國科會專題研究計劃成果報告

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計畫中文摘要：

關鍵詞：第八型人類泡疹病毒, 骨髓移植

第八型人類泡疹病毒是人類泡疹病毒最新的一個成員。目前的數據顯示有一部份的成人已感染過此病毒。在某些地區感染過此病毒者最高可達 50%。我們曾經完成一個第八型人類泡疹病毒血清流行病學研究，結果顯示大約百分之十五到二十的台灣成人已感染過此病毒。到目前為止此病毒跟一些惡性腫瘤有高度的相關性。但是此病毒引起的臨床症狀我們仍所知有限。本研究的目的將要前瞻性地追蹤五十個兒童及成人骨髓移植病人，每個星期抽一次血，檢驗血中第八型人類泡疹病毒 IgG 及 IgM 抗体，並記錄其臨床表現。此外將利用聚合酶鏈反應來偵測血液周邊淋巴球及血清中第八型人類泡疹病毒核酸。我們希望了解在骨髓移植時第八型人類泡疹病毒感染或再發的機率，及感染的時間與臨床症狀。在第二年中我們也將發展定量型的聚合酶鏈反應，用來更精確的監視第八型人類泡疹病毒的活性。並了解定量型的聚合酶鏈反應是否在臨床上有應用的價值。

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計畫英文摘要：

Keywords：HHV8, bone marrow transplantation, reactivation

HHV8 is the newest member of human herpesviruses. Accumulative data indicates that a proportion of healthy adults is infected with HHV8. The prevalence could be as high as 50% in some countries. We have finished a seroprevalence study of HHV8 infection in Taiwanese adults and our data indicated that 15-20% of Taiwanese are infected with HHV8. Up to now, a variety of malignancies has been linked to HHV8. However, the clinical illness caused by HHV8 is almost unknown to us. This study is aimed to understand HHV8 infection or reactivation in adults and children undergoing bone marrow transplantation (BMT). We will prospectively study 50 BMT patients; their blood samples will be collected every week and their clinical data will be recorded. HHV8 IgG and IgM antibodies in serum samples will be measured every week. Polymerase chain reaction (PCR) will be used to detect HHV8 genome in peripheral blood mononuclear cells and sera. We expect to delineate the frequency of HHV8 infection or reactivation during the course of bone marrow transplantation. The timing, clinical manifestation and outcome of HHV8 infection in BMT patients can be learned. In the second half of this two-year project, quantitative PCR will be developed to more accurately monitor the activity of HHV8 and see whether this is a valuable diagnostic tool.

研究計畫之背景及目的(1)：

The herpesvirus family consists of at least 12 different DNA viruses. In 1994, Chang et al. found a new member of the gammaherpesvirus family in Kaposi's sarcoma (KS) tissues from AIDS patients using a technique called representational difference analysis (1). The virus was first designated as KS-associated herpesvirus (KSHS) and later renamed human herpesvirus 8 (HHV 8) because the DNA sequences of KSHV were most close to other herpesviruses and can be found in tumors other than KS (2).

Soon after the discovery of HHV 8 DNA in AIDS-associated KS, numerous laboratories confirmed and extended these findings. This viral DNA is also present in other forms of KS, including classic European KS in elderly men, endemic African KS, and iatrogenic KS in immunosuppressed recipient of organ transplant (3-7).

HHV 8 DNA was also detected in some lymphoproliferative lesions such as body cavity-based lymphoma (BCBL) (8), multicentric Castleman's disease (9), and multiple myeloma (10). However, data regarding the presence of HHV 8 in some endothelial (11,12) or epithelial tumors (13,14) were controversial.

Studies showed that HHV 8 is also present in various tissues of healthy individuals; the reported tissues included peripheral blood mononuclear cell (15), prostate tissue and human semen (16), and nasal secretions and saliva (17).

Reports regarding prevalence of HHV 8 in human population remain limited and vary widely (16-23). In central Africa 22.5% of adults have been infected with HHV8. In Honduras, the infection rate was 11.3%. The rate could be as high as 50% in Ugandan. We also tested HHV8 IgG antibody of sera from 1040 Taiwanese from infant to adult. Our results revealed that about 15% of Taiwanese adults have been infected with HHV8. HHV8 infection in Taiwan started to be seen in older children or adolescents (24). To sum up, all the studies indicated that a proportion of people is infected with HHV8 in their lifetime. However, there has been no report concerning the clinical manifestations of HHV8 infection. The only thing known is that HHV8 is highly connected with a variety of tumors. Hence, although scientists are gathering more and more information about the basic virologic knowledge, our understanding of the clinical behavior of HHV8 is almost blank. It is likely that HHV8 infection may be subclinical; hence, we know very little about clinical HHV8 infection. Still, we feel it to be very important to fully understand the clinical manifestations of HHV8 infection in order to better control HHV8 infection.

Organ transplantation has become a mature medical technology to save some patients who were untreatable before. The key to successful organ transplantation is to avoid graft rejection. To achieve this goal, iatrogenic immune suppression usually is practiced. However, immune suppression not infrequently leads to virus reactivation, which has become a major cause of morbidity and mortality of patients undergoing transplantation (25-27). Being a human herpesvirus, HHV8 can establish latency after primary infection and may reactivate under suitable circumstances. Hence, one may expect to witness HHV8 infection or reactivation in patients undergoing transplantation. Under this situation, we can observe clinical illness caused by HHV8. In support with this reasoning, one report showed that one patient seroconverted to HHV8 and developed Kaposi's sarcoma following kidney transplantation (28). We believe bone marrow transplantation (BMT) provides a unique occasion to observe HHV8 infection or reactivation because patients undergoing BMT receive the most intensive immunosuppressive therapy (29). To date, there has been no report concerning HHV8 infection in bone marrow transplantation.

研究計畫之背景及目的(2)：

This prospective project is aimed to systemically study HHV8 infection or reactivation during the course of bone marrow transplantation. We tried to define the frequency of HHV8 infection in BMT. Furthermore, we also tried to define the risk factor, temporal sequence, and clinical manifestations of HHV8 infection or reactivation. Because the HHV8 infection in young children may not be common, the age spectrum of our study subjects spanned from children to adults.

Another aim of this project is to evaluate the usefulness of molecular diagnosis in HHV8 infection. Since we are to collect serial blood samples from patients undergoing BMT, HHV8 viral activity can be measured by conventional serologic methodology as well as polymerase chain reaction (PCR) of blood cells and serum. This provides a situation whereby different diagnostic methods can be compared and the significance of molecular diagnosis like PCR can be examined with confidence.

研究方法及進行步驟(1)：

In this prospective study, we will recruit 50 patients undergoing bone marrow transplantation (BMT). Half of them will come from Department of Internal Medicine and half from Department of Pediatrics, National Taiwan University Hospital.

BMT patients: In the coming two years, blood of about 50 patients undergoing BMT, regardless of their underlying disease, will be collected before and weekly after the BMT, during the period of admission. The blood samples will be separate into sera and peripheral blood mononuclear cell (PBMC) by centrifuge on a Ficoll-Hypaque gradient immediately after collection and stored at -70 ℃.

The sera of BMT patients will be tested for IgG and IgM antibodies against HHV8 using indirect immunofluorescence assay. On the other hand, DNA will be extracted from serum and PBMC samples of BMT patients and assayed for the presence of HHV8 DNA by polymerase chain reaction (PCR).

The clinical manifestations of BMT patients will be observed and recorded every week and analyzed with the laboratory data of HHV8 infection.

Indirect immunofluorescence assay

The sera will be tested in a blinded fashion for HHV 8 antibody by a previously described indirect immunofluorescence assay that measures antibodies to a latency-associated and lytic cycle- associated antigens (30).

IgG anti-HHV8 determination

The BCBL-1 cells, which are latently infected with HHV 8 but not infected with the Epstein-Barr virus, are used in this assay. Cells are activated by treatment with TPA (20 ng/ml) for 5 days. Thereafter, these cells are fixed onto glass slides by cold acetone and blocked by incubation with PBS containing 5% BSA for 30 min in a humidified chamber. This slide is then overlaid with patient serum diluted 1:10 in blocking solution (5%BSA in PBS) and incubate for 1 hr. Unbound serum is washed away with PBS for 10 min for 3 times. The secondary antibody, mouse anti-human IgG_{123}, diluted 1:2000 in blocking solution is then added and incubated for 1 hour. Washing with PBS for 3 times is performed again followed by adding FITC-conjugated goat anti-mouse IgG diluted 1:4000 in blocking solution and incubate for another 1 hour. After final wash, the slide is observed under immunofluorescence microscope with mounting medium (PBS:glycerol=1:9). Serum titration will be done with 4-fold serial dilution. Titer of a specific serum is defined as the reciprocal of highest dilution, which gives positive fluorescence. A titer of 10 of more is considered positive.

IgM anti-HHV8 determination

The procedure is similar to that of IgG determination, except that the incubation time of serum on slide is extended to be 3 hours and the secondary antibody is mouse anti-human IgM.

研究方法及進行步驟(2)：

Extraction of nucleic acid

Nucleic acid of PBMC and serum will be extracted by phenol/chloroform treatment followed by ethanol precipitation.

PCR assay

Nested PCR approach will be adopted. Primer set of KS-1: 5'-AGCCGAAAGGATTCCACCAT-3' and KS2: 5'-TTCGTGTTGTCTACGTCCAG-3' (nucleotide 987 to 1,006 and nucleotide 1,200 to 1219) were used as outer primers to amplify a DNA sequence of 233 bp. (1). The conditions for PCR are as follows: 94℃ for 2 min (1 cycle); 94 ℃ for 1 min, 57℃ for 1 min, 72℃ for 1 min (25 cycles); 72℃ for 10 min (1 cycle). Each PCR mixture contains 0.2 μ g of genomic DNA, 0.5 λ Tag polymerase (DynaZyme^{R}), 20 pmol of each primer, 100 μ M of each deoxynucleotide triphosphate, 10 mM tris-HCl (pH9.0), 50mM KCl, 1.5 mM $MgCl_2$ and 0.1% Triton X-100 in a final volume of 50 λ . One λ of the PCR products obtained from the first round PCR is then added to the PCR mixtures containing the inner primers and amplified under the same conditions described above. The premier set of H8NS1: 5'-ACGGATTTGACCCCGTGTTC-3' and H8NS2: 5'-AATGACACATTGGTGGTATA-3' were used as inner primers for nested PCR. A product of 160 bp DNA sequence was produced. (16)

The sensitivity of detection of this nested PCR, as determined by end-point dilution of a positive sample, has been estimated to be 100 copies.

研究結果及討論：

本計劃進行至此，總共收集了 657 人次的血液檢體，通通是由 BMT 的病患身上取得。此項工作耗費許多時間及人力，其中只有部分檢體可以分離出足夠的單核球(PBMC)，供做抽取 DNA 進行 PCR 檢驗。

我們優先檢驗了收集次數大於等於 4 次的病患檢體，總共有 15 位病患 70 份血清及 PBMC 檢體。其檢驗結果如表一。其中第一次是骨隨移植前，其餘是骨隨移植後。

整體而言免疫螢光反應的陽性率是 54.3% (38/70)，PCR 的陽性率是 41.4% (29/70)。然而 IFA 與 PCR 的結果並不完全平行，有部分檢體呈現 IFA 陽性卻是 PCR 陰性，或是 IFA 陰性而 PCR 陽性。就單一病患來說抗體的變化，亦沒有一定的趨勢出現。例如抗體濃度逐漸上升或下降都可以觀察到。病例 1, 8, 12, 15 的抗體 titer 似乎有逐漸升高之趨勢，而病例 3, 6, 7, 10 的抗體 titer 卻似乎有逐漸降低之趨勢。

在 IFA 的部分，15 位病患中有 8 位，在骨隨移植前便已呈現陽性，比率為 53.3%。此比率與我們先前作的研究結果相符(　)。而總共有 10 位病例在全程追蹤之中，至少有一次的檢體呈現陽性，整體陽性率為 66.7% 。若進一步比較抗體的 titer 有 4 位病例呈現抗體 titer 上升的趨勢(case 1, 8, 12, 15)。有 4 位病例呈現抗體 titer 下降的趨勢(case 3, 6, 7, 10)。為何出現這樣相反的結果原因並不清楚，可能的解釋包括: HHV8 屬 herpesviruses group，這類的病毒有一共同的特徵，就是它一旦感染人體,會保持 latent 終生。再人體免疫力低下時，再伺機再活化。而這一些病患所罹患的疾病並不一致，其免疫系統的缺陷也不盡相同，部分病例可能因為免疫抑制的緣故，抗體濃度無法上升(case 3, 6, 7, 10)；另外的病例可能 B-cell 功能尚可，在病毒因免疫抑制而 reactivation 時，抗體濃度隨之上升(case 1, 8, 12, 15)。

在 PCR 方面，在骨隨移植前已有 6 位病例呈現陽性(40%, 6/15)。在全程追蹤之中，至少有一次的檢體呈現陽性的病例有 12 位，整體陽性率為 80% 。換句話說，有 6 位病患在 BMT 之後 PCR 結果轉陽。這樣的結果符合我們的假說，那就是有部分病例在 BMT 之前雖偵測不到 HHV8 的 DNA 或抗體，但是他們事實上已經被感染過，而且 HHV8 病毒潛伏在體內，在 BMT 破壞原有免疫力以後, HHV8 病毒便又活化起來。

這些病例中有一位(case 12) 全程的 PCR test 皆為陰性，而 IFA 的結果卻是陽性。相反的，病例 2, 5, 11, 14 的 IFA 雖是陰性，他們的 PCR 結果則是陽性。這代表 PCR 的敏感性高於 IFA。但是, PCR 結果與 IFA titer 之間並無明顯的相關性。本研究所使用的 PCR 為定性 PCR，若要解釋並進一步探究為何 PCR 結果與 IFA titer 之間無明顯相關的問題，必須進行定量 PCR 的檢驗，以探討 HHV8 genome 的量，在 BMT 前後是否有任何的變化。

由於本計劃目前只完成 15 名病例，對於在血清學或 DNA 上有病毒再活化證據的病例，病毒再活化是否有任何臨床症狀？或是否有任何內在或外在的因素和病毒再活化有關?仍無法獲致結論。本實驗將繼續進行，以累積更多病例數，並設法進行 quantitatie PCR，以設法回答上述問題。

Case no.		1	2	3	4	5	6	7
1	IFA	<10	<10	40	40	40	40	160
	PCR	N	P	N	N	N	N	N
2	IFA	<10	<10	<10	<10	<10		
	PCR	N	N	N	N	P		
3	IFA	40	<10	<10	<10			
	PCR	N	N	N	N			
4	IFA	160	160	160	160			
	PCR	N	P	N	P			
5	IFA	<10	<10	<10	<10			
	PCR	P	N	N	N			
6	IFA	40	40	40	20	<10		
	PCR	P	P	P	N	N		
7	IFA	640	320	<10	<10			
	PCR	N	P	P	P			
8	IFA	<10	<10	40	40			
	PCR	P	P	N	N			
9	IFA	640	640	640	640			
	PCR	P	P	N	N			
10	IFA	40	<10	<10	<10			
	PCR	P	P	P	P			
11	IFA	<10	<10	<10	<10			
	PCR	N	N	N	P			
12	IFA	<10	40	40	160	160		
	PCR	N	N	N	N	N		
13	IFA	40	40	40	40	40	40	40
	PCR	P	P	P	P	N	N	N
14	IFA	<10	<10	<10	<10	<10		
	PCR	N	N	P	P	N		
15	IFA	160	160	160	640			
	PCR	N	P	P	P			

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