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人類巨細胞病毒感染與冠狀動脈疾病之相關性

廖永樑[1]　楊義爵[1]　洪鼎鈞[1]　葉慧儀[2]　陳家鋒[3]　廖東南[3]　林靖南[1]

奇美醫院　[1]病理部　[2]內科部　中華醫事學院　[3]醫事技術學系

摘　要

人類巨噬細胞病毒(Cytomegalovirus; CMV)可造成無症狀感染，對於免疫不全的個體，更是危及生命的病原。近年有學者證實：冠心症(Coronary artery disease; CAD)屬慢性發炎性疾病，繼而提出慢性感染可能是病因之一，其中又以CMV感染最受矚目。在此篇研究中，我們收集了60位健康正常人和93位冠心症患者共153人，分別偵測其血清中發炎物質濃度、CMV抗體含量、全血中CMV核酸定性及定量。結果顯示：1.冠心症患者體內發炎物質遠高於正常對照組。2.兩組間CMV抗體陽性率並無差別，但若以抗體效價分級，則冠心症組有較高的趨勢。3.CMV PCR陽性率在兩組間並無明顯差異，然而陽性者在兩組之病毒量確有顯著的不同($p<0.05$)。結論：CMV抗體、CMV nested PCR陽性率與冠心症無關，而在CMV PCR陽性個體，冠心症組血中CMV病毒量較對照組顯著增加。

關鍵詞：人類巨噬細胞病毒(Cytomegalovirus; CMV)
冠狀動脈疾病(Coronary artery disease; CAD)
介白素-6(Interleukin-6; IL-6)
高感度C反應蛋白(High sensitivity C-reactive protein; Hs-CRP)

引言

由於國人生活型態的改變以及飲食習慣逐漸西化，以致心臟病患人數與日俱增。經醫學研究發現，隨著年紀增長，每個人都會出現某種程度的動脈硬化，進而演變成冠狀動脈疾病(coronary artery disease; CAD)[1-3]。根據衛生署統計，因心血管疾病而致死的案例，近年來有增加的趨勢，其中又以冠狀動脈疾病最爲重要。雖然明確的致病機轉並不清楚，對其高危險致病因子卻已有深度了解。最常被廣泛討論的危險因子包括：高血壓、有菸癮者、糖尿病、膽固醇過高和缺乏運動者[4-6]，以及數種發炎因子與生化數值包括：C-reactive protein: CRP、IL-6 homocysteine..等等[7-10]。除此之外，近年來研究發現，受到微生物和病毒感染所引發的發炎反應，如巨細胞病毒(cytomegalovirus; CMV)等，亦能導致心臟疾病的發生[11-14]。

巨細胞病毒(cytomegalovirus; CMV)：屬疱疹病毒科，可感染多種細胞並造成人類相關疾

聯絡人：林靖南　通訊處：710台南縣永康市中華路901號　奇美醫院病理部

病，然而大多屬於無症狀感染，無論所造成的疾病種類爲何，最後皆轉變成潛伏性感染[15-18]。由於CMV 感染具有很高的盛行率，因此易於免疫不全 (immuno-compromised) 的病人身上發病，例如器官移植患者、癌症及AIDS 患者等。近年研究顯示：CMV 感染在冠狀動脈粥狀化、動脈狹窄等疾病中，扮演一個非常重要的角色，主要是因爲原本潛伏在體內的CMV 被活化，使得血管產生發炎反應，進而形成冠狀動脈疾病[19-23]。此外，CMV 感染會導致脂質代謝改變，因而使膽固醇累積在動脈中，最後誘發CAD[24, 25]。而氧化態的LDL (low density lipoprotein) 亦能活化上皮細胞、巨噬細胞及平滑肌肉細胞，使得血管發生粥狀化及血栓化效應[26-29]。然而是否引發動脈硬化的關鍵，除了和病毒本身的生物特性有關之外，也與宿主免疫狀態和接受感染的敏感度有密切關係[30-32]。

雖然CMV 感染與CAD 形成的關聯性已被廣泛的研究，由於該病毒在台灣的盛行率非常高，而且利用血清學方法檢測抗體存在與否，仍會因爲個體的變異不同，而產生僞陰性的現象，再加上CMV 難以利用細胞進行培養，根據1999 年Douglas 發現，僅60% 的陽性檢體能被培養成功[33-35]，實在不足以提供臨床診斷與預防醫學之需求。目前臨床上主要以pp65 antigenemia assay 來診斷CMV 感染而造成的疾病，雖然專一性高，但敏感度卻不甚理想[36-38]。因此，有很多研究CMV 造成的疾病改以PCR 來做診斷，雖然提高了敏感度，但與疾病的專一性卻也降低[39-42]，而且PCR 標定放大基因的種類左右了此方法的敏感度[40, 43, 44]。再者，有報告指出：在CMV 抗體陽性及部分陰性的患者血中可檢測出CMV DNA 的存在[45-48]。然而，亦有相反的研究陳述：在血中以PCR 方式很少將CMV DNA 檢測出來[49-53]。本研究將利用目前分子生物學上最新技術—即時聚合酶鏈鎖反應分析儀，(real-time PCR)，偵測不同CAD 病程血液中CMV-DNA 含量，藉以探討病毒量與疾病程或與疾病治療依據之相關。由於real-time PCR 具有快速、高再現性、高敏感性且具有定量功能等優點[54-56]，冀望能對於診斷心血管疾病或癒後有所助益。

材料及方法

所有的採檢樣本分爲二組；對照組60 人，包括男性45 人，女性15 人，均爲奇美醫院健康檢查的成人，他們在經過病史理學檢查、一般心電圖和心肌酵素篩檢後，無心絞痛或心肌梗塞之跡象。CAD 組共93 人，男性64 人，女性29 人，爲經心導管、運動心電圖或persantin thallium heart scan 證實爲冠心病患者。所有採檢樣本的年齡及性別分佈，綜合於 (表一)。

IL-6 的測量是採用商業用的成套試劑ELISA (R＆D system)， 取100 μl血清和100 μl的分析稀釋液加入每個well 內 (R＆D system ELISA)，

表一：採樣個體分組資料

	Control (n=60)	CAD (n=93)	p value
Age ，mean (range)	62.1 (41-83)	64.6 (40-85)	NS
sex ，male/female	45/15	64/29	NS
Risk factor ，n (%)			
Smoke	12 (21.4)	32 (39.5)	<0.05
Diabetes	10 (16.7)	29 (31.2)	<0.05
Hypertension	21 (35.0)	50 (53.8)	<0.05
Cholesterol ，mg/dL	219.8 ± 37.5[a]	213.0 ± 48.6	NS
LDL ，mg/dL	143.3 ± 79.3	125.5 ± 36.8	NS
TG ，mg/dL	117.8 ± 82.7	196.9 ± 126.6	<0.001
HDL ，mg/dL	56.9 ± 19.1	44.6 ± 11.8	<0.001
Bilirubin ，mg/dL	0.95 ± 0.36	0.48 ± 0.21	<0.001

NS ：not significant. p>0.05

[a] ：mean ± SD

室溫培育兩個小時。以Wash Buffer洗去未結合的物質，再加入抗IL-6的多株抗體200 μl (conjugated with Horseradish peroxidase)，於室溫培育兩個小時，以Wash Buffer洗去未結合的物質，再加入200 μl Substrate Solution [H_2O_2 +tetramethylbenzidine mixture (1:1)] 到每一個well內，室溫避光培育20分鐘，最後加入50 μl Stop solution (2N sulfuric acid) 終止呈色反應。以光學比色計測定吸光值 (OD450nm)，求得已知濃度樣品之標準曲線，再定量待測物濃度。每個檢體測量兩次而分析時的變異性小於10%。

High sensitivity C-reactive protein (hs-CRP) 實驗方法，利用Particle-enhanced technology，以scattered light (Behring BN II nephelometer) 直接偵測抗原抗體複和物濃度而換算出hs-CRP含量。

血清中Bilirubin、uric acid、HDL-C、TG、cholesterol及BUN的測量是利用商業用的成套試劑 (Wake Pure Chemical Industries, Ltd) 以HITACHI 7070自動分析儀檢測。

DNA萃取採用Puregene DNA purification Kit (Gentra, Minnesota, USA) 抽取全血 (0.3ml) 中的DNA，最後溶在0.1ml純水，保存在-80℃。Nested PCR採用標的基因爲UL122，外部引子分別爲MIE-4 (5'-CCAAGCGGCCTCTGATAACCAAGCC-3'), MIE-5 (5'-CAGCACCATCCTCCTCTTCCTCTGG-3') 可放大435bps的產物[57]，內部引子依據美商應用生命公司 (ABI) Primer express軟體設計，針對此產物設計出內部引子，分別爲NS1 (5'-TGAAGGTCTTTGCCCAGTACAT-3'), NAS1 (5'-TGGCCAAAGTGTAGGCTACAAT-3')，可放大出129bps的產物。1st-PCR試劑調配如下：在50 μl的反應總體積內包含有30pmol外部引子、5 μl DNA和1.5U Taq polymerase (Promega, Madison, WI)。2nd-PCR試劑調配如下：在50 μl的反應總體積內包含有30pmol內部引子、1 μl 1st-PCR產物和1.5U Taq polymerase。兩次PCR反應條件相同：加熱95℃10分鐘，三溫度40個循環 (95℃，1分鐘、55℃，1分鐘、72℃，1分鐘) 最後在72℃作用10分鐘。Nested PCR產物最終以7％PAGE進行電泳後，以ethidium bromide染色，再UV呈色後照相存檔。

Real time PCR定量分析方面採用ABI PRISM 7000偵測系統，以UL83爲標的基因，引子分別爲pp549s (5'-GTCAGCGTTCGTGTTTCCCA-3')、pp812as (5'-GGGACACAACACCGTAAAGC-3')，探針爲pp770s (5'FAM-CCCG-CAACCCGCAACCCTTCATG-3'TAMRA)。標準品備製乃以pp549s, pp812as兩引子批出PCR產物，轉殖入大腸桿菌內 (TOPO TA Cloning Kit, Invitrogen) 大量繁殖，最後進行質體DNA萃取，以260nm測定標準品濃度。Real time PCR試劑調配如下：在25 μl的反應總體積內包含有12.5pmol外部引子、5 pmol探針、5 μl DNA和1U platinum taq。反應條件：加熱95℃10分鐘，二溫度45個循環 (95℃，15秒、65℃，1分鐘)[54]。

在統計分析方面：在非常態分布的實驗結果，我們使用無母數統計方式。Spearman's rank test用來評估相關性，而Chi-Square test用來評估不連續資料的相關性。以multiple logistic regression models調整CAD危險因子對生化數值的影響並依序計算出勝率比 (odds ratio, OR) 和95%信賴區 (confidence interval, CI)。有意義的顯著值設爲 $p<0.05$。

結果

CAD的危險因子包括有性別、年齡、肥胖、遺傳、糖尿病、抽煙、高血壓、膽固醇血脂過高、缺乏運動、精神緊張、壓力大。我們隨機選取對照組；健康成人共60人：其中男性45人，女性15人。實驗組；CAD患者共93人：男性佔64人，女性29人。其中二組的年齡、性別、膽固醇和LDL並無顯著的差異，然而二組間之糖尿病、抽煙、高血壓、TG和HDL皆有明顯的差異 (表一)。爲避免這些危險因子影響後續的生化數值統計，我們以multiple logistic regression models調整這些變數的影響。

發炎分子、CMV抗體與PCR之表現

在CAD組中IL-6、hs-CRP之濃度較健康對

表二：採樣個體CMV 感染與發炎指標之表現

	Control n (%)	CAD n (%)	p value
Hs-CRP ， mg/dL	2.51 ±0.63[a]	11.81 ±2.23	<0.001
IL-6 ， pg/ml	1.09 ±0.40	13.69 ±4.82	0.002
α CMV IgG ， EU/ml	49.2 ±2.4	56.0 ±2.3	0.097
Positive>15 EU/ml	59(98.3)	91(97.8)	0.703
Nested PCR	15(25)	29(31.2)	0.409

NS ：not significant. p>0.05
[a] ：mean ± SEM

表三：以CMV 抗體效價分成四等分，分析冠心症相對危險性

	Control (n=60)	CAD (n=93)	relative risk (95% CI)	p value
α CMV IgG (EU/ml)				
mean (range)	49.2 (2.8~98.7)	56.0 (9.18~135.4)		
Quartiles distribution ， n (%)				
1 (15~30 EU/ml)	8 (13.6%)	6 (7.7%)		
2 (30~60 EU/ml)	35 (59.3%)	49 (53.8%)	1.58 (0.55~4.49)	0.395
3 (60~90 EU/ml)	14 (23.7%)	27 (29.7%)	2.17 (0.69~6.86)	0.187
4 (≧90 EU/ml)	2 (3.4%)	9 (9.9%)	5.06 (0.83~30.75)	0.078

p values were calculated by logistic regression analysis.
CI denotes confidence interval.

照組呈現顯著增加，然而anti CMV IgG 抗體效價只有微幅增加且未達顯著意義。同時，兩組間CMV 抗體陽性率皆高達97 %以上，未有差別。此外，兩組間的CMV DNA PCR 陽性率分別爲25 %與31.2 %，CAD 組略高於對照組(表二) 。

CMV 抗體效價對罹患冠狀動脈疾病的影響

依CMV 抗體效價由低至高分成4 等分(level 1~4)，並分析相對危險性(relative risk) 。結果顯示，CAD 組CMV IgG 抗體效價level 2~4 與level 1 相較，其相對危險性分別爲1.58 (p=0.395) 、2.17 (p=0.187) 、5.06 (p=0.078) 有逐漸增加的趨勢(表三) 。再以迴歸統計分析CMV 抗體效價、IL-6 、hs-CRP 、PCR 之相關性，結果顯示：在對照組中CMV 抗體效價與IL-6 呈現相關趨勢(r=0.249, p=0.055) 。在CAD 組中，僅IL-6 與hs-CRP 呈現高度相關(r=0.634, p<0.001) 。但就整體而言：IL-6 與CMV 抗體效價和hs-CRP 皆呈現顯著的相關(r=0.178, p=0.028 ； r=0.503, p<0.001) (表四) 。

兩組間CMV 病毒量之表現

爲檢查病毒量在兩組間是否有差異，我們以定量系統分析血中CMV 病毒量，結果顯示：nested PCR 陰性的檢體，病毒定量依然爲陰性(Data not shown) 。然而nested PCR 陽性的檢體，病毒量表現的多寡就有差異。在對照組中，15 個nested PCR 陽性檢體皆測不到CMV 病毒量；而在CAD 組中，29 個nested PCR 陽性檢體就有14 個測得CMV 病毒量 (p<0.05) ，範圍由數百至數萬不等 (圖一) 。

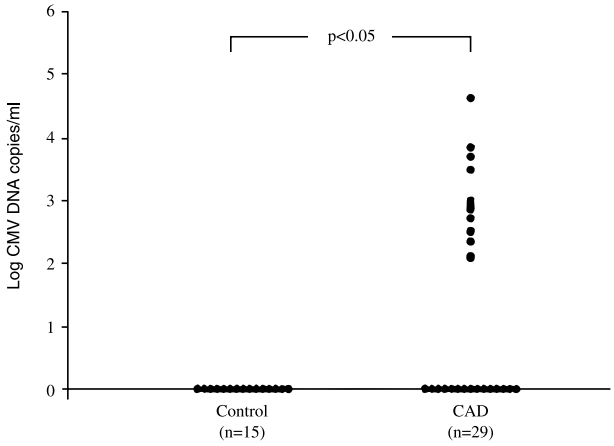
討論

我們的研究證實：1 . 以抗體有無來顯示CMV 盛行率高低狀況與罹患CAD 與否無關。2 . CMV PCR 陽性率在兩組間無顯著差異。3.CAD 組CMV 病毒量比對照組顯著增加。

一個從未暴露於CMV 存在的環境下之健康個體，是不會產生對抗CMV 抗體，故此抗體可作爲感染CMV 的指標。體內產生的抗體主要又分成IgM 和IgG ，分別代表首次感染或再復發以及二次感染或持續性感染。由於CAD 屬於慢性

表四：比較冠心組與對照組CMV IgG 、hs-CRP 、IL-6 之相關性

Group	Item			
	CMV IgG and IL-6	CMV IgG and hs-CRP	CMV IgG and PCR	IL-6 and hs-CRP
Control				
r value	0.249	0.045	0.044	0.119
p value	0.055	0.737	0.737	0.370
CAD				
r value	0.102	0.077	-0.128	0.634
p value	0.335	0.467	0.222	<0.001
Total				
r value	0.178	0.127	-0.047	0.503
p value	0.028	0.119	0.561	<0.001



圖一：CMV nested PCR 陽性個體血中CMV 病毒表現量

發炎性疾病，整個致病流程是個緩慢的過程，若CMV 感染與CAD 有關，應屬於長期感染CMV所誘發引起的疾病，與首次感染無關。故我們僅選取anti-CMV IgG 抗體來代表個體的感染狀況。在不同族群裡，CMV 的盛行率皆隨年齡增加而上升，然而在高度開發國家，盛行率較低，反之則較高[58-61]。在台灣，CMV 盛行率高達90%以上，且隨著年齡，盛行率有顯著增加的情形[62, 63]。本次實驗數據對照組、CAD 組的CMV抗體陽性率皆高達97% ，符合前人論述，兩組間並無差異。然而若將抗體效價劃分四分區評估勝率比，則可發現CAD 組在高抗體效價區的百分比有逐漸提高的趨勢（ 表三 ），這可能因爲CAD 持續著感染個體，造成CMV 抗體效價偏高的緣故。雖然抗體的存在代表個體曾經感染過CMV ，但正常人多數是無症狀感染，CMV 轉而潛伏在體內，伺機而動。爲釐清這個問題，我們以nested PCR 檢測個體血中CMV 是否存在，然而陽性率分別只有25% (對照組) 與31.2% (CAD組)，未達統計學意義。雖然，研究個體大部分都感染過CMV ，然而由PCR 的結果看來，與CMV 抗體表現並不一致，類似的結果亦出現在學者的研究中。可能因爲CMV 潛伏細胞種類太廣、或是PCR 使用的標的基因敏感度不夠，甚者是CMV 潛伏感染時僅嵌入部分基因而導致DNA 萃取過程中流失PCR 標靶基因[15, 64-67]。

IL-6 、hs-CRP 咸認爲與CAD 有關，因此我們亦比對了CMV 抗體、CMV PCR 結果在兩組間的表現，並進一步分析相關性。一如預期，IL-6 與hs-CRP 在CAD 組呈現高度相關 (r=0.634, p<0.001)。另外，在對照組中，CMV 抗體與IL-6 相關性也有明顯增加 (r=0.249, p=0.055)，且就總體而言，CMV 抗體與IL-6 相關性已有顯著的意義 (r=0.178, p=0.028)。這可能是因爲CMV 感染誘發宿主產生免疫反應的結果，然而hs-CRP由於是發炎反應的下游物質，因此相關性就不若IL-6 來得顯著。

CMV 感染與CAD 疾病之間到底有無關連，相關研究各有數據佐證[52, 68-72]。爲釐清這個問題，本實驗除了顯示出抗體效價的差別外，亦將CMV 在血中的病毒量納入衡量範圍。臨床上，用以定量CMV 的方式不外乎三種，一爲CMV

pp65 antigenemia，敏感度低、專一性高。二爲real time PCR，敏感度高 (視標的基因種類而有不同的敏感度)、專一性較低。三爲NASBA 偵測法測pp67 mRNA，敏感度低、專一性較最高。本實驗採用標的基因爲UL83 之 real time PCR 方式檢測 CMV 病毒量，UL83 爲 pp65 抗原的來源基因，因此與pp65 antigenemia test 相關性相當高，敏感度或許不若nested PCR 但是就專一性而言，絕對比nested PCR 來的強。結果顯示：25 個nested PCR (+) 對照組，偵測不到CMV 病毒量。反之，29 個nested PCR (+) CAD 組，有 14 個可偵測到病毒量，範圍由數百至數萬間。這表示CAD 組中，CMV 病毒量較對照組顯著增加 (圖一)。至於其他nested PCR (-) 檢體，則完全測不到病毒量 (Data not shown)。

儘管CMV 感染與CAD 疾病的關係，紛紛擾擾、眾說紛紜。但是藉由病毒定量的方式，本實驗證實CAD 患者體內帶有CMV 病毒的量的確比對照組爲高。這是因爲CMV 長期感染再加上其他危險因子而導致CAD 疾病產生，還是CAD 患者本身免疫力相對於對照組爲低，而使潛伏性感染的CMV 有可趁之機，再度活化感染宿主，因而造成CMV 病毒量增加？若能釐清此問題，或許CMV 在CAD 疾病所扮演的角色就有柳暗花明的一天。

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The Correlation Between Cytomegalovirus Infection and Coronary Artery Disease

Yung-Liang Liao[1], Yi-Chueh Yang[1], Ting-Chun Hung[1], Hui-Yi Yap[2], Chia-Fong Chen[3], Tung-Nan Liao[3], and Ching-Nan Lin[1]

[1]Department of Pathology, Chi-Mei Foundation Hospital, Tainan, Taiwan,
[2]Department of Internal Medicine, Chi-Mei Foundation Hospital.
[3]Department of Medical Technology, Chung-Hwa College of Medical Technology

Primary CMV infection is usually asymptomatic and results in latent, lifelong persistence of the viral genome. Periodically, the virus might reactivate from latency and regain its ability to multiply. CMV is a major cause of life-threatening illness in immunocompromised patients. Various lines of evidence suggest that chronic inflammation is involved in the initiation and progression of atherosclerosis and its complications including coronary artery disease (CAD). Several infectious agents, particularly CMV, have been suggested to play a role in the development of atherosclerosis. In this study, we measured serum levels of IL-6, anti-CMV IgG (α CMV IgG), high sensitivity C-reactive protein (hs-CRP) by ELISA or nephelometry, CMV qualitative analysis by nested PCR, and CMV viral load quantitative analysis by real time PCR in 93 CAD patients and 60 normal healthy controls. Our study showed that the levels of IL-6, and hs-CRP were significantly increased in patients with CAD (p=0.002 and p<0.001, respectively). There was no statistical significance in α CMV IgG positive rate in both groups, however, there was a trend for higher antibody titers in the CAD group according to the quartile levels of analysis (p=0.078). In nested PCR positive cases, the CMV viral loads were significantly elevated in CAD patients than in normal controls (p<0.05); meanwhile, there was no difference in CMV positive rates between the two groups using nested PCR qualitative assay. Conclusion: In CMV nested PCR positive cases, CMV viral loads were significantly elevated in CAD patients than in normal controls. However, there were no differences in CMV nested PCR and αCMV IgG positive rates between the two groups. (J Intern Med Taiwan 2007; 18: 88-96)