ด้วยพระนามของพระผู้เป็นเจ้า
ผู้ทรงยิ่งในความเมตตา ผู้ทรงยิ่งในความกรุณา

สัญญาเลขที่ BRG/4780016

รายงานวิจัยฉบับสมบูรณ์

เรื่อง การศึกษากลุ่มโรค Peripheral T-cell Proliferative Disease ที่มีความสัมพันธ์กับการติดเชื้อไวรัส Epstein-Barr (EBV): การศึกษาจำนวน EBV ในพลาสม่าและทางเข้าสู่ T-cell ของ EBV

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คำนำ

โครงการวิจัยเรื่อง “The studies of Epstein-Barr virus (EBV)-associated peripheral T-cell proliferative disease: EBV viral load and route of EBV infection” ได้รับการสนับสนุนด้านการเงินจากสำนักงานกองทุนสนับสนุนการวิจัย (สกว.) จำนวน 2,000,000 บาท จากที่ขออนุมัติไป 3,000,000 บาท และได้รับการสนับสนุนเพิ่มเติมจากคณะแพทยศาสตร์ มหาวิทยาลัยสงขลานครินทร์อีกจำนวน 500,000 บาท นอกจากนี้ยังได้รับการสนับสนุนด้านวิชาการและสารเคมีบางประเภทจาก Core University System Exchange Programme under Japan for the Promotion of Science (JSPS) และ Ministry of Education, Culture, Sports, Science and Technology, Japan.

การวิจัยได้บรรจุผลประมาณร้อยละ 90 จากเป้าหมายที่ตั้งไว้ ทั้งนี้เนื่องจากงบประมาณที่ถูกตัดลด และปัญหาด้าน cell culture ของ T-cell ที่มาจากผู้ป่วย ซึ่งไม่สามารถเลี้ยงเซลล์เหล่านี้ได้ดี มีเซลล์ตายจำนวนมาก จึงต้องมีการปรับวิธีการวิจัยในบางเรื่อง ผลงานวิจัยครั้งนี้ก่อให้เกิดองค์ความรู้ใหม่มากมาย งานวิจัยโครงการแรกได้รับการยอมรับให้ตีพิมพ์ในวารสาร Journal of Clinical Virology แล้ว ส่วนอีก 3 โครงการกำลังรวบรวมเรียบเรียงเพื่อส่งตีพิมพ์ นอกจากนี้ยังมีผลงานที่เกี่ยวข้องกับโครงการนี้ได้รับการตีพิมพ์แล้วอีก 2 เรื่อง

ความรู้ที่ได้จากงานวิจัยครั้งนี้ก่อให้เกิดการกระตุ้นงานวิจัยอีกมาก อาทิเช่น การหาทางกำจัด EBV ที่อยู่ภายในเซลล์มะเร็งโดยการใช้ iRNA ไปทำลาย EBNA1 gene ของ EBV, การศึกษา CD21 mRNA ในเซลล์มะเร็งชนิด T-cell โดยวิธี quantitative analysis และการศึกษา CD21 protein expression ใน T-cell โดยวิธี quantitative analysis เป็นต้น

คณะผู้วิจัย

กันยายน 2550

บทนำ วิจัยในภาพรวม

Peripheral T-cell and NK-cell lymphoma (PTCL) และ peripheral T-cell proliferative disease (PTPD) เป็นกลุ่มโรคที่มีการเพิ่มขึ้นแบบ monoclonal proliferation ของ mature T-cell และ/หรือ NK-cell ในร่างกาย ที่ส่วนหนึ่งเกิดจากการติดเชื้อ Epstein-Barr virus สู่ T-cell, กลุ่มโรคทั้งสองนี้พบบ่อยในทวีปเอเชียและอเมริกาใต้ พบน้อยในทวีปยุโรปและอเมริกาเหนือ

Epstein-Barr virus (EBV) หรือที่เรียกว่า human herpesvirus 4 (HHV4) อยู่ใน subfamily "gammaherpesviruses" และใน genus "Lymphocryptovirus", ไวรัสชนิดนี้ประกอบด้วย DNA เป็นเส้นพันกัน 2 สาย (linear double stranded DNA) พันรอบแกนโปรตีน และหุ้มด้วยกลุ่มโปรตีนรูปก้อน nucleocapsid, รอบนอกห่อหุ้มโดย envelope ที่มี gp spike จำนวนมาก spike เหล่านี้คือ glycoprotein gp350/220 ที่ใช้จับกับ CD21 (CR2 ซึ่งเป็น complement C3d receptor) ที่อยู่บนผนังเซลล์ของ host cell, ไวรัสนี้มีขนาดประมาณ 180-200 นาโนเมตร

การติดเชื้อ EBV สู่ B-cell ผ่านทาง CD21 แต่การติดเชื้อนี้สู่ T-cell ยังไม่ทราบแน่ชัด T-cell ที่ไหลเวียนในกระแสเลือดมี CD21 บนผนังเซลล์น้อยกว่า B-cell ถึง 10 เท่า ในคนปรกติพบว่าส่วน extracellular domain ของ CD21 ถูกย่อยกลายเป็น soluble CD21 (sCD21) ที่ไหลเวียนในกระแสเลือด sCD21 ยังคงคุณสมบัติในการจับกับ EBV และ complement C3d ส่วนใหญ่ของ sCD21 สร้างมาจาก mature B-cell และเพียงส่วนน้อยเท่านั้นที่สร้างมาจาก mature T-cell, sCD21 ที่ไหลเวียนในกระแสเลือด อาจมีปริมาณเพิ่มขึ้นหรือลดลงในโรคแต่ละอย่าง sCD21 อาจรวมกับ circulating EBV และติดเชื้อเข้าสู่ T-cell หรืออาจเกิดจากการติดเชื้อโดยตรงผ่านทาง CD21 molecule

วัตถุประสงค์ของการวิจัย

1. เพื่อศึกษา EBV viral load ในผู้ป่วยกลุ่มโรค PTCL และ PTPD จำนวนประมาณ 50 ราย เทียบกับคนปรกติจำนวนเท่ากัน (prospective case-control study) เพื่อหาความสัมพันธ์ของจำนวนไวรัสกับการดำเนินโรค
2. ศึกษาทางเข้าของ EBV สู่ T-cell
3. ศึกษาหาปริมาณ sCD21 ในคนปรกติเทียบกับกลุ่มผู้ป่วย PTCL และ PTPD

4. ศึกษาหาชนิดของ EBV genome (episomal form และ/หรือ integrated form) ที่อยู่ใน peripheral blood T-cell

ระเบียบวิธีวิจัย

1. ศึกษาหา EBV viral-load (cell-free EBV-DNA) ในผู้ป่วย 45 ราย และ control 45 ราย โดยวิธี real-time PCR
2. ศึกษาหาชนิดของ EBV DNA ที่ไหลเวียนในกระแสเลือดว่าเป็นชนิด naked form หรือ complete virion โดยใช้การย่อยด้วยเอนไซม์ DNase I และตามด้วยการศึกษาโดยวิธี real-time PCR
3. การศึกษาหา EBV genome (EBER) ในกลุ่มเซลล์มะเร็งของผู้ป่วยโดยวิธี *in situ hybridization*
4. ศึกษาหาปริมาณ sCD21 ในผู้ป่วย PTCL และ PTPD จำนวน 45 ราย และกลุ่มควบคุม 45 รายโดยวิธี ELISA
5. แยก $CD3^+$ cell และ $CD3^-$ cell จาก peripheral blood lymphocyte ของผู้ป่วยและนำไปทำ cell culture และนำไปศึกษาว่า
   5.1 $CD3^+$ cell, $CD3^-$ cell สามารถสร้าง mRNA ของ CD21 ได้หรือไม่ โดยใช้วิธี RT-PCR
   5.2 ศึกษาหาปริมาณ sCD21 ใน supernatant ของ cell culture ข้างต้นว่า $CD3^+$ cell สามารถ shedding sCD21 ได้หรือไม่ และมีปริมาณมากน้อยเพียงใดเมื่อเทียบกับ $CD3^-$ cell โดยใช้วิธี ELISA และ Western blot
   5.3 ใน $CD3^+$ cell ที่มี EBV genome นำไปศึกษาว่ายีนของ EBV อยู่ในสภาวะ episomal form หรือ integrated form โดยวิธี Southern blot

6. การศึกษาเพิ่มเติมนอกเหนือจากที่เสนอไว้ในโครงการคือ การศึกษาความถี่ของการพบ EBV genome ใน nodal และ extranodal peripheral T-cell lymphoma ในประชากรเขตภาคใต้ตอนล่าง

สรุปผลการวิจัย

ผู้ป่วยกลุ่มโรค PTCL และ PTPD ที่นำมาศึกษามีจำนวน 45 ราย (เป้าหมาย 50 ราย) เป็นชาย 32 ราย หญิง 13 ราย มีอายุระหว่าง 2-80 ปี โดยมีชนิดของโรคต่างๆ คือ extranodal NK/T-cell lymphoma = 8 ราย, peripheral T-cell lymphoma, unspecified = 20, angioimmunoblastic T-cell lymphoma = 7 ราย, subcutaneous panniculitis-like T-cell lymphoma = 3 ราย และ PTPD = 7 ราย กลุ่มควบคุม 45 ราย โดยเป็นเพศเดียวกันและมีอายุไม่มากหรือน้อยกว่าผู้ป่วยเกิน 5 ปี กลุ่มควบคุมมีอายุระหว่าง 3-77 ปี

การศึกษาจำนวน EBV DNA ในพลาสม่าพบว่าในกลุ่มควบคุมไม่พบว่ามีรายใดมี EBV DNA ในกระแสเลือด 37 ใน 45 ราย ของกลุ่มผู้ป่วยพบ EBV DNA ในพลาสม่าโดยมีค่าอยู่ระหว่าง 23 ถึง 6,329,500 copies/ml โดยมีค่าเฉลี่ย = 283,560 copies/ml, และค่ามัธยฐาน = 4,200 copies/ml, ผู้ป่วยที่พบ EBV genome ใน peripheral blood T-cell มีจำนวน EBV DNA ในพลาสม่ามากกว่าผู้ป่วยที่ไม่พบ EBV genome ใน peripheral blood T-cell อย่างมีนัยสำคัญทางสถิติ นอกจากนี้ยังพบว่า จำนวนของ EBV DNA ในพลาสม่ามีความสัมพันธ์กับการพบหรือไม่พบ EBV genome ในกลุ่มเซลล์มะเร็งของผู้ป่วย, ปริมาณ EBV DNA ในพลาสม่าไม่มีความสัมพันธ์กับการพยากรณ์โรคของผู้ป่วย ผู้ป่วยที่ได้รับการรักษาโดยเคมีบำบัดส่วนใหญ่จะมีปริมาณของ EBV DNA ในพลาสม่าลดลง EBV DNA ในพลาสม่าที่พบเกือบทั้งหมดเป็นชนิด naked form ซึ่งเชื่อว่าเกิดจากการเน่าสลายของเซลล์มะเร็งและมีการปล่อยสารพันธุกรรมของ EBV เข้าสู่กระแสเลือด

การศึกษาหาระดับของ sCD21 ในกระแสเลือดผู้ป่วยเทียบกับกลุ่มควบคุมพบว่ามีการลดลงของ sCD21 ในกลุ่มผู้ป่วยอย่างมีนัยสำคัญทางสถิต ปริมาณ sCD21 ของผู้ป่วยไม่มีความสัมพันธ์กับจำนวนของ EBV ในกระแสเลือด และไม่มีความสัมพันธ์กับการพบสารพันธุกรรมของ EBV ในกลุ่มเซลล์มะเร็งของผู้ป่วย

การศึกษาการ express ของยีนและโปรตีน CD21 ใน peripheral blood T-cell ของผู้ป่วย PTPD และ PTCL พบว่ามีการ express ของ CD21 mRNA ใน T-cell และ T-cell เหล่านี้สามารถ shedding sCD21 เข้าไปใน supernatant ของ cell culture ซึ่งแสดงว่ามี CD21 molecule บนผนังเซลล์ของ T-cell ซึ่งเป็นทางเข้าสู่ T-cell ของ EBV นอกจากนี้ยังพบว่า EBV ที่อยู่ใน peripheral blood T-cell อยู่ในสภาวะ episomal form

รายงานฉบับที่ 1

Quantitative Analysis of Cell-free Epstein-Barr Virus DNA in Plasma of Patients with Peripheral T-cell Lymphoma and Peripheral T-cell Proliferative Disease

บทคัดย่อ

วัตถุประสงค์ของงานวิจัยนี้เพื่อหาปริมาณและรูปแบบของไวรัสเอ๊ปสไตน์-บาร์ (EBV) ที่อยู่ในพลาสม่าของผู้ป่วยกลุ่มโรค Peripheral T-cell and NK-cell lymphomas (PTCL) และกลุ่มโรค Peripheral T-cell proliferative disease (PTPD) จำนวน 45 ราย เทียบกับกลุ่มควบคุมที่มีอายุใกล้เคียงกันและเพศเดียวกันจำนวน 45 ราย ศึกษาหาความสัมพันธ์ระหว่างปริมาณของ EBV DNA ในพลาสม่า กับการพบยีนของ EBV ใน T-cell ที่ไหลเวียนในกระแสเลือดและการพบยีนของ EBV ในเนื้องอกของผู้ป่วยแต่ละราย นอกจากนี้ยังศึกษาว่าปริมาณของ EBV DNA ในพลาสม่าของผู้ป่วยมีความสัมพันธ์กับอัตราการอยู่รอดของผู้ป่วยหรือไม่

การหาปริมาณ EBV DNA ในพลาสม่าของผู้ป่วยและกลุ่มควบคุมใช้วิธี real-time PCR, การแยกชนิดของ lymphocytes ($CD3^+$, $CD3^-$) ในกระแสเลือดใช้วิธีแยกโดย anti-human CD3 antibody conjugated magnetic beads, การหายีนของ EBV ใน $CD3^+$ cell และ $CD3^-$ cell ใช้วิธี PCR และการค้นหายีนของ EBV ในเซลล์มะเร็งใช้วิธี in situ hybridization ของ RNA ชนิด EBER, การระบุรูปแบบของ EBV DNA ในพลาสม่าว่าเป็นรูปแบบ complete virion หรือเป็นรูปแบบ naked ใช้เอนไซม์ DNase I ย่อยก่อนนำไปหาโดยวิธี real-time PCR

ผลการศึกษาพบว่าในกลุ่มควบคุม 45 รายไม่พบ EBV DNA ในพลาสม่าเลยแม้แต่รายเดียว ร้อยละ 84 และร้อยละ 71 ของผู้ป่วย PTCL และ PTPD พบ EBV DNA ในพลาสม่าโดยมีจำนวน EBV DNA พบอยู่ระหว่าง 23 ถึง 6,329,500 ต่อมิลลิลิตร (ค่ามัธยฐาน = 4,200 ต่อมิลลิลิตร) และพบว่า EBV DNA เกือบทั้งหมดเป็นรูปแบบ naked, ปริมาณของ EBV DNA ในพลาสม่ามีความสัมพันธ์กับการพบยีนของ EBV ใน $CD3^+$ cell ที่ไหลเวียนในกระแสเลือดและการพบยีนของ EBV ในเซลล์มะเร็ง แต่ไม่มีความสัมพันธ์กับอัตราการอยู่รอดของผู้ป่วย งานวิจัยนี้จึงสรุปว่าปริมาณของ EBV DNA ในกระแสเลือดไม่สามารถนำมาใช้เป็นตัวแสดงถึงการพยากรณ์โรคของผู้ป่วย

ABSTRACT

Purpose: To investigate the detectability and nature of Epstein-Barr virus (EBV) DNA in the plasma and to evaluate whether the plasma concentrations of EBV DNA correlate with the EBV genomic status in peripheral blood T-cells and neoplastic cells, and the clinical outcome among patients with peripheral T-cell and NK-cell lymphomas (PTCL) and peripheral T-cell proliferative diseases (PTPD).

Experimental Design: The quantitative EBV DNA in the plasma of samples was measured by using real-time PCR with SYBR green I. All of 90 samples, 38 samples were obtained from patients with PTCL, 7 samples were obtained from patients with PTPD, and 45 samples were obtained from healthy volunteers. Lymphocytes were isolated from peripheral blood by using Ficoll solution and anti-human CD3 antibody conjugated magnetic beads. The presence of the EBV genome, internal repeat-1 region (IR-1), in the isolated $CD3^+$ and $CD3^-$ cells was analysed by PCR. Detection of the EBV expression (EBV-encoded early RNA, EBER) in corresponding tumor tissues was carried out using *in situ* hybridization. DNase I digestion was applied to determine encapsidated and naked EBV DNA. Statistical analysis of the concentrations of EBV DNA in the plasma, EBV genomes in peripheral blood lymphocytes and in tumor cells, and disease outcome was performed.

Results: Using real-time quantitative cell-free EBV DNA in the plasma was detectable in 32/38 (84.2%) of PTCL patients and 5/7 (71.4%) of PTPD patients. The mean EBV DNA concentration in the positive cases was 283,560 copies/ml (range, 23-6,329,500 copies/ml). EBV DNA in the plasma was not detected in any of the controls. Patients with EBV genome (IR-1) in peripheral blood $CD3^+$ cells and EBV genome (EBER) in the tumor cells had a significantly higher plasma EBV DNA levels than those with negative EBV genomes ($P=0.0001$ and $P<0.00005$, respectively). After DNAse I treatment in ten representative patients with various levels of EBV DNA in their plasma, the majority showed that circulating EBV DNA molecules were naked form with minimal amount of encapsulated virions. Nine of the 11 patients showed a reduction of plasma EBV DNA levels after chemotherapy. The patients in this study had a very poor prognosis; the median survival time was only three months. The plasma EBV DNA levels were not related to survival of the patients ($P=0.08$).

Conclusion: The concentration of EBV DNA in the plasma had a significant relationship to the EBV-positive PTCL and PTPD patients but was not a reliable marker for predicting the outcome of disease. The majority of the circulating cell-free EBV DNA was naked DNA.

Key Words: Epstein-Barr virus (EBV), peripheral T-cell and NK-cell lymphomas (PTCL), peripheral T-cell proliferative diseases (PTPD), real-time quantitative PCR, cell-free EBV DNA.

INTRODUCTION

Latent Epstein-Barr virus (EBV) infection is associated with a heterogeneous group of malignancies, including nasopharyngeal carcinoma, gastric carcinoma, smooth muscle tumor in immunocompromised patient, thymic epithelial carcinoma, Hodgkin's lymphoma, malignant B-cell lymphoproliferative diseases, post-transplant lymphoproliferative disorders, peripheral T-cell and NK-cell lymphomas (PTCL), and peripheral T-cell proliferative diseases (PTPD) [1-10].

PTCL represent 7% to 40% of all non-Hodgkin's lymphoma with the ratio depending largely on the geographic area[11]. They are more commonly found in Asia and Central and South America than in western countries [6, 12, 13]. PTCL consist of various groups of lymphoid neoplasms, which have heterogeneous clinical, histologic, immunophenotypic, and molecular features. The World Health Organisation (WHO) classification of neoplastic diseases of hematopoietic and lymphoid tissues was established in 2001[14]. The peripheral (mature) T-cell and NK-cell neoplasm classification in the WHO scheme is separated into 16 subtypes, including extranodal NK/T-cell lymphoma, nasal type, peripheral T-cell lymphoma, unspecified, angioimmunoblastic T-cell lymphoma, and subcutaneous pannicutitis-like T-cell lymphoma. We have reported a separate entity of the peripheral T-cell neoplasm, which is not included in the WHO classification scheme defined as Peripheral T-cell Proliferative Diseases (PTPD) [7, 8, 15]. Clinical and laboratory findings of the PTPD patients include prolonged fever, weight loss, lymphadenopathy, hepatosplenomegaly, anemia, jaundice and high serum levels of alkaline phosphatase and lactate dehydrogenase. Liver involvement was demonstrated by the infiltration of T-cells into the hepatic sinusoids and portal tracts. The morphology of the T-cell infiltrates varies from the mature cells to the definite malignant lymphoid cells. The monoclonal band of the T-cell receptor genes rearrangement study was detected in about 90% of patients [7]. The majority of antigenic phenotypes of these T-cell infiltrates were $CD3^+$, $CD4^-$, $CD8^+$, $CD20^-$ $CD45R0^-$, $CD56^-$, $CD57^-$, $\beta F1^-$ and $TIA\text{-}1^+$. The median survival of the PTPD patients was only two months despite chemotherapy.

Specific subtypes of PTCL, such as extranodal NK/T-cell lymphoma, nasal type, peripheral T-cell lymphoma, unspecified, and angioimmunoblastic T-cell lymphoma were highly associated with EBV infection, ranging from 40% to more than 95% of the cases [6, 16, 17]. The EBV genome, by an *in situ* hybridization study, was also demonstrated in 30% to 40% of PTPD patients [7, 15]. These

findings confirmed the significant association between EBV infection and peripheral T-cell proliferative diseases/lymphomas.

A high level of cell-free EBV DNA in plasma was detected in patients with EBV-associated disease such as nasopharyngeal carcinoma, Hodgkin's lymphoma, extranodal NK-T-cell lymphoma, nasal type, post-transplant lymphoproliferative disease, Burkitt's lymphoma, lymphoepithelial carcinoma, and infectious mononucleosis [18-25]. The concentration in positive samples varied from 24 to more than 600,000 copies/ml, while the samples from healthy individuals were usually negative. In nasopharyngeal carcinoma and extranodal NK-T-cell lymphoma, nasal type, and Hodgkin's lymphoma patients, the EBV viral load shows promise as a marker of tumor burden that will facilitate monitoring of patients after therapy [16, 21-24].

In this study, we investigated whether EBV DNA levels in the plasma measured by real-time quantitative PCR correlate with the presence of EBV genomes in peripheral blood lymphocytes and in tumor tissues, chemotherapy, and survival of patients. DNase I treatment was designed to determine circulating cell-free EBV DNA as naked EBV DNA.

MATERIAL AND METHODS

Study population. A case-control study of patients with peripheral T-cell proliferative diseases/lymphomas was conducted in Songklanagarind University Hospital, a 900-bed hospital in Songkhla Province, Thailand. The subjects were recruited from August 2004 to July 2006 (a two-year period). There were 45 patients included in this study, of which 38 patients were cases of peripheral T-cell and NK-cell lymphomas (PTCL) and 7 were peripheral T-cell proliferative diseases (PTPD). The PTCL patients were classified according to the WHO classification[14]. The group comprised of 8 extranodal NK/T cell lymphoma, nasal type (NK/T), 20 peripheral T-cell lymphoma, unspecified (PTCL-u), 7 angioimmunoblastic T-cell lymphoma (AITL), and 3 subcutaneous panniculitis-like T-cell lymphoma. PTPD cases were classified according to our previous reports [7, 15]. Clinical follow-up and outcome were derived from patient case notes. Follow-up blood samples were taken at three month intervals. Forty-five healthy volunteers who were residents of Songkhla province in Thailand were recruited as a control group. Their selection was based on matching with the patients according to sex and age (± 5 years). Volunteers with a previous history of serious illness, hematological disease, or fever within one month prior to the study were all excluded from the study. This study was approved by the Hospital Ethics Committee, and all the patients and controls were informed.

***In situ* hybridization (ISH) for EBV**. The fluorescein-conjugated EBV oligonucleotides complementary to portions of the EBV-encoded early RNA transcripts (EBER) (EBER1/2; EBV probe ISH Kit, Novocastra, UK) that are actively transcribed in latently infected cells, as previously described [26], were used in this study. The ISH assay was performed in tumor tissues derived from lymph node, bone marrow, skin, nasopharynx, and nasal cavity of 42 patients. Briefly, sections (5µm) from formalin fixed, paraffin-embedded blocks of the tumor tissue were mounted on Histogrip-coated slides (Zymed labs, San Francisco, CA, USA) and baked for 60 min at 55°C. They were then deparaffinised in xylene, hydrated in ethanol, and digested in proteinase K. Each slide was hybridized with a fluorescein-conjugated EBER probe, washed, and reacted with 5-bromo-4 chloro-3-indolylphosphate (BCIP) and nitro blue tetrazolium (NBT) for visualisation.

White blood cell preparation. Twenty milliliters of heparinized peripheral blood samples were obtained from 45 patients and 45 healthy controls. Lymphocytes were isolated by centrifuge using Ficoll solution (Ficoll-Paque Plus, Amersham Pharmacia Biotech AB, Uppsala, Sweden). Next, the isolated lymphocytes were washed with saline and complete RPMI and then resuspended in 0.5-1.0 ml of complete RPMI supplemented with 10% fetal calf serum. By using anti-human CD3 antibody conjugated magnetic beads (Dynabeads M-450 CD3, Dynal AS, Oslo, Norway) the cells were fractionated into non-T cells ($CD3^-$ cells) and T cells ($CD3^+$ cells). The steps for the isolation procedure followed those of the manufacturer's protocol.

DNA extraction and PCR analysis. DNA was extracted from isolated $CD3^-$ and $CD3^+$ cells using the QIAamp DNA blood Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The extracted DNA was eluted with 50-100 µl of elution buffer prior to the PCR reaction.

The PCR for EBV DNA was performed using the oligonucleotide primers for amplification of IR-1, E1:3 (1087-1106) and E2:3 (1196-1215) as previously described [27]. The amplification included positive control with DNA from Raji cells. The amplified fragment length was 129 bp.

Plasma preparation and DNA extraction. The heparinized plasma from the patients and controls was centrifuged at 6,000 rpm for 10 minutes and the supernatants were passed through a 0.45 µm filter using spin X centrifuge tube filters (Corning, NY, USA) to remove any cellular fragments. The plasma samples were then stored at -20°C until further processing. DNA was extracted from 200 µl of each filtrated plasma using the QIAamp DNA blood Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's process. A final elution volume of 200 µl was stored prior to the PCR reaction.

Real-time PCR for quantitative EBV DNA with SYBR green I. The PCR primers set was derived from the BamHI W region, the internal repeat 1 (IR-1) of EBV [27] . The upstream and downstream primers sequences were 5'-CCC GGA CGA TAT TGA ACA-3' and 5'-GTT TCC GTC TGG GCT TCT-3', respectively. The real-time PCR amplification was carried out in a COBAS TagMan 48 Instrument (Roche, USA). The reaction mixture contained 10 µl of plasma DNA, 20 µl of QuantiTect SYBR Green PCR Master Mix (Qiagen GmbH, Hilden, Germany), 0.5 µM each primer, and nuclease-free water to a final volume of 50 µl. The amplification reaction consisted of a preincubation step at 95°C for 10 min to activate the HotStarTaq DNA polymerase. This was followed by 50 cycles of amplification including denaturation at 95°C for 20 s, annealing at 55°C for 20 s cycle delay for 5 s and extension at 72°C for 20 s. Real-time fluorescence measurements were taken and a threshold cycle (CT) value for each sample was calculated by determining the point at which the fluorescence exceeded a threshold limit. A calibration curve was run in parallel with each analysis, using serial 10-fold DNA extracted from an EBV-positive cell line (Raji) as a standard. Raji was a diploid cell line containing EBV viral genome 50 copies/cell [28]. A conversion factor of 6.6 pg of DNA per diploid cell [29] was calculated for the amount of EBV. The CT values from plasma DNA were plotted on the calibration curve, and the copy number was calculated automatically by a software package for data analysis. Each sample was tested in duplicate, and the mean of the two values was shown as the copy number of the sample. The results were expressed as copies of EBV genome/ml of plasma. Samples were defined as undetectable if the CT values exceeded 50 cycles.

After amplification, melting curve analysis was performed by increasing the temperature form 40°C to 95°C. The melting temperature (T_m) of each sample was used for identification of the EBV DNA. Samples that showed the same T_m point with a Raji cell were interpreted as containing the EBV genome.

DNase I Treatment

To further distinguish all-free circulating naked EBV DNA from encapsidated EBV virions**,** the plasma samples from ten representative patients (4 NK/T, 3 PTCL-u, 2 PTPD, and 1 AITL) with various levels of EBV viral load were digested by DNase I (DNase RQI, Promega, Medison, WI, USA). The supernatant from B95-8 cell line was included as a positive control for the presence of EBV DNA. The samples (200 µl, each) were incubated with 25 µl DNase I for 6 hours at 37°C, and then an additional 25 µl DNase I was added followed by a second incubation for 6 hour at 37°C. Stop buffer was added to inactivate the enzyme with incubation at 65°C. Both digested and

undigested samples were assayed for EBV viral load by the real-time PCR as described above. The real-time PCR of β–globin DNA in post digested samples was performed as a control to confirm the efficiency of DNase I degradation.

Statistical analysis

The data were analysed with Stata statistical software (Stata version 7.0). The correlation of the plasma EBV DNA level in each group was determined using Student's t test. A Kaplan-Meier plot was used to estimate the survival of patients.

RESULTS

The patients were 32 males and 13 females, ranging in age from two to 80 years. The sex-matched controls ranged in age from three to 77 years. The mean ± SD ages of the patients and controls were 43.8 ± 20 years and 44.8 ± 20.1 years, respectively. Of the 45 patients, two of the NK/T subtype and one PTCL-u subtype had insufficient tumor tissue for detection of EBER by ISH assay and were excluded from ISH assay. Additionally, two cases of NK/T subtype had lymphopenia and gave inadequate samples for cell isolation, resulting in only 43 patients being available for PCR detection of EBV genome (IR-1) in peripheral blood lymphocytes.

TABLE I. EBV *In Situ* Hybridization (EBER) Study in the Tumor Cells

Type of tumors	Positive cases/total no. tested cases	Percent positive
NK/T	6/6	100
PTCL-u	13/19	68.4
AITL	3/7	42.9
Subcut	0/3	0
PTPD	3/7	42.9
Total	25/42	59.5

NK/T, extranodal NK/T-cell lymphoma, nasal type; PTCL-u, peripheral T-cell lymphoma, unspecified; AITL, angioimmunoblastic T-cell lymphoma; Subcut, subcutaneous panniculitis-like T-cell lymphoma; PTPD, peripheral T-cell proliferative diseases.

Table I shows the ISH study of EBER in 42 patients with various subtypes of peripheral T-cell proliferative diseases/lymphomas. EBERs were identified in 25/42 (59.5%) of patients. In 25 of these samples, the EBERs were shown in 6/6 (100%) of NK/T, in 3/7 (42.9%) of AITL, in 13/19 (68.4%) of PTCL-u, and in 3/7 (42.9%) of PTPD. None of the three cases of subcutaneous panniculitis-like T-cell lymphoma was EBER positive.

Table II shows the EBV genome (IR-1) in the peripheral blood lymphocytes of 45 controls and 43 patients. Among the control group, the EBV genome in the peripheral blood $CD3^+$ cell was not detected in any subject, whereas 42.2% of controls contained the EBV genome in the peripheral blood $CD3^-$ cells. In contrast to the controls, 65.1% of the patients showed EBV genome in the peripheral blood $CD3^+$ cells, and 83.7% in the peripheral blood $CD3^-$ cells. All cases of NK/T subtype showed EBV genome in both $CD3^+$ and $CD3^-$ cells. All patients with positive EBV genome in $CD3^+$ cells also showed EBV genome in $CD3^-$ cells.

TABLE II. EBV Genomic (IR-1) Study in Peripheral Blood Lymphocyte of the 45 Controls and 43 Patients

Type of cells	No. of cases with IR-1 positive													
	Control		NK/T		PTCL-u		AITL		Subcut		PTPD		Total cases	
	(n=45)		(n=6)		(n=20)		(n=7)		(n=3)		(n=7)		(n=43)	
		%		%		%		%		%		%		%
$CD3^+$ cells	0	0	6	100	14	70.0	2	28.6	2	66.6	4	57.1	28	65.1
$CD3^-$ cells	19	42.2	6	100	17	85.0	5	71.4	2	66.6	6	85.7	36	83.7

NK/T, extranodal NK/T-cell lymphoma, nasal type; PTCL-u, peripheral T-cell lymphoma, unspecified; AITL, angioimmunoblastic T-cell lymphoma; Subcut, subcutaneous panniculitis-like T-cell lymphoma; PTPD, peripheral T-cell proliferative diseases

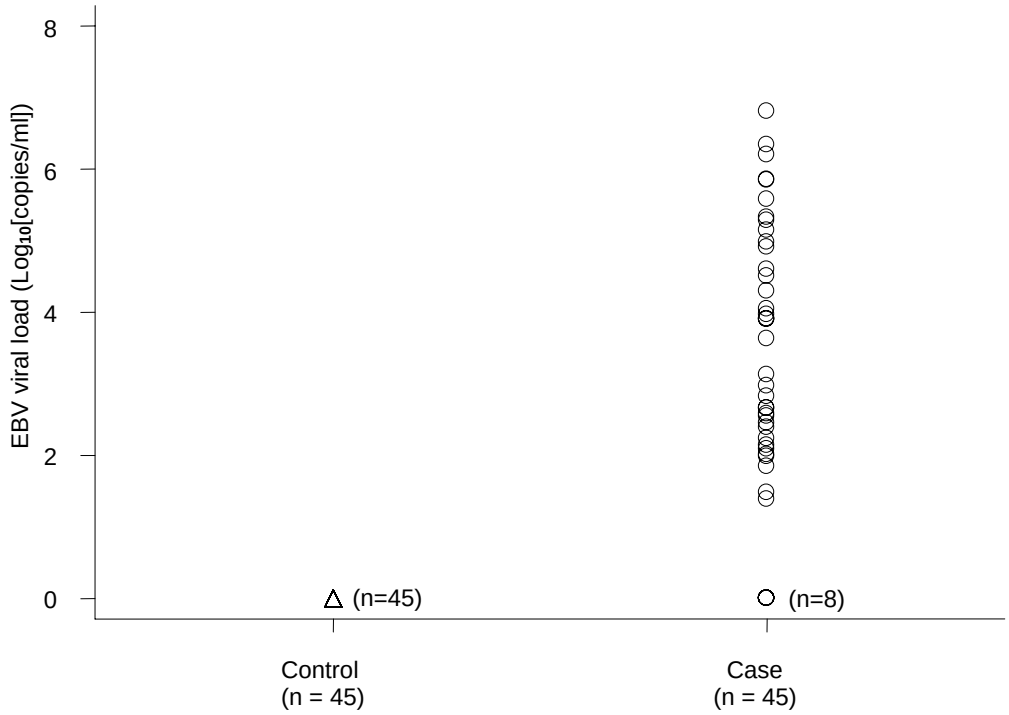

Figure 1. EBV DNA levels in the 45 controls and 45 patients.

The results of the quantitative analysis of EBV DNA in the plasma of the controls and patients are shown in Figure 1. EBV DNA in the plasma was detected in 37/45 (82.2%) of the patients, but none was detected in the controls. The concentration of EBV DNA in the plasma of the positive samples varied from 23 to 6,329,500 copies/ml (mean = 283,560 copies/ml). Figure 2 shows the EBV DNA levels in each of the subtypes of PTCL, and PTPD. All the cases of NK/T showed EBV DNA positive in the plasma with the load varying from 120 to 2,184,000 copies/ml. The highest concentration was detected in a patient with PTPD (6,329,500 copies/ml).

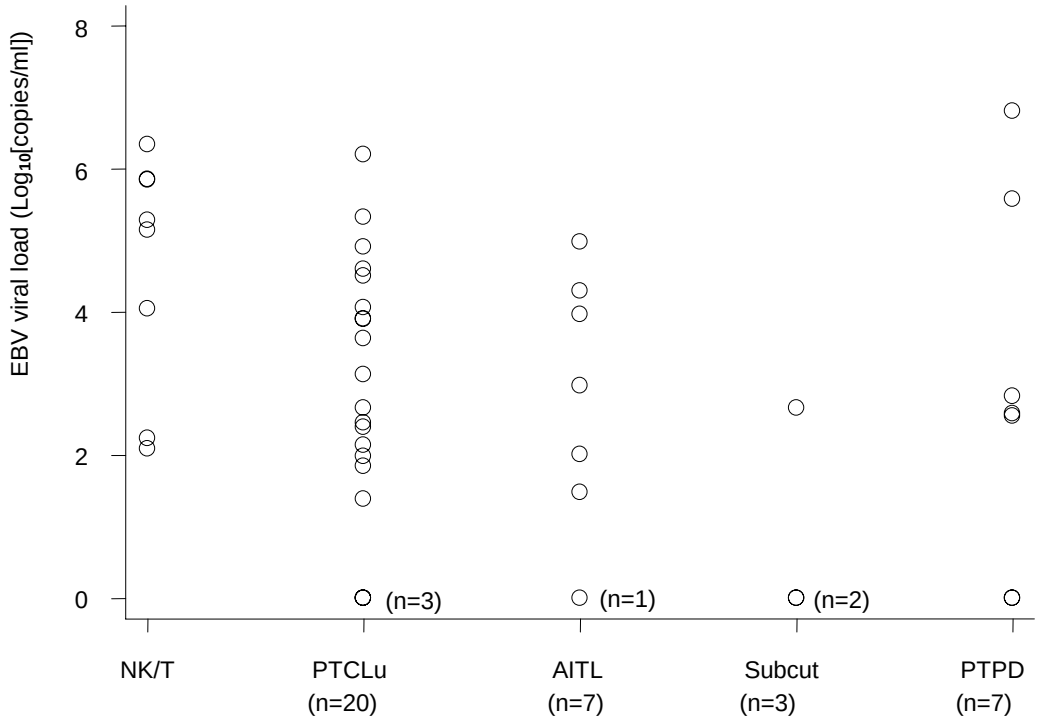

Figure 2. EBV DNA levels classified by subtypes of disease (NK/T, extranodal NK/T-cell lymphoma, nasal type; PTCL-u, peripheral T-cell lymphoma, unspecified; AITL, angioimmunoblastic T-cell lymphoma; Subcut, subcutaneous panniculitis-like T-cell lymphoma; PTPD, peripheral T-cell proliferative diseases)

With DNase I treatment in ten plasma samples, the levels of EBV DNA were undetectable in the plasma samples of 5 patients and other five patients showed marked reduction of the levels of EBV DNA. The EBV DNA detection in the supernatant of the B95-8 cell line after DNase I treatment was 6.44% (Table III). To confirm that DNase I enzyme was efficiently degrading DNA in these samples, a real time PCR of the β-globin gene post-DNase I treatment was performed. The β-globin DNA was completely degraded by DNase I treatment in all of the five tested samples and the supernatant of the B95-8 cell line.

TABLE III. Cell-free EBV DNA Levels Before and After DNase I Digestion in Ten Patients.

Sample	Type of tumor	EBV DNA (copies/ml)		% EBV DNA remaining
		Before DNase I	After DNase I	After DNase I
1	NK/T	2.67×10^5	undetectable	0
2	NK/T	5.91×10^5	42	0.01
3	NK/T	1.35×10^5	20	0.13
4	NK/T	1.86×10^2	undetectable	0
5	PTCL-u	2.13×10^6	1.84×10^3	0.09
6	PTCL-u	1.27×10^5	3.17×10^2	0.25
7	PTCL-u	1.70×10^4	undetectable	0
8	AITL	4.99×10^2	undetectable	0
9	PTPD	9.69×10^5	9.65×10^2	0.10
10	PTPD	3.45×10^4	undetectable	0
control	B95-8	1.02×10^7	6.55×10^5	6.44

NK/T, extranodal NK/T-cell lymphoma, nasal type; PTCL-u, peripheral T-cell lymphoma, unspecified; AITL, angioimmunoblastic T-cell lymphoma; PTPD, peripheral T-cell proliferative diseases

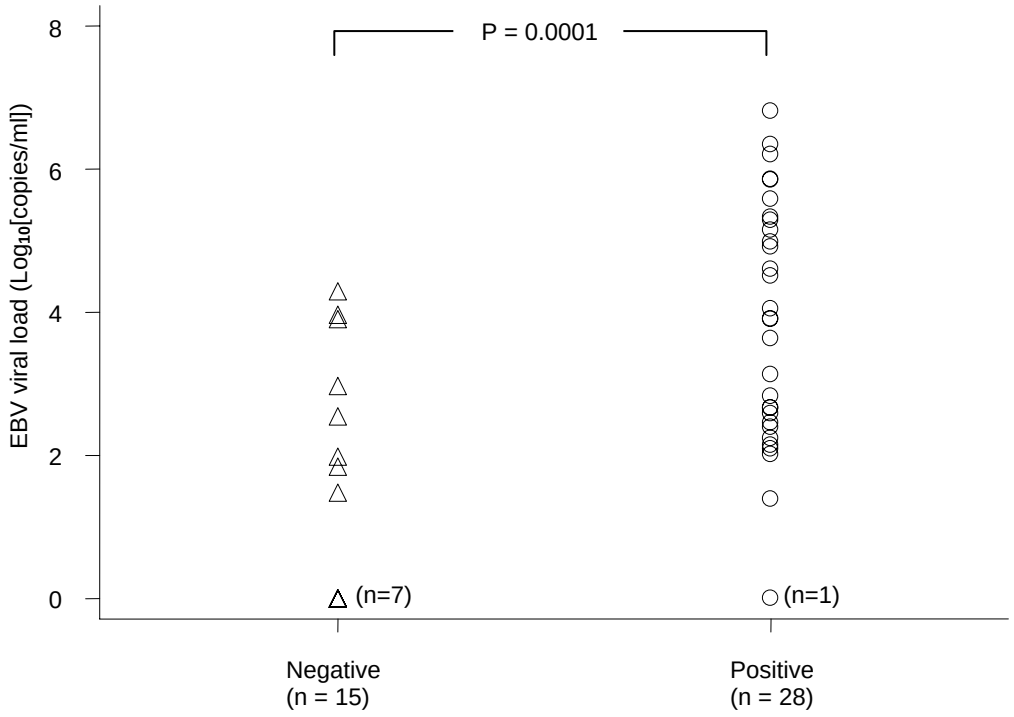

Figure 3. EBV DNA levels in 28 patients who showed EBV DNA in peripheral blood $CD3^+$ cell, and 15 patients with absence of EBV DNA in peripheral blood $CD3^+$ cells.

The relationship of the concentration of EBV DNA in plasma between the presence and the absence of EBV DNA in $CD3^+$ cells is shown in Figure 3. The EBV DNA levels in the EBV-positive $CD3^+$ cells were significantly higher than those in the EBV-negative $CD3^+$ cells (logarithmically transformed followed by t-test, $P = 0.0001$). Figure 4 shows the relationships of the EBV DNA levels in the plasma between EBER-positive and EBER-negative tumor cells. Significant difference of EBV DNA levels was detected between the EBER-positive tumor cells and the EBER-negative tumor cells (logarithmically transformed followed by t-test, $P < 0.00005$).

Ten patients with EBER-negative tumor cells showed EBV DNA in the plasma ranging from 23 to 9,164 copies/ml. Of these ten patients, four showed the EBV genome in peripheral blood $CD3^+$ and $CD3^-$ cells, three showed EBV genome only in $CD3^-$cells, and three showed no EBV genome in either the $CD3^+$or the $CD3^-$ cells.

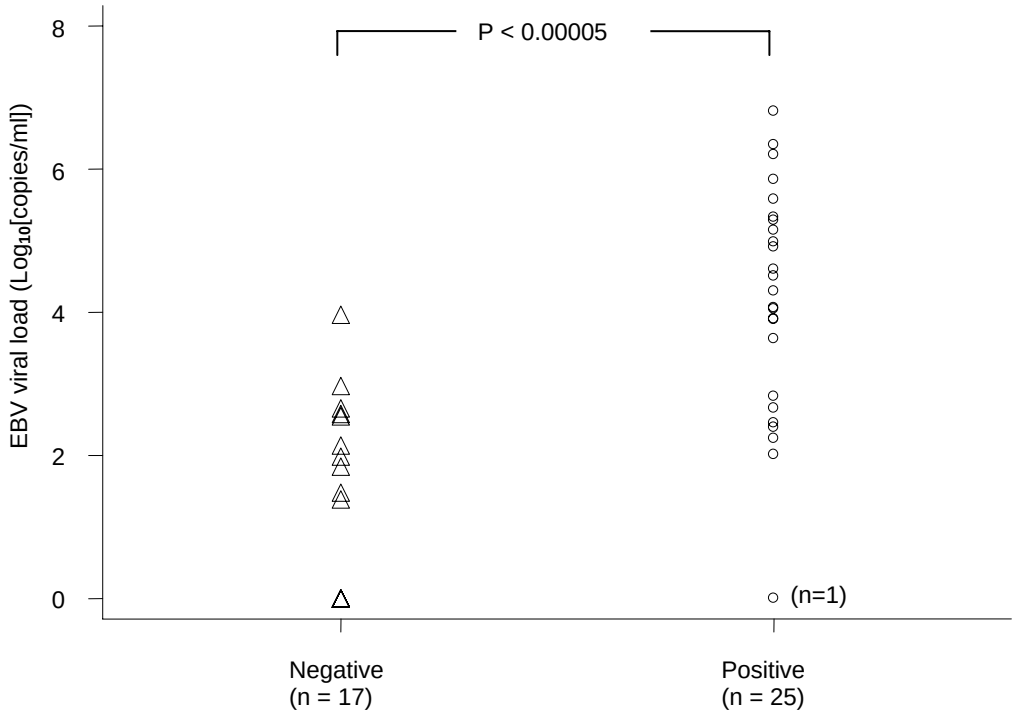

Figure 4. EBV DNA levels in 17 patients with EBER negative in the tumor cells, and 25 patients with EBER positive in the tumor cells.

As for the patients with multidrug chemotherapy (CHOP regimen), the follow-up of plasma EBV DNA levels at three-month intervals was taken from 11 patients as shown in Figure 5. Nine patients demonstrated a reduction of plasma EBV DNA levels after therapy. However, seven of these nine patients died after follow-up at the second three-month interval.

Follow-up data and outcome of the 45 patients are represented by Kaplan-Meier analysis (Figure 6). Thirty-six (80%) patients died at the median survival time of 3 months. Nineteen patients received multidrug chemotherapy and 15 of them died at a median survival time of 5.7 months. The plasma EBV DNA concentration was not related to the survival time of the patients (logarithmically transformed followed by t-test, P=0.08).

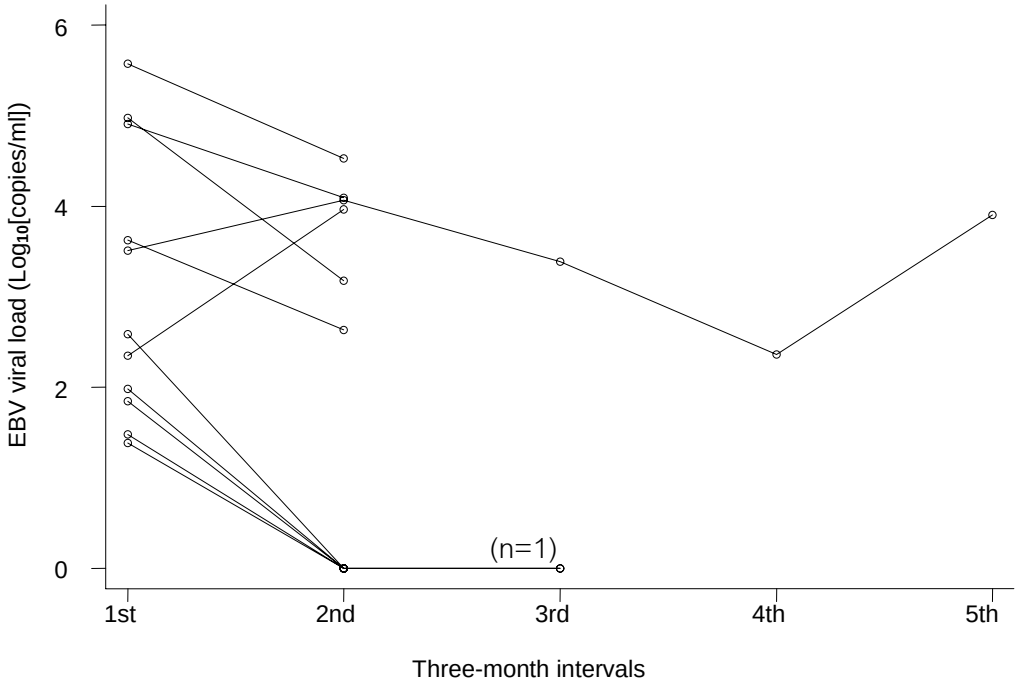

Figure 5. Serial measurement (three-month intervals) of EBV DNA levels in 11 patients who received chemotherapy and died.

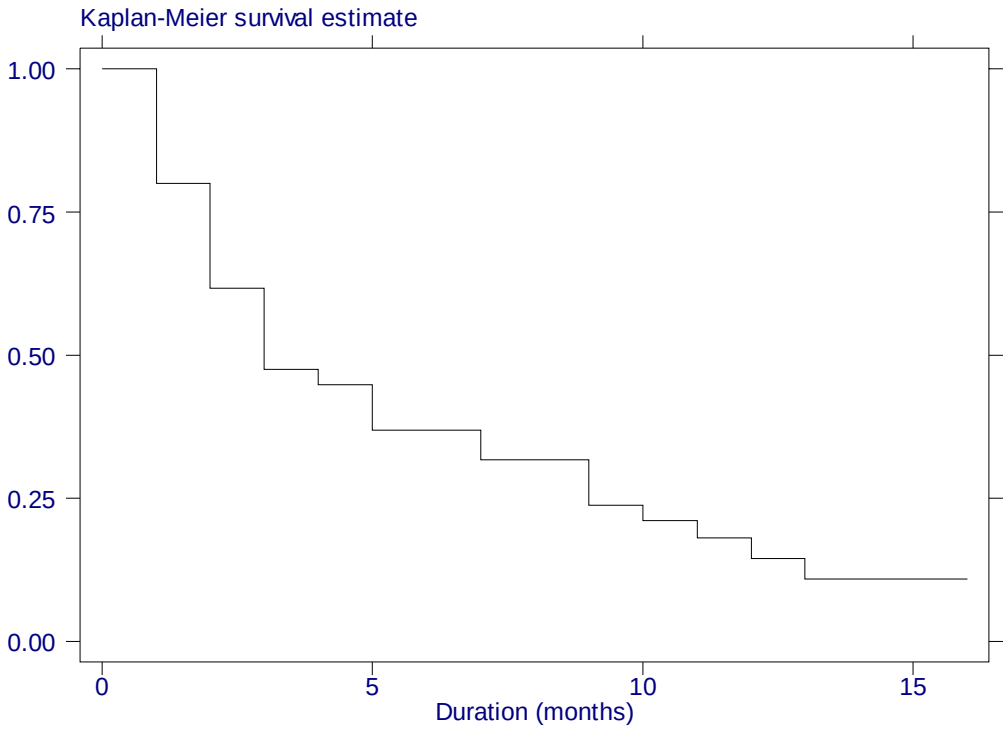

Figure 6. Kaplan-Meier survival estimates of 45 patients with peripheral T-cell and NK-cell lymphomas and peripheral T-cell proliferative diseases.

DISCUSSION

Quantitative analysis of plasma EBV DNA levels has been shown to be clinically important in the diagnosis, therapeutic response and monitoring of EBV-associated malignancy patients [21-24, 30]. Circulating EBV DNA may exist in different forms in different clinical entities. Chan., *et al* [18] showed that the circulating EBV DNA molecules in patients with nasopharyngeal carcinoma (NPC), Hodgkin's lymphoma (HL) and NK/T-cell lymphoma are naked DNA fragments instead of being encapsidated EBV DNA. However, Shotelersuk K, *et al* [31] and Gallagher A *et al* [19] reported that half of the NPC patients but none of HL patients had EBV DNA in the serum after DNase I digestion. These results indicate that the circulating EBV DNA could be mixture of naked and encapsidated EBV DNA. Ryan *et al* [32] have shown that EBV DNA in the plasma of AIDS-related lymphoma and immunosuppressed/transplant patients was almost always naked viral DNA, whereas infectious mononucleosis patients often have a mixture of encapsidated and naked EBV DNA. In this study, we showed that the nature of circulating EBV DNA in patients with PTCL and PTPD exists as the naked form, whereas the intact virions may be found in minimal amount in some patients. It has been reported that in most EBV-associated malignancies, EBV gene expression is rather limited and does not involve lytic genome replication or production of new virions[33]. The EBV genome is present in individual tumor cell in its latent episomal form and the viral genome is replicated during each tumor cell division by host DNA polymerase together with host chromosomes[34]. In some EBV-associated malignancies, the EBV genome may be present in a latent integrated form and also be replicated during each tumor cell division[34]. The presence of circulating naked EBV DNA is possible due to the necrosis and apoptosis of neoplastic cells and release of free DNA to the circulation. Previous study has shown that cellular DNA was degraded into nucleosomal fragments during apoptosis and necrosis[35]. The circulating latently EBV-infected memory B-cells in the immunocompromised patients may differentiate into plasma cells and enter the lytic program of EBV replication[36, 37]. Our finding of the presence of encapsidated virions in the plasma of some patients is suggested to be due to this mechanism.

In the present study, we detected circulating EBV DNA in the plasma samples from all 8/8 of patients with NK/T, 17/20 of patients with PTCL-u, 6/7 of patients with AITL, 1/3 of patients with subcutaneous panniculitis-like T-cell lymphoma, and 5/7 of patients with PTPD. Eight patients (11.8%) and all of the controls did not show circulating EBV DNA. There was a wide variation of plasma EBV DNA concentration in the positive cases with the results varying from 23 to more than six million copies/ml. This might be a reflection of the tumor load or type of neoplasm in individual cases. Our data also showed the reduction of plasma EBV DNA level following chemotherapy. The

correlation between plasma EBV DNA level and the therapeutic response suggests that tumor cells were the principal source of circulating EBV DNA. This hypothesis is confirmed by a previous report on EBV typing between primary tumors and sera showing identical results [38].

Two thirds of the patients showed the EBV genome in the peripheral blood $CD3^+$ cells, which was not detected in any of the controls. This concurs with our previous report on EBV-associated T-cell and NK-cell proliferative diseases/lymphomas [8]. By analyzing the levels of plasma EBV DNA from patients with presence and absence of EBV genome in the peripheral blood $CD3^+$ cells, our data indicate that patients with EBV genome in peripheral blood $CD3^+$ cells have a significantly higher concentration of plasma EBV DNA copies than those negative cases. All patients with EBV genome in the peripheral blood $CD3^+$ cells also have EBV genome in the peripheral blood $CD3^-$ cells. This raises the possibility that infected B-cells could be the source of the viral infection in T-cells.

Our data have demonstrated that the EBV genome (EBER) in tumor cells was found in 60% of the cases and the EBER-positive patients also showed significantly higher concentrations of the plasma EBV DNA than the EBER-negative patients. However, some of our patients with EBER negative tumor cells demonstrated low levels of EBV DNA in their plasma. This finding corresponds to a previous report showing a slight increase in plasma EBV DNA in a significant proportion of patients with EBER-negative squamous cell carcinoma of the head and neck regions [25]. Additionally, three patients in our study who had undetectable EBV genome in tumor cells and circulating lymphocytes showed a positive EBV DNA detection in the plasma (29, 69, and 920 copies/ml). The presence of circulating EBV DNA in these patients remains unclear.

Plasma EBV DNA was shown to be a predictor for the outcome of treatment in patients with nasopharyngeal carcinoma [39]. The patients with detectable plasma EBV DNA had a significantly worse overall survival and relapse-free survival than those with undetectable EBV DNA after radiotherapy. On the contrary, the overall survival of the patients in our reported series was not related to the plasma EBV DNA concentration. This may be attributed to the very short survival time of the patients with PTCL and PTPD.

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รายงานฉบับที่ 2

Reduction of Soluble Complement Receptor 2/CD21 in Patients with Peripheral T-cell and NK-cell Lymphoma, and Peripheral T-cell Proliferative Disease

บทคัดย่อ

Soluble CD21 (sCD21) เป็นโปรตีนที่พบในพลาสม่า เกิดจากการหลุดของส่วน extracellular domain ของโมเลกุล CD21 ซึ่งเกาะอยู่บนผนังเซลล์ของ B-cell การศึกษาก่อนหน้านี้พบว่าโรคบางชนิดได้แก่มะเร็งหลังโพรงจมูก และ B-cell chronic lymphocytic leukemia มีการเพิ่มขึ้นของ sCD21 ในพลาสม่า และในทางตรงข้าม ผู้ป่วยกลุ่มโรค autoimmune ซึ่งได้แก่ systemic lupus erythematosus, Sjogren syndrome และ rheumatoid arthritis พบการลดลงของ sCD21 ในพลาสม่า

การศึกษาหาระดับของ sCD21 ในพลาสม่าของผู้ป่วย PTCL และ PTPD จำนวน 45 ราย โดยเทียบกับกลุ่มควบคุมจำนวน 45 ราย การศึกษาพบว่ามีการลดลงของ sCD21 ในกลุ่มผู้ป่วยอย่างมีนัยสำคัญทางสถิติ ปริมาณ sCD21 ในพลาสม่าของผู้ป่วยไม่มีความสัมพันธ์กับจำนวน EBV ในพลาสม่า และไม่มีความสัมพันธ์กับการพบหรือไม่พบ EBV genome ในเซลล์มะเร็งของผู้ป่วย

ABSTRACT

Background: The soluble form of complement receptor 2/CD21 (sCD21) is spontaneously released by B and T cells upon shedding of the extracellular domain of the molecule. Reduction of sCD21 levels was detected in systemic lupus erythematosus, Sjogren syndrome and rheumatoid arthritis

Objective: To investigate plasma sCD21 level in patients with peripheral T-cell and NK-cell lymphoma (PTCL), and peripheral T-cell proliferative disease (PTPD).

Study design: The case-control study was conducted on 45 patients with PTCL and PTPD. Detection of plasma sCD21 levels was based on a solid phase sandwich enzyme link-immuno-sorbent assay (ELISA).

Results: Plasma sCD21 concentration of the patients was significantly lower than the controls. There was no significant difference between PTCL and PTPD group, and also in the presence or obsence of EBV genome in the tumor tissues. There was no correlation between plasma sCD21 concentration and the EBV viral load in this group of patients.

Conclusion: PTCL and PTPD patients have significant lower sCD21 levels than healthy controls. Plasma sCD21 level is not related to the presence or absence of EBV genome in the plasma and in the tumor tissues.

INTRODUCTION

CD21 (complement receptor type 2, CR2) is a 145 kDa type 1 transmembrane glycoprotein that was described as the receptor for fragments of complement component C3 (C3dg, C3d and iC3b) on the immune complexes and as the receptor for Epstein-Barr virus (EBV) (1-3). The CD21 protein consists of an extracellular domain of 15 or 16 short concensus repeats (SCR1 to SCR16) followed by a transmembrane region and a short intracytoplasmic domain (4). The EBV glycoprotein gp350/220 attaches to the two most membrane-distal CD21 repeats, SCR1 and SCR2 (5,6). CD21 is expressed in peripheral blood B cells and B cell lines, but not on early pre B cells and late developmental stages (7). In addition it is expressed in low amount on human follicular dendritic cells, peripheral blood T cells and a subpopulation of immature T cells (8-10). Thirty to 40 per cent of normal peripheral blood T cells express CD21 and the intensity of CD21 expression is approximately 10-fold lower than peripheral blood B cells (9). CD21 also expressed on basophils and astrocytes (11, 12).

Soluble CD21 (sCD21) is the ectodomain part of membrane bound CD21 generated by shedding from the cell surface and is found in human plasma, synovial fluid and in cell culture supernatant of human lymphoid cells (B cells and T cells) (13-15). There are several forms of sCD21 in the circulation, and a 126 kDa form is considered to be a major component (16, 17). Peripheral blood B cells, but not T cells, contribute to a major source of plasma sCD21-pool (18). Soluble CD21 retains the capacity to bind complement fragements (iC3b), EBV and CD23. Functionally sCD21 can activate monocyte through binding to membrane CD23 and abrogate B cell/follicular dendritic cell interaction, there by inhibiting antibody production by antigen primed B cells (14, 19). Levels of sCD21 vary in age and in several clinical conditions. Serum sCD21 concentration decreases with age and is independent of gender (18). Decreased levels of serum sCD21 were seen in patients with systemic lupus erythematosus, Sjogren syndrome and rheumatoid arthritis (13-14, 20). Elevated levels of sCD21 were observed in patients with infectious mononucleosis, rubella infection, B cell chronic lymphocytic leukemia (B-CLL) and nasopharyngeal carcinoma (21-22). To our knowledge, there is no substantial report on sCD21 levels in patients with malignant lymphoma of various types. It has been shown that recombinant sCD21 can cause inhibition of EBV infection of B cell in vitro and in vivo (23;24). Soluble CD21 may has poteintial therapeutic value in the treatment of EBV-induced malignancies.

Peripheral T cell lymphoma (PTCL) represent 7% to 40% of all non-Hodgkin's lymphoma with the ratio depending largely on the geographic area (25). They are more commonly found in Asia and Central and South America than in western countries (26, 27). PTCL consist of various

groups of lymphoid neoplasms, which have heterogeneous clinical, histologic, immunophenotypic, and molecular features. We have reported a separate entity of the peripheral T cell neoplasm, which is not included in the WHO classification scheme defined as Peripheral T cell Proliferative Diseases (PTPD) (28-30). Clinical and laboratory findings of the PTPD patients include prolonged fever, weight loss, lymphadenopathy, hepatosplenomegaly, anemia, jaundice and high serum levels of alkaline phosphatase and lactate dehydrogenase. Liver involvement was demonstrated by the infiltration of monoclonal T cells into the hepatic sinusoids and portal tracts.

Specific subtypes of PTCL, such as extranodal NK/T cell lymphoma, nasal type, peripheral T cell lymphoma, unspecified, and angioimmunoblastic T cell lymphoma, were highly associated with EBV infection, ranging from 40% to more than 95% of the cases (28, 31). The EBV genome, by an *in situ* hybridization study, was also demonstrated in 30% to 40% of PTPD patients (28, 29). These findings confirmed the significant association between EBV infection and peripheral T cell proliferative diseases/lymphomas.

Cell-free EBV DNA was detected in 84% of PTCL patients and 71% of PTPD patients, but not in the control (32). Patients with EBV genome (EBER) in the tumor cells had significantly higher plasma EBV DNA levels than those with negative cases. The majority of circulating EBV DNA molecule was naked form.

In this study, we investigated sCD21 levels in the plasma by the ELISA method in patients with PTCL and PTPD as compare to the healthy controls. We also investigated whether sCD21 levels correlate with the presence of EBV genome (EBER) in tumor tissues and EBV DNA concentration in the plasma.

MATERIALS AND METHODS

Study population

A case-control study of patients with peripheral T-cell proliferative diseases/lymphomas was conducted in Songklanagarind University Hospital, a 900-bed hospital in Songkhla Province, Thailand. There were 45 patients included in this study, of which 38 patients were cases of peripheral T-cell and NK-cell lymphomas (PTCL) and 7 were peripheral T-cell proliferative diseases (PTPD). The patients were 32 males and 13 females, ranging in age from two to 80 years. The sex-matched controls ranged in age from three to 77 years. The median ages of the patients and controls were 47 and 48 years, respectively. This study was approved by the Hospital Ethics Committee, and all the patients and controls were informed.

Quantification of sCD21 from plasma by ELISA

The detection of human sCD21, was based on a solid phase sandwich Enzyme Linked-Immuno-Sorbent Assay (ELISA), was performed follow the manufacturer's protocol (Abcam, Cambridge, United Kingdom). Briefly, 100 mcL of plasma sample, was added into the microtiter plate well, followed by adding 50 mcL of biotinylated anti-sCD21. The mixture was incubated for 1 hour at room temperature. After washing 5 times with washing buffer, Strepatavidin conjugated with horseradish peroxidase enzyme was added 100 mcL into the well and incubated for 30 minutes. The reaction well was washed 5 times as above then 100 mcL of tetramethylbenzidine (TMB) chromogen was added and incubated in the dark for 15 minutes. The reaction was stopped by adding 100 mcL of H_2SO_4 and measured the optical density (OD) at 450/620 nm with automate ELISA analyzer (BEP III, Dade-Behring, Marburg, Germany). The 2-fold serial dilution of standard 100 to 3.12 U/ml were added every batch tested. The calibration curve was constructed to calculate the concentration of human sCD21 in the sample.

In situ hybridization (ISH) for EBV

The fluorescein-conjugated EBV oligonucleotides complementary to portions of the EBV-encoded early RNA transcripts (EBER) (EBER1/2;EBV probe ISH Kit, Novocastra, United Kingdom) that are actively transcribed in latently infected cells, as previously described (33), were used in this study. The ISH assay following the manufacturer's instructions was performed in tumor tissues derived from lymph node, bone marrow, skin, nasopharynx, and nasal cavity of 42 patients.

Real-time PCR for quantitative EBV DNA with SYBR green I

Real-time PCR method and the results of quantitative analysis of EBV DNA in the plasma of the patients are referred to Ref. 32.

RESULTS

Fig.1 shows plasma concentration of sCD21 in 45 healthy controls and 45 patients with PTCL or PTPD. The plasma levels of the control group ranged from 23.1 to 527.3 U/ml with the geographic mean/median values of 94.9/94 U/ml, and for the patients, ranging from 20.1 to 111.3.2 U/ml with the geographic mean/median values of 62.6/50.4 U/ml. The sCD21 plasma level of the patients was significantly lower than the control group (Mann-Whitney, P=0.0005). There was no

correlation between plasma concentration of sCD21 and the cell-free EBV DNA plasma concentration in this group of patients (Spearman correlation coefficient = -0.0545, P=0.7285)

According to patients with PTCL and PTPD, there was no significant difference in the plasma sCD21 concentration (Mann-Whitney, P=0.925). Of those 42 patients with adequate tumor sample taken for EBER analysis; 24 showed EBER positive and 18 showed EBER negative in their tumor cells Significant difference of the plasma sCD21 levels was not detected among this two groups (Mann-Whitney, P=0.4926)

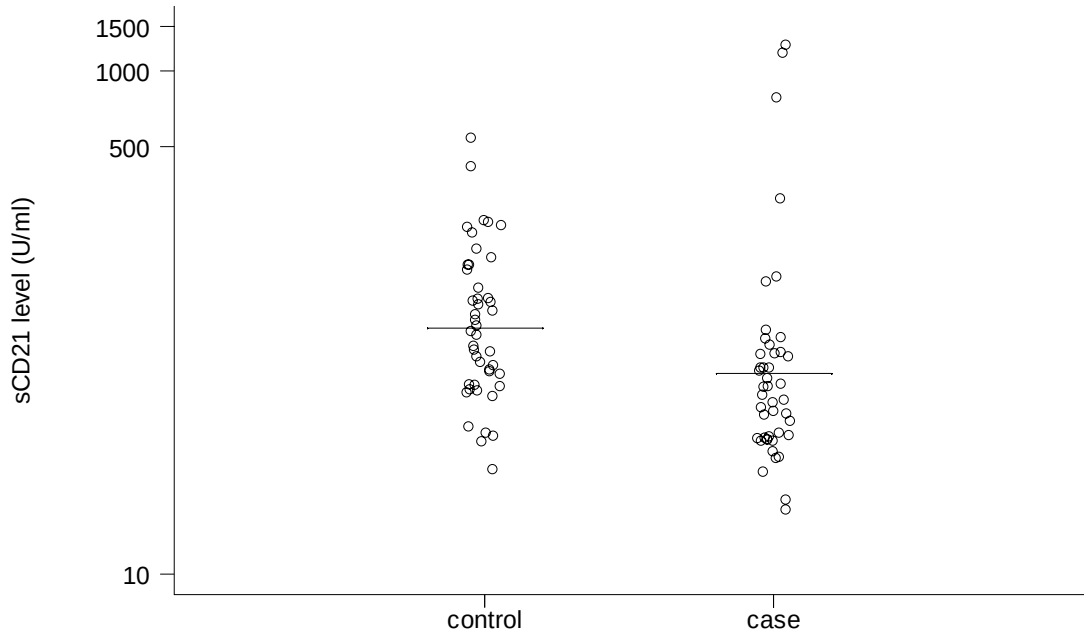

Fig. 1 Plasma soluble CD21 (sCD21) concentration in the 45 healthy controls and 45 patients.

DISCUSSION

The study presented here demonstrates that the levels of sCD21 in the plasma of PTCL and PTPD patients were significantly lower than in a control group of age- and sex-matched healthy individuals. This finding was similar to the previous report on patients with systemic lupus erythematosus. Sjogren syndrome and rheumatoid arthritis (13-14, 20). The sCD21 plasma concentration in this group of patients was not related to the presence of EBV genome in their tumor tissues and the numbers of plasma cell-free EBV DNA. We believe that the reduction of sCD21 level is not caused by EBV infection but rather than causing by the disease mechanisms.

There are several possible reasons for this reduction. First, a decreased amount of circulating lymphocytes in PTCL and PTPD patients. The median value of circulating lymphocytes in this group of patients was 643 cells/mm^3 with the range of less than 50 to 2470 cells/mm^3. Secondly, an increased in amount of complement activation products could bind to sCD21, leading to increased clearance from plasma. Thirdly, circulating EBV DNA may bind to sCD21 and inhibited sCD21 detection by the ELISA method.

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รายงานฉบับที่ 3

The Study of Episomal and Integrated Epstein-Barr Virus DNA in the Peripheral Blood Lymphocytes of Peripheral T-cell Proliferative Diseases/Lymphomas Patients

บทคัดย่อ

การศึกษาหารูปแบบของ Epstein-Barr virus (EBV) ที่อยู่ในเซลล์ลิมโฟไซท์ชนิด T-cell และ non-T-cell ที่ไหลเวียนในกระแสเลือดของผู้ป่วยกลุ่มโรค Peripheral T-cell proliferative diseases/lymphomas จำนวน 10 ราย โดยวิธี DNA probes และ Southern blotting โดยใช้เซลล์ Raji และเซลล์ B95-8 เป็นตัวควบคุม ผลการศึกษาพบว่า เซลล์ Raji มีรูปแบบของ EBV DNA ที่อยู่ในเซลล์เป็นชนิด episomal form ส่วนเซลล์ B95-8 มีรูปแบบ EBV DNA เป็นชนิด episomal form และ integrated form ร่วมกัน การศึกษา peripheral blood T-cell ของผู้ป่วยจำนวน 10 ราย ไม่สามารถทำได้ ทั้งนี้เนื่องจากไม่สามารถเลี้ยง T-cell ได้ เซลล์พวกนี้ตายเร็วมาก และไม่สามารถหาเซลล์ในระยะ metaphase ที่จะนำมาศึกษาโดยวิธี FISH ได้ ส่วนใน peripheral blood lymphocytes ชนิด non-T-cell สามารถศึกษารูปแบบของ EBV DNA ได้ 4 รายจากจำนวนที่นำมาศึกษา 10 ราย ซึ่งทั้ง 4 รายเป็นชนิด episomal form ของ EBV DNA

INTRODUCTION

Epstein-Barr virus (EBV) is a lymphocryptovirus belonging to the subfamily of gammaherpesvirinae. It is an etiologic agent of infectious mononucleosis, which is a self-limiting disease, and a fatal form is an uncommon presentation of primary infection and is characterized by an uncontrolled B-cell proliferation due to a defect in T-cell-mediated immune regulation (1,2). EBV infection has also been implicated in the development of a variety of malignancies, including nasopharyngeal carcinoma, gastric carcinoma, smooth muscle tumor in immunocompromised patients, thymic lymphoepithelial carcinoma, Hodgkin's disease, and non-Hodgkin's lymphoma of B-cell and T-cell (NHL-B, NHL-T) origins(3-10).

EBV DNA is detected as a linear form in cells at the lytic phase of EBV infection, but during latent infection the viral genome is maintained in the episomal form. For many EBV-associated diseases/malignancies, it is still a matter of controversy whether infected cells harbor episomal or chromosomally integrated EBV genomes of both. Whether EBV integrates into the human genome randomly or non-randomly is still debatable. It is well established that the expression of EBV genes per se carries oncogenic potential, but the discrimination between the episomal form and integrated form is of great relevance because integration events can contribute to

the ocogenic propertics of EBV, whereas host cells that harbor episomes may not carry the risks mediated by chromosomal integration (11).

The EBV genomic integration into the host cell chromosome was reported in many diseases such as chronic active EBV infection (12), and EBV-associated nasopharyngeal carcinoma (13). Oshima K et al (14) reported 104 cases of EBV-associated disease/malignancies and showed the integrated EBV was demonstrated in 11 cases (10%); one of 12 cases with nasopharyngeal carcinoma (8%), 5 of 14 cases with B-cell lymphoma (36%), 4 of 31 cases with NK cell leukemia/lymphoma (13%), and one of 11 cases with chronic active EBV infection (9%). However, none of the 24 T-cell lymphoma, 0/7 of Hodgkin's lymphoma, and 0/5 of acute EBV infection cases showed integrated EBV. Seven of the eleven cases with EBV integration showed both integrated and episomal forms. Majority of the EBV-associated malignancies carried the episomal form of EBV DNA. The integrated form was frequently found in B-cell lymphoma and none was detected in T-cell lymphoma.

In contrast to the EBV-associated malignancies, the integrated form was almost always found in the EBV-associated tumor cell lines (15-18). Burkitt lymphoma cell line (Raji cell line) showed EBV integration frequently in chromosome 4q, 2q, 1q and 7q, and no viral integration occurred in chromosome 16-22 and sex chromosomes (18). This finding support the notion that EBV integrates into the Raji cell genome non-randomly. The aim of this study is to investigated whether EBV DNA in the peripheral blood lymphocyte ($CD3^+$ cells, $CD3^-$ cells) of the PTPD and PTCL patients is the episomal or integrated forms, or both.

MATERIALS AND METHODS

DNA probes and Southern blotting analysis

DNA was extracted from Raji and B95-8 cell lines and isolated $CD3^-$ and $CD3^+$ cells of the PTPD and PTCL patients using the QIAamp DNA blood Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions.

DNA probes: The extracted DNA from Raji or B95-8 cell lines were labeled with DIG-dUTP in the PCR (PCR DIG probe synthesis kit) according to the manufacturer's procedure (Roche Diagnostics, IN, USA). EBV1 and EBV2 probe were developed at the left and the right of terminal repeats of viral genome. The following specific primers based on GenBank database sequence EBV/V01555 as previously described (19) were: for EBV1 probe (895 bp),
5'-AGGCATTTACGGTTAGTGTG-3',

and 5’-CGGTCAGGATAGCAAGAAT-3’, for EBV2 probe (620 bp),
5’-ATCCTCAGGGCAGTGTGTCAG-3’,
and 5’-CAAGCCGCAGCGACTTTC-3’.

Southern blotting analysis: For each cell line and sample, 1-5 µg of DNA was digested with BamHI and the fragments were separated by electrophoresis on 0.8% agarose gel and transferred to a Hybond N+ nylon membrane. The filters were prehybridized with DIG Easy Hyb (Roche Diagnostics) for 2 h at 42°C, then hybridized with EBV1 and EBV2 probe overnight at 42°C. Washings were performed in 2X SSC, 0.1% SDS at room temperature for 10 min, 0.5X SSC, 0.1% SDS for 30 min at 68°C, and maleic acid buffer (washing buffer) for 2 min at room temperature. To detect probe-target hybrids, the membrane was blocked with blocking solution (Roche Diagnostics) for 30 min, then incubated with anti-Digoxigenin-AP antibody solution (Roche Diagnostics) for 30 min. After washing with washing buffer, the chemiluminescent substrate, CDP-Star (Roche Diagnostics) was added to the blot for 5 min. Finally the membrane was sealed in the enveloped and the light emission was recorded on the Lumi-Imager.

RESULTS

Ten PTPD and PTCL patients with the EBV genome in the peripheral blood lymphocytes; $CD3^+$ cells and $CD3^-$ cells were included in the study. The study was performed on $CD3^-$ cells only because difficulty in cell culture of those $CD3^+$ cells. Raji and B95-8 cell lines were used as controls. Results showed that, the episomal form of EBV DNA was detected in Raji cell line and the mixed forms of EBV DNA, episomal form and integrated form, were detected in B95-8 cell line. Four of ten PTPD and PTCL patients showed episomal form of EBV DNA in their peripheral blood $CD3^-$ cell. There was no integrated form of EBV DNA detected. Six Patients did not have enough DNA for Southern blotting analysis.

DISCUSSION

This study has demonstrated EBV DNA in Raji cell line to be a epsiomal form instead of being integrated form as previously described (18). The EBV genome in the circulating lymphocytes (non-T-cells) was presented as an episomal form in the PTPD and PTCL patients which is similar to the nature of EBV in the circulating B-cell of the latently EBV-infected healthy

individuals. In this study are can not determine the form of EBV DNA in the peripheral blood T-cell because of the difficulty in cell culturing of T-cells and the inadequacy of the extracted DNA.

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รายงานฉบับที่ 4

CD21 mRNA Expression and Shedding of Soluble CD21 from Peripheral Blood T Lymphocytes of Patients with Peripheral T-cell Proliferative Diseases/Lymphomas.

บทคัดย่อ

การศึกษานี้เพื่อยืนยันว่ามี CD21 อยู่บนผนังเซลล์ของ T-cell ของผู้ป่วย peripheral T-cell proliferative disease (PTPD) และ peripheral T-cell lymphoma (PTCL) หรือไม่ ผลการศึกษาพบว่ามีการแสดงของ CD21 mRNA ใน T-cell แสดงว่ามีการ transcription ของยีนนี้ และพบ soluble CD21 (sCD21) ใน supernatant ของ cell culture แสดงว่ามี CD21 บนผนังเซลล์ของ T-cell และมีการ shedding ของ CD 21 เป็น sCD21 เข้าไปยัง supernatant ของ cell culture ผลการศึกษานี้สรุปว่า peripheral blood T-cell ของผู้ป่วย PTPD และ PTCL มี CD21 บนผนังเซลล์ ซึ่งเป็นทางเข้าสู่เซลล์ของไวรัสเอ็ปสไตน์-บาร์ และเป็นสาเหตุของการเกิดมะเร็งของ T-cell

ABSTRACT

CD21 mRNA expression in the peripheral blood lymphocytes ($CD3^+$ cells, and $CD3^-$ cells) and the analysis of soluble CD21 (sCD21) of the cell culture supernatant of those lymphocytes were conducted in the peripheral T-cell proliferative disease (PTPD) and peripheral T-cell lymphoma (PTCL) patients by RT-PCR and Western blot analysis techniques, respectively. Results showed that half of $CD3^+$ cells and $CD3^-$ cells express CD21 mRNA, and those with positive results, sCD21 shedding was detected in all of $CD3^+$ and $CD3^-$ cell cultures. This confirms that peripheral blood T-cell of PTPD and PTCL patients have considerable amount of CD21 molecules on the cell surface.

INTRODUCTION

Peripheral T-cell proliferative diseases (PTPD) and peripheral T-cell lymphomas (PTCL) are highly associated with Epstein-Barr virus (EBV) infection (1-5). The EBV genome was detected in 30% to more than 90% of PTPD and PTCL cases. By contrast, it was detected in about 10 to 15 percent B-cell non-Hodgkin's lymphomas in the subtypes of Burkitt lymphoma and diffuse large B-cell lymphoma (2).

CD21 is a 145 kDa type 1 transmembrane glycoprotein that was described as the EBV receptor and as the receptor for fragments of complement component C3 (C3d, c3dg, iC3b) (6-8). It consists of an extracellular domain of 15 or 16 short concensus repeats (SCR1 to SCR16) followed by a intramembranous region and a short intracytoplasmic domain (9). The EBV glycoprotein gp350/220 attaches to the most membrane-distal CD21 repeats, SCR1 and SCR2, which is the route of EBV entering into host cell and causes EBV infection (10, 11). CD21 is expressed in peripheral blood B-cells and is expressed in the lower intensity (10-fold lower than peripheral blood B-cells) in the peripheral blood T-cell (12). Peripheral blood B-cells and peripheral blood T-cells ($CD4^+$ and $CD8^+$) from healthy individuals have similar amout of CD21 mRNA, but only B-cells, not $CD4^+$ or $CD8^+$ T-cells, expressed detectable amount of CD21 glycoprotein on their cell surface (13). A case control study on PTPD and PTCL patients demonstrated EBV genome in the peripheral blood T-cell ($CD4^+$ cells, $CD8^+$ cells, or both) in 50% of cases which was not found in any of the controls (14). This confirms the EBV infectivity to the peripheral blood T-cells, but the route of infection is uncertain.

Soluble CD21 (sCD21) is ectodomain part of membrane bound CD21 generated by shedding from the cell surface and is found in human plasma, synovial fluid and in cell culture supernatant of human lymphoid cells (B-cells and T-cells) (15-17). There are several forms of sCD21 in the circulation, and a 126 kDa form is considered to be major component (18, 19). Peripheral blood B-cells, but not T-cells, contribute to a major source of plasma sCD21-pool (20).

In this study, we investigated the CD21 mRNA expression in the peripheral blood lymphocytes ($CD3^+$ and $CD3^-$ cells) by RT-PCR technique and indirect measurement of CD21 glycoprotein expression by Western blot analysis of sCD21, in 10 PTPD and PTCL patients.

MATERIALS AND METHODS

White blood cells preparation

Lymphocytes from the peripheral blood were isolated by centrifugation using Ficoll solution (Ficoll-Paque plus, Amersham Pharmacia Biotech AB, Uppsala, Sweden). Isolated lymphocytes were washed with saline and medium, and then suspended in fetal calf serum supplemented 0.5-1.0 ml of medium. By using anti-human CD3 antibody conjugated magnet beads (Dynabeads M-450 CD3, Dynal AS, Oslo, Norway), cells were fractionated into non-T cells ($CD3^-$ cells) and T cells ($CD3^+$ cells). The steps of isolation were followed by the manufacturer's protocol.

Western blot analysis

The culture medium was harvested from $CD3^+$ and $CD3^-$ cell culture and Raji cell line. The media were added to sample buffer and heated at 90°C for 5 min. An equal volume of each sample was loaded onto gradient polyacrylamide gels (4-9%) together with broad range prestained standards as a marker (Bio-Rad, CA, USA). The protein was electrophoresed in SDS-PAGE running buffer for 2 h at 100V and transferred to Hybond ECL nitrocellulose membrane by electrophoresis at 35 V overnight in transfer buffer. The membrane were blocked for 1 h in a solution of 5% w/v skim milk powder in 1M Tris-buffered saline pH 7.4, containing 0.1% Tween-20 (TBS-T). Rabbit anti-CD21 (1:400 in TBS-T) (Santa Cruz) was added to the membrane for 3 h and subsequently incubated with peroxidase- conjugated anti-rabbit IgG (1:10,000) (Sigma) for 1 h. After each antibody incubation, the membranes were washed 3 times with TBS-T for 10 min. Immunoreactive protein was detected using the enhanced chemiluminescence (ECL) system according to the manufacturer's instructions (Amersham Biosciences, UK).

RT-PCR detection of CD21 mRNA expression by $CD3^+$ and $CD3^-$ cells

Total cellular RNA was extracted from cultured $CD3^+$ and $CD3^-$ lymphocytes, Raji, and Molt4 cell lines using RNeasy mini kit (QIAGEN), according to the manufacturer's protocol. Total RNA (1ug) was reverse transcribed using SuperscriptTM II and random oligo (dT) primers in a total volume of 20 µl as recommended by the manufacturer (Invitrogen). The cDNA of each sample was amplified using the following primers: 21S2761: 5'-GGAACCTGGAGCCAACCTGCC-3' and 21R3360: 5'-CTGGGCTCCCATCTTTAC-3'. PCR amplification was performed in a 25 µl of total volume containing 2µl cDNA, o.4 units Taq polymerase, 2.5 µl of 10XPCR buffer containing 25 mM $MgCl_2$, 2.5 µl of 2 mM dNTP, 20 pmol of sense and antisense primers, and water. The PCR

reaction was amplified with initial denaturation at 95°C for 5 min, followed by 35cycles of 1 min denaturation at 95°C, 1 min annealing at 57°C and 1 min extension at 72°C, with a final extension of 10 min at 72°C. The PCR products were run on a 1.5% agarose gel and stained with ethidium bromide for size verification.

RESULTS

CD21 mRNA expression

Cell cultures of $CD3^+$ cells and $CD3^-$ cells from the peripheral blood of 10 patients with PTPD and PTCL were evaluated for the presence of CD21 mRNA by the RT-PCR analysis. Five of 10 samples of each $CD3^+$ cells and $CD3^-$ cells showed the expression of CD21 mRNA. Four patients showed the expression on both $CD3^+$ and $CD3^-$ cells, and 4 patients did not showed any expression on both $CD3^+$ and $CD3^-$ cells.

Shedding of Soluble CD21 (sCD21)

Supernatants of the cell culture of $CD3^+$ and $CD3^-$ cells from three patients with CD21 mRNA expression, plasma samples of the other 3 PTPD and PTCL patients, and a supernatant from Raji cell culture were evaluated for the presence of soluble CD21 (sCD21) by the Western blot analysis. All of these 10 tested sample showed the presence of sCD21 in the supernatant/plasma sample with the molecular weight of 130-140 kDa. Soluble CD21 from the ELISA KIT (Abcam, Cambridge, United Kingdom) was used as a control and resulted in the same molecular weight product.

DISCUSSION

Our study has shown that half of the peripheral blood lymphocytes ($CD3^+$ cells and $CD3^-$ cells) in PTPD and PTCL patients expressed CD21 mRNA. The CD21 mRNA positive samples also showed the sCD21 in the supernatant of cell cultures. This confirms that peripheral blood T-cell of the PTPD and PTCL patients have CD21 molecule on the cell surface and have ability to shed sCD21. This finding was different from a previous report that in the healthy individuals, peripheral blood T-cells expressed CD21 mRNA and did not express detectable amounts of CD21 molecule on their cell surface (13). Fischer E et al (12) found that, 30 to 40 per cent of normal

peripheral blood T-cells expressed CD21 molecules on their cell surface, and the intensity is approximately 10-fold lower than peripheral blood B-cells.

From the above findings, we conclude that peripheral blood T-cell of PTPD and PTCL patients express considerable amounts of CD21 molecules on the cell surface, and is much more than the peripheral blood T-cells from healthy individuals. We believe that over expression of CD21 molecules on the cell surface of peripheral blood T-cells causes EBV infection to those T-cells and contribute to the pathogenesis of PTPD and PTCL.

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การประยุกต์การใช้ผลงานวิจัย

เชิงพาณิชย์: ไม่มี เนื่องจากเป็นการวิจัยแบบ basic science research

เชิงนโยบาย: กลุ่มโรค PTPD และ PTCL พบบ่อยในประเทศไทย ส่วนใหญ่มีความสัมพันธ์กับการติดเชื้อไวรัสเอ็ปสไตน์-บาร์ (EBV) การศึกษาวิจัยในกลุ่มโรคดังกล่าวมีน้อยมาก แพทย์ไม่ค่อยให้ความสนใจเท่าที่ควร รัฐและองค์กรของรัฐควรสนับสนุนงานวิจัยเพื่อให้ทราบการเกิดและการดำเนินโรค เพื่อจะนำไปสู่การรักษาอย่างมีประสิทธิภาพ

เชิงสาธารณะ: โรคกลุ่มนี้มีพยากรณ์ที่หลากหลาย บางรายเสียชีวิตในเวลาอันรวดเร็ว ควรต้องมีการศึกษาด้านการรักษาเพื่อลดอัตราตาย ตลอดจนถึงหาแนวทางป้องกันเช่น การทำวัคซีน เป็นต้น

เชิงวิชาการ: การวิจัยครั้งนี้พบว่า T-cell ของผู้ป่วยกลุ่มโรค PTPD และ PTCL มี EBV receptor ชนิด CD21 บนผนังเซลล์ และเป็นทางเข้าของไวรัสสู่ T-cell และทำให้ T-cell กลายเป็นมะเร็ง นอกจากนี้ยังพบ DNA ของ EBV ไหลเวียนในกระแสเลือดจำนวนมาก ยีนของไวรัสนี้เกือบทั้งหมดเป็นชนิด naked form ซึ่งเข้าใจว่าเกิดจากการสลายของเซลล์มะเร็งที่มีไวรัสชนิดนี้อยู่ภายในเซลล์ จำนวนของ EBV DNA ในกระแสเลือดไม่มีความสัมพันธ์กับการพยากรณ์โรค จากข้อมูลดังกล่าวทำให้ได้ข้อคิดว่าไม่ควรศึกษาหาจำนวนของ EBV ในกระแสเลือด และ EBV DNA ในกระแสเลือดก็จะไม่ก่อให้เกิดการติดเชื้อต่อไป ทั้งนี้เนื่องจากเป็น naked EBV DNA

ผลที่ได้จากการวิจัย

รายงานฉบับที่ 1 : Accepted for publication in J Clin Virol

รายงานฉบับที่ 2 : กำลังจะส่งตีพิมพ์

รายงานฉบับที่ 3 : จะมีการปรับปรุงก่อนส่งตีพิมพ์

รายงานฉบับที่ 4 : กำลังจะส่งตีพิมพ์

ภาคผนวก 1 และ 2 : ได้รับการตีพิมพ์แล้วใน Asian Pacific J Cancer Prev

ภาคผนวก

1. Epstein-Barr virus-associated nodal malignant lymphoma in Thailand
2. Epstein-Barr virus-associated extranodal non-Hodgkin's lymphoma of the sinonasal tract and nasopharynx in Thailand.