UNIVERSITI PUTRA MALAYSIA

PREVALENCE OF HUMAN T-CELL LYMPHOTROPIC VIRUS TYPE 1 AND CHEMOSENSITIVITY OF LEUKAEMIA AND LYMPHOMA CELLS IN ADULT PATIENTS IN MALAYSIA

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By

VIKNESVARAN SELVARAJAN

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

August 2007

To my parents ...
mentors and friends

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

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August 2007

Chairman: **Zamberi Sekawi, PhD**

Faculty: **Medicine and Health Sciences**

The elucidation of virus-cancer associations is of particular importance since large numbers of people are potentially exposed to cancer. The first link relates to the causation of adult T-cell leukaemia/lymphoma (ATL), a highly malignant haematological malignancy of mature activated T cells with a poor prognosis, by a retrovirus called human T-cell lymphotropic virus type-1 (HTLV-1). The HTLV-1 tax oncoprotein plays an integral role in productive viral replication and disease progression. Seroprevalence studies demonstrated that the distribution of HTLV-1 is heterogeneous worldwide and not specific to a particular region only. Patients with this disease have a very poor prognosis because of intrinsic chemoresistance and severe immunosuppression. Hence, the general objective of the present study is to establish the prevalence of HTLV infections in leukaemia and lymphoma adult patients. The experimental design consists of two folds: screening for the presence of HTLV-1 *tax* gene and chemosensitivity profiles of patient cells treated with clinical chemotherapeutic agents. A total of 140 subjects

consisted of lymphoid leukaemia (12%), myeloid leukaemia (26%) and lymphoma patients (62%) were included in this study. First line screening was performed using ELISA and PCR was used to detect HTLV-1 *tax* gene followed by confirmation using direct DNA sequencing. Mononuclear cells were isolated using density gradient centrifugation from bone marrow or peripheral blood samples of adult patients admitted to Universiti Malaya Medical Centre (UMMC), Ward 6TD. Patient cells were treated based on standard chemotherapeutic regimen for 96 hours and assessed using 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) cytotoxicity assay. Initial ELISA screening showed 9 samples were initially reactive and 7 patients were classified indeterminate due to inconsistency of immunoassay replicates. Further confirmation by PCR validated all seropositive patients and only four of the indeterminate samples, which yields a prevalence of 9.29% in 140 adult patients. Concurrently, the HTLV-1 *tax* positive patient's chemosensitivity profiles were compared with the seronegative samples. However, a distinct relationship between the presence of HTLV-1 *tax* gene and chemosensitivity between these groups were not obtained. This preliminary study provided a baseline data on the prevalence of HTLV-1 infections in leukaemia and lymphoma adult patients. However, the lack of direct association of HTLV-1 *tax* gene with the chemotherapy resistance was mainly due to the limited sample size used in this study. Further studies should be performed in a larger cohort of patients and healthy subjects to further substantiate the preliminary data.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PREVALENS HUMAN T-CELL LYMPHOTROPIC VIRUS TYPE 1 DAN CHEMOSENSITIVITI SEL-SEL PESAKIT DEWASA LEUKEMIA DAN LIMFOMA DI MALAYSIA

Oleh

VIKNESVARAN SELVARAJAN

Ogos 2007

Pengerusi: **Zamberi Sekawi, PhD**

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Penakrifan dalam hubungan virus-kanser adalah sangat penting memandangkan semakin ramai yang terdedah kepadanya. Bukti pertama dapat dikesan melalui kewujudan sel T leukemia/lymphoma (ATL), iaitu suatu keadaan kanser yang malignan dalam hematologi, hasilnya dari sel T matang, yang telah diaktifkan dengan prognosis yang lemah oleh retrovirus yang dikenali sebagai Human T-cell lymphotropic virus type-1 (HTLV-1). HTLV-1 *tax* onkoprotein memainkan peranan secara integrasi dalam replikasi virus secara produktif dan pengaktifan penyakit. Kajian seroprevalens menunjukkan bahawa taburan HTLV-1 adalah secara heterogeneous di seluruh dunia dan bukannya khusus kepada sesuatu kawasan sahaja. Para pesakit menunjukkan penampilan fizikal yang kurang daya tenaga akibat kesan dalaman terhadap rintangan kemo serta hilang daya tahan penyakit yang serius. Berdasarkan hakikat ini, objektif umum kajian ini ialah untuk menetapkan prevalens jangkitan HTLV untuk pesakit-pesakit leukemia dan limfoma dewasa. Rekabentuk kajian terdri daripada dua bahagian:

penyaringan gen *tax* HTLV-1 dan profil kemosensitiviti sel pesakit selepas dirawat menggunakan agen-agen kemoterapeutik. Sejumlah 140 sampel merangkumi limfoid leukaemia (12%), myeloid leukaemia (26%) dan juga kes-kes limfoma (62%) telah diambil kira. Penyaringan dasar adalah menggunakan ELISA dan PCR digunakan untuk mengesan gen *tax* HTLV-1 serta diikuti oleh DNA sekuensing sebagai kaedah pengesanan. Sel-sel mononuklear daripada sampel darah dan sum-sum tulang pesakit dewasa yang menerima rawatan di Hospital Universiti Malaya (UMMC), Wad 6TD diasingkan melalui centrifugasi kecerunan tumpat. Sel-sel pesakit dirawat melalui regimen umum untuk 96 jam meggunakan kaedah 3-(4, 5-dimetiltiazol-2)-2, 5-difeniltetrazolium bromida (MTT) sitotoksik. Penyaringan dasar menggunakan teknik ELISA menunjukkan 9 sampel adalah reaktif dan 7 sampel dikategorikan sebagai ketidakpastian kerana ulangan kaedah yang tidak konsisten. PCR menunjukkan semua seropositif pesakit dan hanya empat daripada sampel-sampel yang tidak tentu adalah positif untuk HTLV-1 dengan prevalens 9.29% dalam 140 pesakit dewasa. Dalam pada itu, profil kemosensitiviti pesakit-pesakit HTLV-1 positf diasas dengan sample-sampel seronegatif. Hakikatnya, tida suatu hubungkait yang kukuh di antara dua kumpulan pesakit. Kajian awal ini telah menunjukkan data dasar mengenai prevalens jangkitan HTLV-1 di kalangan pesakit-pesakit leukemia dan limfoma dewasa. Hubungkaitan yang kukuh dengan gen *tax* HTLV-1 dan kesan kemoterapeutik adalah kurang jelas kerana saiz sampel yang sangat kecil. Kajian lanjut perlu dijalankan di dalam kohort sampel yang lebih besar dari golongan pesakit serta subjek-subjek sihat untuk menegaskan data awal dari kajian ini.

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I certify that an Examination Committee has met on 14 August 2007 to conduct the final examination of Viknesvaran Selvarajan on his Master of Science thesis entitled "Prevalence of Human T-Cell Lymphotropic Virus Type 1 and Chemosensitivity of Leukaemia and Lymphoma Cells in Adult Patients in Malaysia" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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Date: 10 July 2008

DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously and is not concurrently submitted for any other degree at UPM or at any other institutions.

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VIKNESVARAN SELVARAJAN

Date: 10 July 2008

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LIST OF ABBREVIATIONS

ALL	Acute lymphoblastic leukaemia
AML	Acute myeloid leukaemia
ATL	Adult T-cell leukaemia/lymphoma
BLV	Bovine leukaemia virus
BMT	Bone marrow transplantation
CLL	Chronic lymphocytic leukaemia
CML	Chronic myeloid leukaemia
CREB	Cyclic AMP responsive element binding protein
CSF	Cerebrospinal fluid
DIC	Disseminated intravascular coagulopathy
EBV	Epstein-Barr virus
ELISA	Enzyme-linked immunosorbent assays
ER	Endoplasmic reticulum
FAB	French-American-British
GLUT1	Glucose transporter type 1
HAM/TSP	HTLV-1-associated myelopathy/ tropical spastic paraparesis
HL	Hodgkin's lymphoma
HTLV-1	Human T-cell lymphotropic virus type-1
HTLV-2	Human T-cell lymphotropic virus type-2
IVDU	Intravenous drug users
LTRs	Long terminal repeats
MTOC	Microtubule organising centre
MTT	3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide
NHL	Non-Hodgkin's lymphoma

ORFs	Overlapping reading frames
PCR	Polymerase chain reaction
PTLVs	Primate T-cell leukaemia viruses
Rex	Regulatory gene of ‘X’ region
SRF	Serum-responsive factor
STLV	Simian T-leukaemia virus
Tax	Transactivating gene of ‘X’ region
WB	Western blot
WBC	White blood cell

CHAPTER 1

INTRODUCTION

1.1 Background

Since the dawn of time, transformation of normal cells of an animal into cancer cells is a process which can be induced experimentally by a variety of agents. A perplexing aspect of these experiments is the difficulty or impossibility, of formulating a unitary mechanism for the various agents. For instance, cancer can be induced equally well by substances which have a strong action on the nucleic acids, or by hormones, or even by completely inert substances, such as sheets of plastic inserted under the skin. The lack of similarity in the properties of cancer-inducing agents has suggested that the similarity lies in the cellular mechanisms that they affect.

In the past 25 years, revelations on the genesis of human cancer have come at an increasing pace. The contributions of knowledge about oncogenic infectious agents, especially viruses, have been instrumental in that understanding because in transforming cells they mirror, often brilliantly, basic cellular processes that culminate in cancer. Infectious agents, chiefly viruses, are accepted causes or candidates as causes of diverse malignancies of people worldwide. From a universal perspective infectious agents especially viruses account for several of the most common malignancies – up to 20% of all cancers (Pagano *et al.*, 2004). Some of these cancers are endemic with high incidence in certain geographic locations, but have sporadic low incidence in other parts of the world. The consistency of association of a given virus and a specific malignancy ranges from

essentially 100% to as low as 15% depending on the virus, the cancer and the geographic location.

The elucidation of virus-cancer associations is of particular importance since large numbers of people are potentially exposed. The possible role of viruses in leukaemogenesis has recently drawn considerable attention, and some links between viruses and leukaemia appear to exist. The first link relates to the causation of adult T-cell leukaemia/lymphoma (ATL), a highly malignant haematological tumour with a poor prognosis, by a retrovirus called human T-cell lymphotropic virus type-1 (HTLV-1). This virus is endemic in Southern Japan, the Caribbean and parts of Central Africa with prevalence in the general population of up to 30% and more (Tajima & Cartier, 1995). The lifetime risk of ATL among infected persons has been estimated to be 3.0% for women and 6.9% for men, with a long latency period of 30 years or more (Kondo *et al.*, 1989). An infection in early period of life is particularly dangerous, and preventive measures in endemic areas should focus on maternal-infant transmission dynamics. In certain geographic areas, about 60%–80% of ATL are being attributed to this retrovirus (Manns *et al.*, 1993), which is not only transmitted *via* breastfeeding, but also through other routes. Among intravenous drug users, HTLV-seropositivity is a common finding (Briggs *et al.*, 1995).

1.2 Research Significance

This research was set to be an initial step on the prevalence study of HTLV infections in this part of the region.

It was a preliminary study of these viruses that could later form a basis for further comprehensive research in the development of routine clinical diagnostics and treatment protocols for HTLV infections.

1.3 Objectives

1.3.1 General Objective

To establish the prevalence of HTLV infections in leukaemia and lymphoma patients.

1.3.2 Specific Objectives

1. To investigate the presence of HTLV in adult patients diagnosed with leukaemia or lymphoma.

2. To identify and characterise HTLV based on serology and genomic techniques.

3. To evaluate *in vitro* chemosensitivity profiles of HTLV-infected as well as non-infected leukaemia or lymphoma cells to standard chemotherapeutic drugs.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Human T-cell lymphotropic virus type 1 (HTLV-1), which is also known as Human T-cell leukaemia virus, belongs to the genus deltaretrovirus, which includes HTLV-2, bovine leukaemia virus (BLV) and simian T-leukaemia virus (STLV). As well as HTLV-1, the latter two viruses cause lymphoid malignancies in the host. Since STLV and HTLV share the same molecular, virological, and epidemiological features, they are designated as primate T-cell leukaemia viruses (PTLVs).

HTLV-1 is closely related to STLV-1, a virus isolated from old world monkeys in Africa. Phylogenic analysis indicates that HTLV-1 originated in Africa, and it seems that interspecies transmission from nonhuman primates to man also occurred in Africa and disseminated to other locations by the migration of Africans to other continents around 27300 ± 8200 years ago (Van Dooren *et al.*, 2001). Nevertheless, it does not explain HTLV-1 isolation from Ainu indigenous people in Japan and aborigines in Melanesia and Australia.

HTLV-1 subtypes include subtype A, also known as the cosmopolitan subtype, which includes the prototype HTLV-1 sequence from Japan (Seiki *et al.*, 1982) and is found in many HTLV-1-endemic areas worldwide, subtypes B, D, and F from Central Africa, subtype E from South and Central Africa (Slattery *et al.*, 1999) and subtype C from Melanesia (Gessain *et al.*, 1991; Sherman *et al.*, 1992). HTLV-1 sequences may also be compared to STLV-1

isolates from non-human primate species (Vandamme *et al.*, 1994). For most HTLV-1 subtypes, STLV-1 isolates from the same geographic region cluster closely with the human HTLV-1, suggesting multiple episodes of simian to human transmission and a probable African origin of HTLV-1 (Koralnik *et al.*, 1994; Vandamme *et al.*, 1994). Close sequence similarity between HTLV-1c and STLV-1 isolated from *Macaca arctoides* in Melanesia is an example of this phenomenon (Mahieux *et al.*, 1997).

2.2 Discovery of HTLV

HTLV-1 was first identified in T-cell lymphoblastoid cell lines and fresh peripheral blood lymphocytes obtained from a patient with cutaneous T-cell lymphoma (mycosis fungoides) (Poiesz *et al.*, 1980). This virus was associated with adult T-cell leukaemia/lymphoma (ATL) (Uchiyama *et al.*, 1977) because it was observed that the cell line established from peripheral blood lymphocytes of a patient with ATL, produced antigens that reacted against sera from ATL patients (Hinuma *et al.*, 1981). It was also associated with HTLV-1-associated myelopathy/ tropical spastic paraparesis (HAM/TSP) due to the prevalence of the antibody against this virus in the serum from TSP patients (Gessain *et al.*, 1985) and, in the serum and cerebrospinal fluid from HAM patients (Osame *et al.*, 1986).

Conversely, HTLV-2 was first identified in a cell line established from the spleen of a patient with hairy-cell leukaemia (Kalyanaraman *et al.*, 1982). This virus has not yet been associated with any specific disease, although some HTLV-2 infected patients have been reported to be affected by atypical

T-cell hairy-cell leukaemia of large granular lymphocyte leukaemia (Rosenblatt *et al.*, 1986), and tropical ataxic neuropathy (Sheremata *et al.*, 1993).

2.3 Genomic Structure

HTLV-1 virions are complex type C particles, spherical, enveloped and 100–110 nm in diameter. The viral genome consists of a linear, positive sense, ssRNA held together by hydrogen bonds. Each monomer has about 9032 nucleotides. The 3' terminal viral genome is polyadenylated and its 5'-terminal is capped. Each unit is associated with a specific molecule of tRNA that is based paired to a region, primer binding site, near the 5' end of the RNA. Proviral forms are flanked at both termini by long terminal repeats (LTRs) of 754 nucleotides.

The genomic structure encodes structural and enzymatic proteins: gag, pol, env, reverse transcriptase, protease, and integrase (Franchini, 1995). In addition, HTLV-1 has a region at the 3' end of the virus, called pX, which encodes four partially overlapping reading frames (ORFs) (Figure 1). These ORFs code for regulatory proteins which impact the expression and replication of the virus. The names, product size and functions of the genes of HTLV-1 are summarised in Table 1.

Table 1: Products of HTLV-1 genes

5' LTR	Contains regulatory elements essential for viral replication				
gag	Group *anti*gen nucleocapsid protein		p19	matrix	
			p24	capsid	
			p15	nucleocapsid	
pol	*Pol*ymerase	reverse transcriptase	RT	transcription of DNA from RNA	
		proteinase		splicing of protein precursors	
		RNaseH		synthesis of RNA	
		integrase		Integration of proviral DNA into host genome	
env	*Env*elope	Surface glycoprotein	gp46	(SU)	
		Transmembrane	gp21	(TM)	
pX	ORF I		$p12^{I}$		
	ORF II		$p13^{II}$		
			$p30^{II}$		
	ORF III		p27	*Rex*	*R*egulatory gene of *'X'* region
			$p21^{rexIII}$		Cytoplasmic protein unknown function
	ORF IV		p40	*Tax*	*T*ransactivating gene of *'X'* region
3' LTR	Contains regulatory elements essential for viral replication				

The $p12^{I}$ protein, which is encoded by ORF I, is a small hydrophobic protein which localizes to the golgi and endoplasmic reticulum (ER) (Ding *et al.*, 2001; Johnson *et al.*, 2001; Albrecht & Lairmore, 2002). Although not necessary for HTLV-1 replication *in vitro*, $p12^{I}$ was shown to contribute to viral infectivity *in vivo* using a rabbit animal model system (Collins *et al.*, 1999; Lairmore *et al.*, 2000; Albrecht *et al.*, 2002). Later studies have linked $p12^{I}$ to T cell activation. $p12^{I}$ has been shown to increase signal transducers and activators of the STAT5 pathway, increasing DNA binding and transcriptional activity in T cell lines as well as primary T cells (Nicot *et al.*, 2001). In agreement with having a role in T cell activation/proliferation, $p12^{I}$ also interacts with both calnexin and calreticulin, ER--resident proteins which regulate calcium storage and increase calcium release (Ding *et al.*, 2001).

The ORF II region of the viral mRNA encodes the p30 and p13 accessory proteins. When the p30 protein is expressed ectopically, it is found to localise to the nucleus and nucleus of transfected cells (Koralnik *et al.*, 1993). The p13 represents the C-terminal 87 amino acids of p30. Ectopic expression of p13 localises it to the nucleus and mitochondria. While little is known about