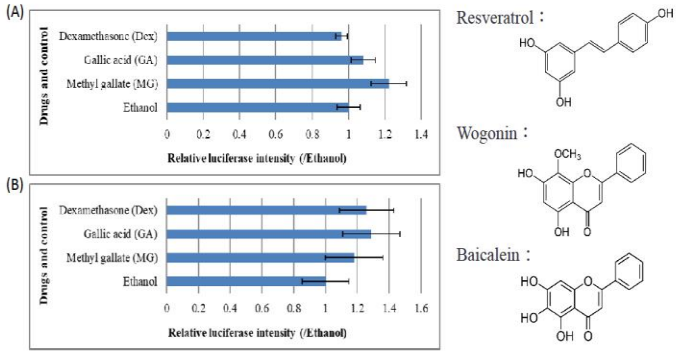


Fig. 2 Transcriptional activity analysis of human *GSTM4* gene promoter-inducer treatment (EtOH)

The EtOH group did not significantly enhance the transcriptional activity of *GSTM4* gene promoter. (A) Firefly/Renilla；(B) Only firefly

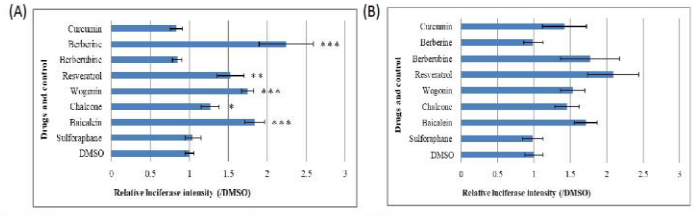


Fig. 3 Transcriptional activity analysis of human *GSTM4* gene promoter-inducer treatment (DMSO)

The Berberine, Resveratrol, Wogonin, and Baicalein of DMSO group induce a significant increase in the transcriptional activity of the *GSTM4* gene promoter compared to the control group, but we find that some phytochemicals would directly impact the Renilla luciferase activity in the dual luciferase reporter assay, which leads to calibration failed. After we independently use firefly luciferase activity for analysis, only three phytochemicals, Resveratrol, Wogonin, and Baicalein, were found to enhance the transcriptional activity of *GSTM4*. *p<0.05, **p<0.01, ***p<0.001.
(A) Firefly/Renilla；(B) Only firefly

Conclusions

- The shortest -5/+93 human *GSTM4* gene promoter fragment has an obviously down-regulated activity in cell assays, indicating that there is a transcription factor important for *GSTM4* gene expression in the human *GSTM4* gene promoter -5 to -88 bp interval.
- The phytochemicals **Resveratrol**, **Wogonin**, and **Baicalein** are indeed inducible for the activity of the *GSTM4* gene promoter, so there is a chance in the future to be a candidate compound that can induce the expression of the intracellular *GSTM4* gene.

國立嘉義大學生命科學院

學生學術研究成果優良海報評選獲獎名單

時間:108 年 6 月 5 日

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Parecoxib synergizes 5-fluorouracil-inhibited migration and invasion in colon cancer

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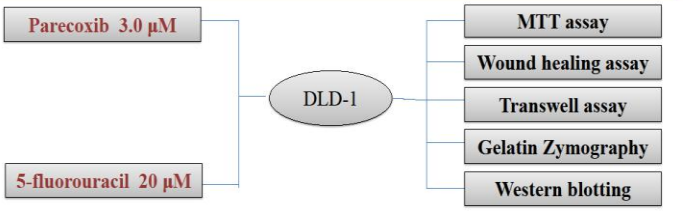
Abstract

It is reported that the incidence of colon cancer is the third highest among all malignant tumors. Patients with advanced colon cancer usually have poor clinical treatment effects and a 5-year survival rate of 20% results from widespread metastasis and drug resistance. 5-fluorouracil (5-FU) has played an important role in the treatment of colon cancer and other cancers. Despite its many advantages, clinical applications have been greatly limited due to the drug resistance. The overall response rate for advanced colorectal cancer of 5-FU alone is still only 24%. Many anti-inflammatory including Non-steroidal anti-inflammatory drugs (NSAIDs) agents can alter the tumors themselves or the tumor microenvironment, potentially decreasing migration, increasing apoptosis, and increasing sensitivity to other therapies. Parecoxib (PC) is a selective cyclooxygenase (COX)-2 inhibitor widely used as an analgesia technique in perioperative period for its potent anti-inflammatory and analgesic effects. The aims of my thesis are focus on the enhancement of parecoxib in 5-fluorouracil treatment in metastatic colon cancers and its underling molecular mechanisms. My results show that the combination of 5-fluorouracil and parecoxib can enhance the inhibition of migration and invasion in colorectal cancer DLD-1 cell line by enhancing the inhibition of matrix metalloproteinases MMP-2, MMP-9 and phosphorylated-Akt, phosphorylated-IKK, phosphorylated-IκB, phosphorylated-p65, and enhancing the inhibition of epithelial-mesenchymal transition (EMT)-related protein, increased E-cadherin (an epithelial marker) expression, and decreased β-catenin expression(a mesenchymal marker).

Materials and Methods



Results

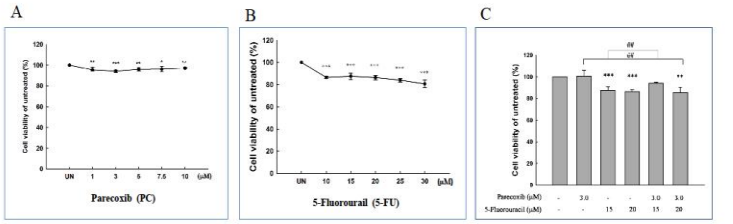
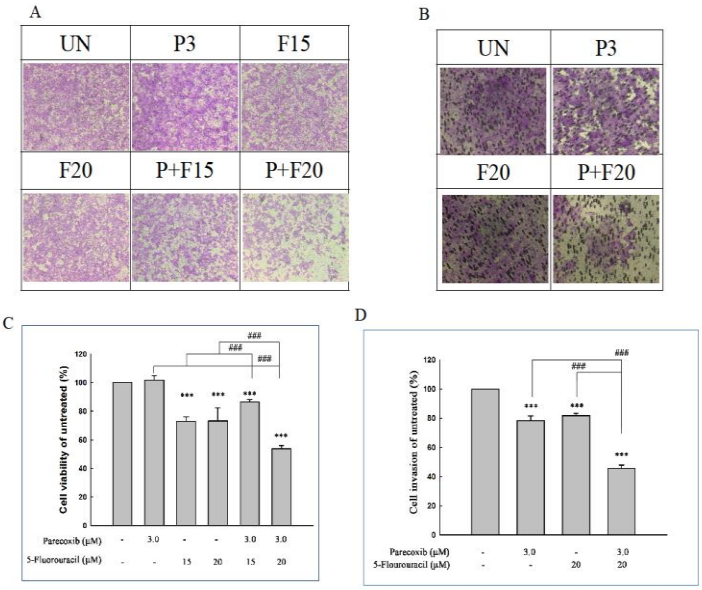


Figure 1 .Effect of Parecoxib and 5-Fluorourail with 48 hours in DLD-1 cell viability by MTT assay
(A) DLD-1 cells were treated with parecoxib in the following concentrations: 0, 1, 3, 5, 7.5, 10 μM for 48 hours. (B) DLD-1 cells were treated with 5-fluorouracil in the following concentrations: 0, 10, 15, 20, 25 , 30 μM for 48 hours. (C) DLD-1 cells were treated with parecoxib (3 μM) and 5-fluorouracil (15, 20 μM) for 48 hours. Viable cells were determined by MTT test. The values shown are expressed as mean ± SD (n=3/group) Significant differences from untreated group are *** p< 0.001 compare with untreated. ### p< 0.001 compare with parecoxib or 5-fluorourail.



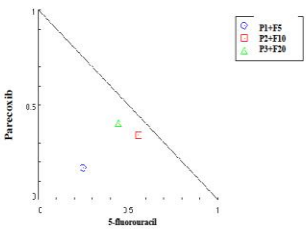


Figure 2. Parecoxib and 5-Fluorourail inhibits cell migration and invasion of DLD-1 cells by Transwell assay. (A) DLD-1 cells were treated with parecoxib (3 μM) and 5-fluorouracil (15, 20 μM). (B) DLD-1 cells were treated with parecoxib (3 μM) and 5-fluorouracil (20 μM). After 48 hr subsequently photographed (x200 magnification) in five random fields. (C) Migration cells. (D) invasion cells were quantified and expressed on the basis of untreated cells (control) representing 1. (E) DLD-1 cells were treated with parecoxib (0, 1, 2, 3 μM) and 5-fluorouracil (0, 5, 10, 15, 20 μM) calculate Normalized Isobologram for Combo by CompSyn. Significant differences from untreated group are *** p< 0.001 compare with untreated. ### p< 0.001 compare with parecoxib or 5-fluorourail.

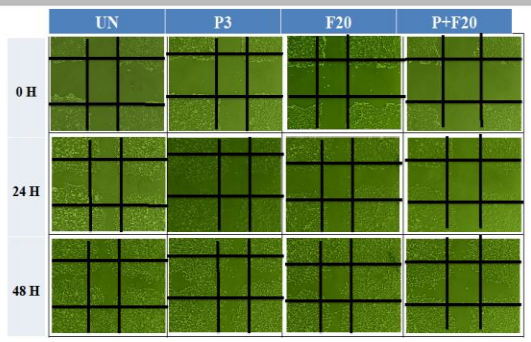


Figure 3. Parecoxib and 5-Fluorourail inhibits cell migration of DLD-1 cells by Wound healing assay. DLD-1 cells were treated with parecoxib (3 μM) and 5-fluorouracil (20 μM). After 24 and 48 hr incubation, cells subsequently photographed (x40 magnification) in five random fields.

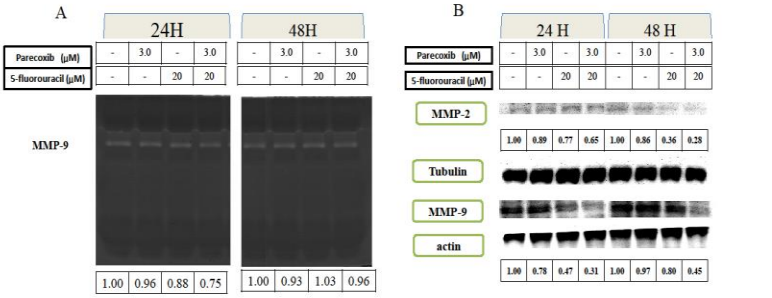


Figure 4. Inhibitory effects of parecoxib and 5-Fluorourail on MMP-2, MMP-9 expression of DLD-1 cells by Gelatin Zymography and Western blotting.(A) DLD-1 cells were treated with parecoxib (3.0 μM) and 5-fluorouracil (20 μM). (A) MMP-9 expression were determined by Gelatin Zymography and (B) by Western blotting. Actin was used as internal control respectively.

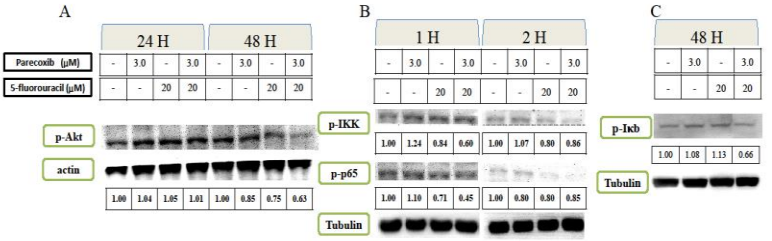


Figure 4. Parecoxib and 5-Fluorourail attenuated pAkt/p-IKK/p-Ikb/p-p65 signaling pathway in DLD-1 cells. DLD-1 cells were exposed to parecoxib (3 μM) and 5-fluorouracil (20 μM)). and cell lysates were thereafter subjected to western blot. (A) Protein expression of p-Akt (B) p-IKK/ p-p65 (C) p-Ikb were determined by western blot. Actin / Tubulin was used as internal control respectively.

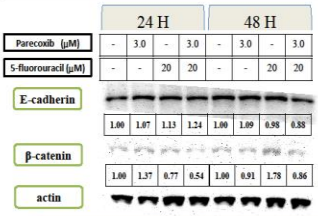
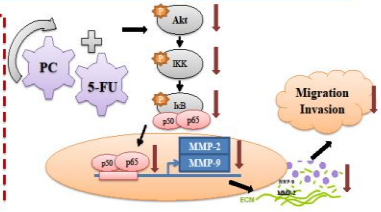


Figure 5. Inhibitory effects of parecoxib and 5-Fluorourail on the EMT-associated protein expression of DLD-1 cells. DLD-1 cells were exposed to parecoxib (3 μM) and 5-fluorouracil (20 μM). and cell lysates were thereafter subjected to western blot, Protein expression of E-cadherin and vimentin were determined by western blot. actin was used as internal control respectively.

Conclusions

1.My studies found that parecoxib can enhance the inhibitory effect of 5-fluorouracil on the metastasis of colorectal cancer.

2.Parecoxib combined with 5-fluorouracil may be a benefit for the research and development of colorectal cancer treatment.



Mechanism of the effect of microsatellite CAI on *Candida albicans* toxicity factors
微衛星 CAI 對白色念珠菌毒性因子影響之機制

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[1] 國立嘉義大學微生物免疫與生物藥學系；[2] 林口長庚紀念醫院內科部感染科；[3] 林口長庚紀念醫院檢驗醫學科

背景

白色念珠菌 (*Candida albicans*) 為人類伺機性真菌感染的病原體，主要分布黏膜表面。已有多種分子方法被應用於分析白色念珠菌基因型，其中微衛星序列分析法指微生物基因體中 2 到 5 個核苷酸所構成之短片段重覆具有高多態性，所以經常被使用作為標記，例如：CAI 和其他 (*HIS3*、*CD3*、*CEF3*)。微衛星 CAI 位於 *RLM1* 基因 ORF 中 3' 端，其為以 glutamine codons (CAA 或 CAG) 單元所構成的重覆區域，可能參與轉錄活化作用。

而 *RLM1* 基因為一個參與酵母菌細胞壁完整性途徑的轉錄因子，其對維持細胞壁完整性扮演重要角色，但目前有關白色念珠菌 *RLM1* 之調控與其下游基因相關研究不多。分析林口長庚醫院菌血症白色念珠菌菌株 CAI 基因型，發現在 clade 17 高死亡率菌群中感染 CAA/G 重複數 ≥29 菌株之 30 天致死率明顯高於 <29 菌株。另外利用顯微注射斑馬魚感染白色念珠菌的研究也顯示，CAI 基因型 31-46 菌株所感染斑馬魚致死率高於 CAI 16-16 菌株感染者，暗示 CAA/G 重複數高的菌株可能毒性越強。

材料與方法

1 菌株

Candida albicans strains:

- Δ*rlm1* mutant: BRY429
- Δ*rlm1* parental wild type: CAF2-1 (Arg^+, Ura^+, His^+) [*ura3*Δ::*imm434*/*ura3*Δ::*imm434*]
- 林口長庚醫院經微衛星 CAI 鑒定基因型菌血症菌株
  - clade 17 CAI genotype clinical strains
  - homologous CAI diploid clinical strains
    - CAI 11-11: C027, C044, C067
    - CAI 25-25: C022, C141, D020, P043
    - CAI 35-35: C068, C091, C155
    - CAI 41-42: C108

2 Germ tube formation assay

菌株培養於含 YPDNP、血清和 Tween 20 之培養液靜置於 37 ˚C，2 小時，誘發 germ tube 生成，分析剔除 *RLM1* 基因及 CAI 基因型對 germ tube 生成之影響。

3 Susceptibility assay

- 菌株培養於含 caspofungin 或剛果紅 (Congo red) 之 YPD broth 以 30˚C，140 rpm 旋轉培養 8 小時，再以 drop assay 稀釋菌液培養於含有壓力劑之 YPD agar plate，觀察菌株生長狀況。
- 菌株培養於 YNB + 2% lactate 以 30 ˚C, 140 rpm 旋轉培養 30 小時，再以 drop assay 稀釋菌液培養於含 caspofungin 之 YNB + 2% lactate agar plate，觀察菌株生長狀況。

4 Real-time RT-PCR

菌株培養於含有 caspofungin 之 YPD broth 以 30 ˚C，140 rpm 旋轉培養 8 小時，再以 real-time RT-PCR 分析 CAI 重覆數對毒力或細胞壁相關基因表達 (*SAP5*、*HWP1*、*ECE1*、*CHT3*) 之影響。

結果與討論

利用 germ tube 生成率分析 *RLM1* 剔除突變株和林口長庚醫院之 CAI 微衛星基因型白色念珠菌菌血症菌株發現 *RLM1* 突變株 (BRY429) 誘發 germ tube 形成率較野生型菌株 CAF2-1 高但兩者之間沒有統計顯著差異。同時，CAI 18-18 菌株 (C026、C074、C104) 以及 CAI 18-41 菌株 (C009、C054、C062) 之間的 germ tube 形成效率亦未發現差異。此外使用具有同源 CAI 二倍體菌株進行實驗發現 CAI 41-42 菌株 C108 germ tube 形成率高於 CAI 11-11 菌株 (C027、C044、C067)、CAI 25-25 菌株 (C022、C141、D020、P043) 和 CAI 35-35 菌株 (C068、C091、C155)，而且 CAI 41-42 菌株與 CAI 11-11、CAI 25-25 或 CAI 35-35 菌株分別比較下皆顯示明顯差異。前述結果顯示 CAI 重複數高可能對 germ tube 生成有一定程度影響。

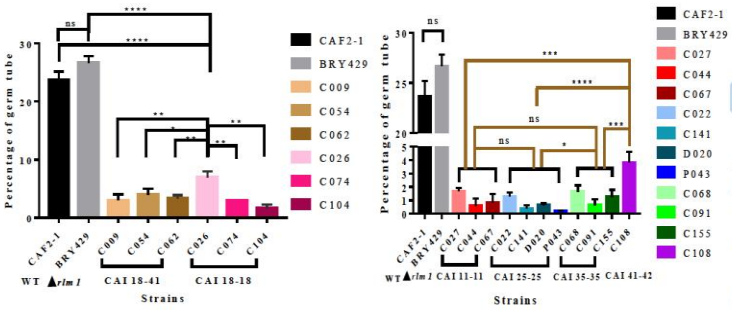

Figure 1. 剔除 *RLM1* 基因及 (A) clade 17 CAI 基因和 (B) 同源 CAI 二倍體臨床菌株對 germ tube 生成之影響。

以 drop assay 分析剔除 *RLM1* 基因及 CAI 基因型菌株對細胞壁壓力劑之影響，結果發現 *RLM1* 突變株 BRY429 對 caspofungin 或剛果紅之敏感性高於 CAF2-1，其中 CAI 11-11 菌株與重複數高之 CAI 35-35 或 CAI 41-42 菌株相比稍微有差異，經菌落計數量化 (colony counting assay) 顯示出 CAI 11-11 菌株對 caspofungin 抗性高於 CAI 25-25 或 CAI 35-35 與 CAI 41-42 菌株；然而 CAI 二倍體菌株培養於含剛果紅之生存率無差異。以 2% lactate 代替 glucose 作為碳源後，經 drop assay 分析後發現 CAI 11-11 菌株或 CAI 35-35 與 CAI 41-42菌株對 caspofungin 之耐受能力高於 CAI 25-25 菌株，推測 *RLM1* 基因上 CAI 區域中重複數會影響白色念珠菌對 caspofungin 之耐受能力。

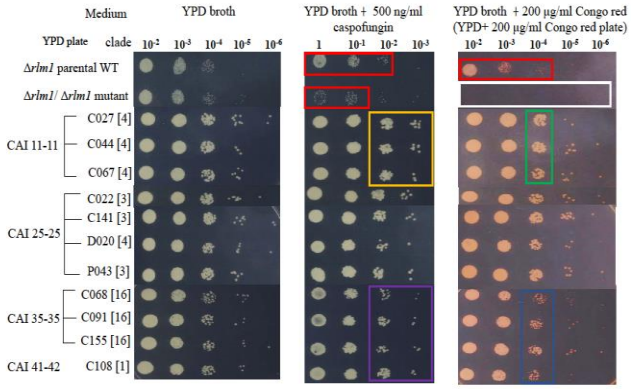

Figure 2. 剔除 *RLM1* 基因及同源 CAI 二倍體臨床菌株對干擾細胞壁完整性之壓力劑 (caspofungin 和剛果紅) 之耐受能力。

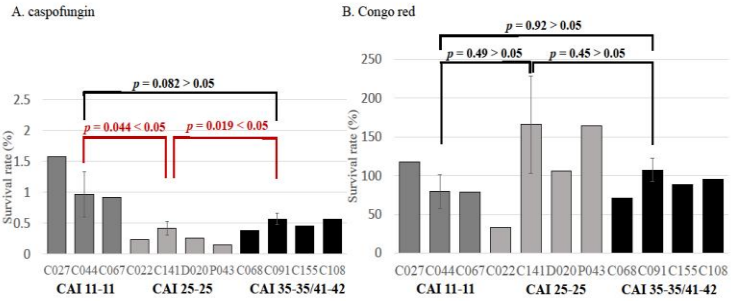

Figure 3. 以菌落計數量化方法 (colony counting assay) 分析同源 CAI 二倍體臨床菌株對抗壓力劑 (caspoungin 或剛果紅) 之生存能力。

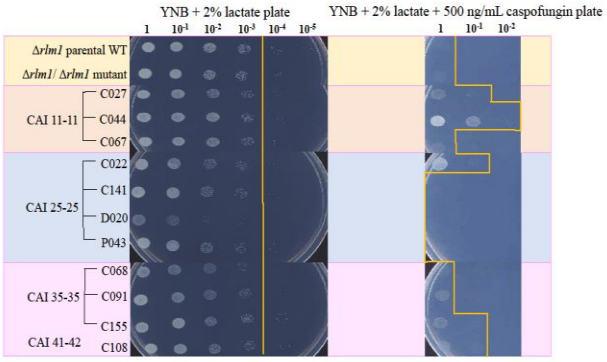

Figure 4. 以 2% lactate 作為碳源培養同源 CAI 二倍體臨床菌株分析對干擾細胞壁完整性之壓力劑 (caspofungin) 敏感性之影響。

結論與未來研究方向

RLM1 基因存在與否確實影響白色念珠菌對細胞壁壓力試劑 caspofungin 與剛果紅之敏感性。不論以 2% glucose 或 lactate 為碳源時發現 CAI 11-11 或 CAI 35-35 與 CAI 41-42 菌株對 caspofungin 之耐受能力高於 CAI 25-25 菌株。CAI 基因型之重複數可能影響其位置之 *RLM1* 基因功能，因而影響其細胞壁完整性進而改變白色念珠菌對細胞壁壓力劑 caspofungin 或剛果紅敏感性。

未來研究方向以 real-time RT-PCR 分析實驗控制組 (SC5314、CAF2-1、BRY429) 和 CAI 二倍體菌株 (CAI 11-11、CAI 25-25、CAI 35-35 和 CAI 41-42) 對壓力反應後 (caspofungin) 相關基因表達 (*SAP5*、*ECE1*、*HWP1*、*CHT3*、*ACT1*) 探討 CAI 基因型 (*RLM1*) 在環境壓力中對毒力或細胞壁相關基因影響。

Cytotoxicity and Immunomodulatory Effects of the Polysaccharides Extracted from *Auricularia polytricha*

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Abstract

Auricularia polytricha (AP), also called as wood ear or Jew's ear, is an edible tree fungus, primarily farming at an industrial level in Chiayi county, Taiwan. Evidences showed that dietary fiber and polysaccharides from fruit body of *Auricularia* species exhibit some biological modifier activities including lowering blood cholesterol level, controlling blood glucose level and decreasing heart vascular diseases. Only few studies addressed their immunotoxicity or immunomodulatory properties. Fresh AP were purchased from local contract farmer and subjected for polysaccharides isolation based on the anatomical portions of fruit body and root (hyphae) to obtain polysaccharide fractionations, denoted as SA, SB, VA and VB. Cytotoxicity was assessed in both myeloid and lymphoid derived cell lines, RAW264.7 and J774A.1, and YAC-1. Immunomodulatory properties were further investigated in macrophages upon activation. Results showed SA and SB stimulated NO production and lysozyme production in RAW264.7 and J774A.1 macrophages, respectively. When SA and SB proportionally mixed (1:1; 1:3 and 3:1), similar immunostimulatory effects were obtained in both macrophagic cell lines. Divergently modulate peritoneal macrophages were demonstrated by treating different mixed samples. Data support polysaccharides derived from fruit body or rood of AP could exert biphasic modulatory properties in macrophages.

Introduction

Evidences showed that polysaccharides extracted from *Auricularia polytricha* have antitumor, antiinflammatory, and antioxidant effects [1,2]. It has been reported that polysaccharides from AP can modulate some immune function by interact with the pattern recognition receptor on surface of antigen presenting cells, but only few studies addressed their immunotoxicity [3] or immunomodulatory properties. Therefore, to investigate the cytotoxicity and its mechanism on macrophage is an important issue.

Materials and Methods

Fresh AP were purchased from local contract farmer. AP Fruit bodies (group A) and roots (group B) of polysaccharides was extracted with distilled water in different temparature and precipipitated with ethanol. The polysaccharides was re-dissolved in phosphate buffer saline and use ultrasonic to homogenization. Their cytotoxicity was measured using the MTT assay, and assessed by murine macrophage cell line RAW264.7 and J774A.1, murine lymphoma cell line YAC-1 and used murine liver cell line BNL CL.2 as a health control group. Griess reaction assay was using to detect the production of NO, DCFH assay was using to detect the reactive oxygen species, and lysozyme test was using to detect the secretion of lysozyme. Reverse transcription-polymerase chain reaction was using to measure the gene expression in primary cell (murine peritoneal macrophage).

Result

Table 1. Cytotoxicity test in cell line for 24h. AP polysaccharides, especially HB, have higher cytotoxicity in RAW264.7 than J774A.1 and BNL CL.2. AP polysaccharide must have immune cytotoxicity.

group		abbreviation	IC50 (μg/mL)		
			RAW264.7	J774A.1	BNL CL.2
cold water exteacted	fruit body	CA	72.4	200.6	175233.3
	fruit body (ethanol precipitation)	CAE	117.2	164.8	410.6
	roots	CB	75.4	62.3	1101.9
	roots (ethanol precipitation)	CBE	74.3	58.2	1547.9
hot water extracted	fruit body	HA	119.1	8.3	862.4
	fruit body (ethanol precipitation)	HAE	63.4	4.6	424.8
	roots	HB	36.1	83.5	630.7
	roots (ethanol precipitation)	HBE	96.7	85.7	1121.5

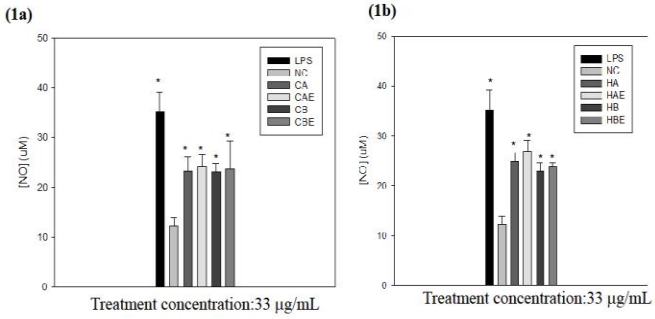

Figure 1. Effect of AP polysaccharide on NO production in RAW264.7 for 24h. (1a) NO production result of the cold water extracted sample. (1b) NO production result of the hot water extracted sample. Both cold water extracted group and hot water extracted group can induce NO production. And consider the polysaccharide yield, we chosed hot water extracted group (HA, HB) for the following experiments. (*, P value < 0.05, when compared with negative control group.)

Table 2. Cytotoxicity test in cell line for 24h. AP polysaccharides, especially SA and SB, have higher cytotoxicity in RAW264.7 than J774A.1, BNL CL.2 and YAC-1. AP polysaccharides must have immune cytotoxicity in macrophage.

		abbreviation	IC50 (μg/mL)			
			RAW264.7	J774A.1	YAC-1	BNL CL.2
hot water exteacted fruit body (HA)	supernatant	SA	58.0	242.1	1329.1	168.4
	viscous layer	VA	82.0	234.5	2731.5	194.5
hot water extracted roots (HB)	supernatant	SB	65.1	260.6	1220.7	357.7
	viscous layer	VB	76.0	255.8	412.5	283.7

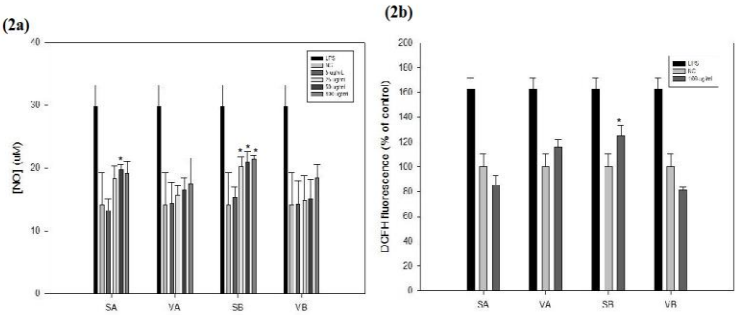

Figure 2. Effect of AP polysaccharide on NO production and ROS in RAW264.7 for 24h. (2a) NO production result. (2b) intracellular ROS level result. SA and SB stimulated NO production significantly, and VA and VB had no sigmificant effect on stimulate NO production on RAW264.7. All sample seem to have no influence in intracellular ROS level. (*, P value < 0.05, when compared with negative control group.)

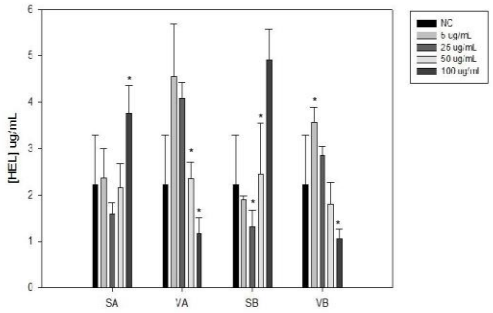

Figure 3. Effect of AP polysaccharide on lysozyme secretion in J774A.1 for 24h. SA and SB stimulated lysozyme secretion while VA and VB reduced lysozyme secretion. (*, P value < 0.05, when compared with negative control group.)

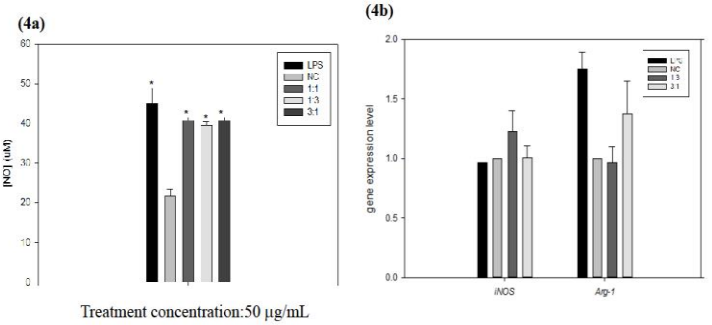

Figure 4. Effect of AP polysaccharide on immune modulatory in murine macrophage. SA and SB are mixed with different proportion (1:3 and 3:1). (4a) NO production result in RAW264.7. (4b) Effects of mixing sample on the expression of iNOS and Arg-1 gene in murine peritoneal macrophage. The mixing sample seem to have immunestimulatory effect in RAW264.7. But in murine peritoneal macrophage, they have different gene regulation effect. (*, P value < 0.05, when compared with negative control group.)

Discussion

AP polysaccharides, especially HB, SA and SB, have immune cytotoxicity on macrophage. SA and SB must have stronger immunomodulatory activity than VA and VB. Moreover, Our result suggest different mixing sample of AP polysaccharides have exerted biphasic modulatory properties in macrophages. When SB mixed with SA in 1:3 ration, it must modulate macrophage in a classical activation, and when in 3:1 ration, it must modulate macrophage in an alternative activation.

References

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