CHARACTERIZATION OF HUMAN IMMUNODEFICIENCY VIRUS TYPE-1 (HIV-1) VIRAL PROTEIN R (VPR) DURING DISEASE PROGRESSION AND PATHOGENESIS

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Human immunodeficiency virus (HIV), which progresses into the disease commonly referred to as Acquired Immunodeficiency syndrome (AIDS), has become one of the world's most destructive epidemics since its discovery in the early 80's. To date, the virus has killed more than 25 million people, with an average of 5 million newly infected cases last year alone. The HIV-1 genome is comprised of structural and enzymatic polyproteins as well as regulatory/accessory, which are essential for viral replication. Viral protein R (Vpr), which is identified as one of the regulatory/accessory genes, is responsible for carrying out several of the virus' life functions, including virus replication, cell cycle regulation, apoptosis, and immune dysregulation. Through research of the virus, the disease has been divided into two very distinctive categories: Rapid Progressors (RPs) and Long Term Non-Progressors (LTNPs). The differences between these categories are due to the varying quasispecies, which infect the population, and ultimately disease progression. Several well-known mutations that occur within *vpr* have been associated with disease progression, linking them to one of the category types. Using a population from the Multicenter AIDS Cohort Study (MACS), patient *vpr* genotypes were analyzed and compared with current findings in research. Several of the patients' deduced amino acid sequences revealed different gene variants, truncations, as well as a number of point mutations. Functional analysis revealed a decrease in cell apoptosis, which could have been

caused by the observed point mutations. Further analysis is needed in order to determine if any other functions of the virus are disrupted due to the observed mutations. Because the virus has the ability to make changes within, as of right now the only hope in counteracting the effects of HIV is through the use of antiviral medication, such as HAART. But studies have shown that not everyone has the same positive effect when these drugs are administered. By understanding the virus and its pathogenesis, researchers will be able to develop new targets for therapeutic interventions. The public health significance of this project is to provide the valuable research that will lead towards such viable HIV-1 therapeutic interventions.

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danielle

1.0 HIV-1/AIDS

Human Immunodeficiency virus type 1 (HIV-1) is the etiologic agent of Acquired Immunodeficiency syndrome (AIDS). As of Dec. 2005, the total number of people living with the human immunodeficiency virus (HIV) reached its highest level: an estimated 45 million [36.7–45.3 million] people are now living with HIV. AIDS has killed more than 25 million people since it was first recognized in 1981, making it one of the most destructive epidemics in recorded history. Despite the recent introduction of highly active antiretroviral treatment (HAART), the AIDS epidemic claimed 3.1 million [2.8–3.6 million] lives in 2005; more than half a million (570 000) were children (1).

HIV belongs to the Retroviridae family under the genus classification of lentivirus (2). Lentiviruses are known for causing immune deficiencies as well as disorders of the hematopoietic and central nervous systems, arthritis, and, autoimmunity (2). To better help with the classification of cases, HIV-1 has been divided into three groups based on geographical location. The first group identified was group M, which stands for main. As its name implies, this group encompasses the majority of the world's HIV/AIDS pandemic (3). It is estimated that that around 42 million people are infected by group M virus (3). The next is group O, which stands for outliers, and can mainly be found in Cameroon. Only tens of thousands of people are estimated to have been infected by this particular virus (3). Lastly is group N, the newest group to be identified. Group N has been identified as a mixture of either M and N, or O and N, but is not comprised of an entirely unique genome. Research has shown HIV is most probably of a zoonotic origin, while the emergence of this virus is suggested to have resulted from three

independent cross-species transmission events, which gave rise to the aforementioned three groups (3). Research has suggested that the source of HIV-1 is the central African chimpanzee. This is supported by the fact that the groups are more closely related to SIV_{cpz} strains that infected central African chimpanzees, *Pan troglodytes troglodytes* (SIV_{cpz}PTT) than the *Pan troglodytes schweinfurthii* strain which infects the east African chimpanzees (3).

Since HIV-1 is a viral agent, it must find a host with the proper mechanics in which to live and reproduce. Viral transmission from one host to another can occur in one of three ways: 1) contact with blood, 2) contact with semen and vaginal secretions, or, 3) perinatal transmission, which is known as the period concentrated around the time of a child's birth. Human $CD4^+$ T-cells and macrophages are the primary targets of the HIV-1 virus. Once inside these cells, the virus begins replicating by using the host's cellular mechanisms. Once the viral RNA is released into the cell it is reverse transcribed into cDNA and ready for integration into the host's DNA. This integrated form of the viral DNA is known as the provirus and once established, this DNA is permanently incorporated into the genome of the infected cell. There is no known mechanism by which it can be efficiently eliminated (2). From there, the proviral DNA is transcribed and RNA transcripts are transported out of the nucleus. Subsequently viral proteins are synthesized in the cytoplasm and assembled into newly formed viral particles. During its life cycle, the viral genome plays an important role in virus replication. The genome of HIV-1 consists of both structural and enzymatic polyproteins (Gag, Pol, and, Env) and the two groups of regulatory/accessory proteins (Figure 1). Tat and Rev comprise the first group and are described as the regulatory proteins. The second group is referred to as the accessory proteins and is comprised of Nef, Vif, Vpu, and, Vpr (virology). The regulatory proteins are essential for viral replication, whereas the accessory proteins aid in enhanced viral production.

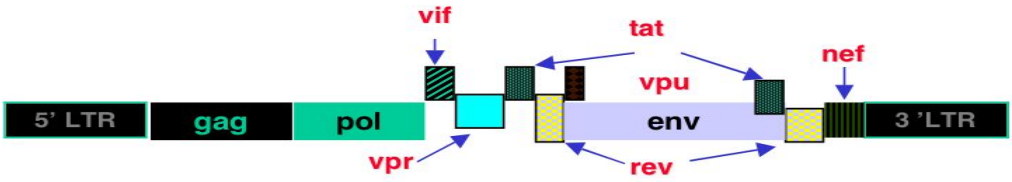


Figure 1: Schematic representation of HIV-1 Virus Genome

2.0 THE VIRAL PROTEIN R (VPR) GENE

2.1.1 Structure of Vpr

HIV-1 *vpr* is a well-conserved viral gene with an average length of 96 amino acids with a predicted molecular weight of 15kDa (4-7). The gene is located in the central region of the HIV-1 genome between *vif* and *vpu* overlapping *vif* at its 5' end and *tat* at its 3' end (8,7). HIV-1 vpr is expressed as a late protein during virus replication. A proposed tertiary structure of Vpr is based on a nuclear magnetic resonance (NMR) analysis which consists of three α-helical domains: an α-helix-turn-α-helix domain in the amino-terminal half from amino acids17 to 46 and a long leucine rich α-helix from 53 to 78 in the carboxy-terminal half (4,6). The three helices, which are connected by loops, are folded about a hydrophobic core surrounded by a flexible negatively charged N-terminal domain and a positively charged arginine-rich C-terminal region (7, 9). The arginine-rich carboxy-terminus of *vpr* has similarities when compared to arginine-rich protein transduction domains, which may explain the transducing capabilities of Vpr including its ability to cross the cell membrane lipid bilayer (7, 10-13). One side of the helix offers a stretch of hydrophobic residues that can form a leucine-zipper-like motif (14). This motif may in fact account for the formation of Vpr dimers and/or for the interaction of Vpr with different cellular proteins (4, 15-20). Both helical domains contribute to the incorporation of Vpr into virions, specifically through the interaction between Vpr and the p6 form of the viral protein, Gag (4). Gag, the structural protein, forms the core, capsid and matrix of the virus particle.

2.1.2 Functional Analysis of HIV-1 Vpr

Detailed analyses of *vpr* have concluded that the gene encodes for a protein that is involved in many functions during the life span of the virus. Vpr is responsible for carrying out several functions including transactivation of the autologous HIV-1 promoter LTR as well as heterologous host cellular promoters, formation of ion selective channels in the lipid bilayers, and regulation of cellular apoptosis (4). Vpr also aids the virus in fulfilling three major functions: (1) regulation of the nuclear transportation of the HIV-1 pre-integration complex (PIC), (2) virion incorporation of newly formed particles, and (3) induction of cell cycle arrest of the infected proliferating cells.

Nondividing cells, such as resting T-cells and terminally differentiated macrophages, are important targets for viral replication during the initial stages of infection, since primary infection of these cell populations contributes to the establishment of viral reservoirs. Such reservoirs are crucial for subsequent virus spread to the lymphoid organs and the T-helper lymphocytes (21). After virus entry into the cell, the viral capsid is rapidly uncoated and the reverse transcription of the genomic HIV-1 RNA leading to the full-length double strand DNA is completed. This viral DNA associates with viral and host cell proteins into the aforementioned PIC. Lentiviruses are unique in the fact that they have evolved a strategy to import their own genome through the envelope of the interphasic nucleus via an active mechanism within four to six hours post infection (22). Data has supported the notion that Vpr enhances the transportation of viral DNA into the nucleus of nondividing cells by promoting direct or indirect interactions with the cellular proteins, especially the nuclear importins, regulating the nucleo-cytoplasmic shuttling (23-30). The exact composition of the PIC has yet to be determined, but what is known is that it is comprised of the viral DNA associated with integrase, cellular factors that participate in both intra-cytoplasmic routing and nuclear translocation of viral DNA, and, Vpr (31-33). The

PIC progresses through the cytoplasm via the cytoplasmic dynein motor and travels along the microtubule network in order to migrate towards the nucleus. The role of Vpr in association with the PIC is still unclear. It is not yet known whether Vpr plays an active role during the movement of the PIC along the microtubules or whether it is only associated with the complex and then actively participates in the subsequent steps of anchoring the PIC to the nuclear envelope and the nuclear translocation of the viral DNA (7).

Cells expressing Vpr have been known to accumulate in the G2/M phase of the cell cycle. It has been shown that Vpr targets a general host cellular mechanism that controls progression of cells from G2 to mitosis. Briefly, in normal cells $p34^{cdc2}$ is phosphorylated, which is one of the first steps in the transition phase from G2 to mitosis (4, 34). However in Vpr-infected cells, $p34^{cdc2}$ is for the most part inactive, leaving the cells suspended in a phase ideal for viral replication. The determinants of the G2 arrest activity are mainly located in the C-terminal unstructured basic region of HIV-1 Vpr and phosphorylation of Vpr protein is required for Vpr-mediated cell cycle arrest (35, 36). Several forms of Vpr, i.e. intracellular, intravirion, and free Vpr, present within the host are capable of inducing cell cycle arrest function upon exposure (5, 37). There are also several lines of evidence in support of the involvement of these various forms of Vpr in pathogenic events as well as in the regulation of HIV-1 replication (5).

2.1.3 *In vivo* analysis of Vpr: Long-Term Nonprogressors (LTNPs) or Slow Progressors and Rapid Progressors (RPs)

Earlier efforts in studying the role of *vpr* in HIV-1 disease progression were for the most part based on nucleotide sequence analysis (6). This method proved very useful in detecting gene deletions, insertions, and, mutations. Even though the *vpr* sequence is one of the most conserved regions of the HIV-1 genome, variations are inevitably found in the quasispecies that affect

patients, ultimately affecting the rate of disease progression. Thus the two category terms Long-Term Nonprogressors (LTNPs) and Rapid Progressors (RPs) were devised in order to describe the progression of viral pathogenesis within the HIV-1 patient (38, 39). LTNPs are defined as asymptomatic with CD4 T-cell counts > 500 X 10^6 cells/1, low levels of spontaneous apoptosis, and low or undetectable viral loads for at least ten years. They also maintain a strong immunity, both humoral and cellular, against HIV-1 without the assistance of any antiretrovirals such as HAART (8, 40, 41). The RPs on the are symptomatic and progress to AIDS within the usual time span of ten years, without the help of antiretroviral therapy. Recently, a non-functional mutation (R77Q) identified in *vpr* has been implicated in readily establishing long-term chronic infection of T-cells, whereas wild type *vpr* is known to increase the rate of viral replication and cytopathic effects of the virus in cell culture (42). Specific mutations within the *vpr* gene include several known changes that seem to be associated with this slow progression to disease.

Mutations within *vpr* include the polymorphism at the 3rd amino acid residue. Normally at this position the presence of glutamine (Q) has been observed, however in LTNPs, it has been replaced by arginine, R. This change has been shown to significantly impair the ability of Vpr to confer its normal cytopathicity. But this polymorphism has no effect on the efficiency of the virus to replicate (6, 43). Similarly the C-terminal domain mutant R80A is another mutation studied in this regard. This particular mutation abolishes the G2/M cell cycle arrest which, as discussed earlier, the virus depends on to suspend cells in this specific transition phase, leading to an increase in viral replication (44). The next mutation in Vpr that has been studied is the R77Q mutation where an arginine (R) residue is replaced by a glutamine (Q) residue. This point mutation does impair the capability of Vpr in inducing cellular apoptosis in the absence of cell cycle arrest, which could overall dramatically affect disease progression (8, 38, 45). However, a recent study disputes this observation indicating that this change may not in fact be only associated with just the LTNPs, since it is also present in the RPs (45). This suggests that R77Q

is just another point mutation and it may not be related to disease progression as thought earlier. Another mutation identified at the onset of HIV-1 research involved the deletion of *vpr* from the virus. The HIV-1 viral strain HxB2 carries this distinctive mutation that leads to the premature termination of the open reading frame (ORF), impairing the ability of Vpr to induce cell cycle arrest and apoptosis (6, 46). More recently reviewed is the insertion of arginine (R) at position 90 in the gene, which alters the highly conserved C-terminal motif. The C-terminal motif, defined as RQRRAR, has previously been indicated as playing a role in nuclear localization as a nuclear localization signal (NLS) (15). This motif is believed to play a role in subtype B Vpr nuclear targeting (15). To date, subtype O is the only other subtype with the R insertion leaving the sequence with a consensus length of 100 amino acid residues in comparison to the usual 96 amino acid residues. Therefore, gene defects that diminish any of the Vpr-specific activities would have a negative impact on viral survival and thus could potentially slow down disease progression (16).

Human leukocyte antigens (HLA) exist in large numbers within the human population. Their alternatives, HLA alleles, are known for their association with disease, some of which have the ability to increase the risk and progression of certain human diseases such as HIV-1 (41). Mutations are known to cluster in certain HLA regions, suggesting an HLA-driven evolution of the gene *vpr*. Studies have documented that HLA-B27 and HLA-B57 as well as others are strongly associated with slower rates of disease progression, making them a protective agent in LTNPs. Certain subtypes of the HLA alleles such as HLA-B35 and HLA-A29 are, however, known for being associated with faster rates of disease progression (47, 48). These findings lead to the conclusion that Vpr protein evolution may in fact be host- driven (15).

Within the last few years, research has been carried out to study the effects that antiretrovirals have on the HIV-1 viral strains within patients. The results reported several HAART-induced mutations. The mutation with the highest frequency was seen repeatedly in LTNPs on HARRT

and involves a serine (S) to asparagines (N) change at position 28 on the vpr. To validate this particular polymorphism, gene analyses were performed and no other defects were found in any of the other HIV-1 genes, thus implying that this mutational change was in fact unique to the *vpr* gene (15).

3.0 PUBLIC HEALTH SIGNIFICANCE

Human immunodeficiency virus (HIV) is still among the top killers of the world population. Throughout the world the virus is broken up into types, i.e. type 1 or type two, as well as different clades or subtypes, i.e. clade B in North America, clade C in India (49). Based on the viral pathogenesis seen *in vivo*, the resulting disease has been divided into to very distinctive categories: Rapid Progressors (RPs) and Long Term Non-Progressors (LTNPs) (38, 50). The difference between the two groups of patients is the time in which HIV develops into full-blown AIDS. $CD4^+$ T-cells and macrophages constitute the main cell targets of the virus. Once inside these cells, the virus begins replicating by using the host's cellular mechanisms. It is through these replicative stages that the virus makes changes in its own genome, resulting in the manufacturing of new/recombinant virus strains that are not only different from the original infecting virus, but also between each other. Throughout its evolution HIV has developed ways to "outsmart" its hosts through techniques such as mutation and its ability to hide for months or even years at a time. Understanding the pathogenesis and the strategies that are employed by the virus using its gene products such as Vpr is greatly needed to develop immune therapeutics and antivirals. Furthermore, lessons learned from *in vivo* analysis of the *vpr* gene and its role in disease status will lead to the identification of domains that play a role in host cellular alterations, thus new targets for therapeutic interventions.

Most patients infected with HIV-1 develop AIDS unless they receive antivirals such as HAART. The only hope in counteracting the effects of HIV is through the use of antiviral medications. But studies have shown that not everyone has the same positive effect when these

drugs are administered. With the new reports of “Super AIDS” in the media, the world is ready for a new approach to battling with this ultimately deadly disease (51). With the development of a working vaccine, scientists hope to be able to lessen the effects that this virulent virus has had on the human population as a whole.

The public health significance of this project is to provide valuable research that will lead towards such a viable HIV-1 vaccine for the general population. By studying the individual genes of the virus, researchers will be able to better understand the mechanisms by which such a complex virus operates. In the history of sexually transmitted diseases, no one has been able to develop a means of eradication, ever. This work, which entails detailed research into the many different aspects of the HIV-1 viral gene *vpr*, encompasses the very beginning stages of vaccine development and of hope for such a cure.

4.0 THESIS AIMS

The HIV-1 genome in infected individuals demonstrates a marked heterogeneity. Even within a single infected individual, the virus exists as quasispecies. The HIV-1 genome codes for the three structural/enzymatic genes *Gag*, *Pol*, and, *Env*, the two regulatory genes *Tat* and *Rev*, and, the four accessory genes *vif, nef, vpu,* and, *vpr*. In addition to their roles in viral replication and pathogenesis, expression of these accessory genes has been implicated in modulation of host cellular events. The *vpr* gene specifically is known to modulate a number of host cellular events, however, it is unknown how the Vpr-mediated cellular events affect viral pathogenesis and disease progression.

HIV-1 patients harbor distinct genotypes and phenotypes of the accessory gene products at various stages of infection, suggesting a correlation between genotypes and disease progression. Using a longitudinal cohort of HIV-1 patient samples, I propose to functionally characterize the Vpr genotypes associated with disease progression. By characterizing Vpr within the individual, naturally occurring mutations and deletions within Vpr can be reviewed to determine if the protein is in fact lethal or defective and further correlate this with disease progression and clinical outcome. This knowledge will also help in future studies involving HIV-1 vaccine development.

4.1.1 Specific Aim #1

Isolation of *vpr* from samples of HIV-1 infected individuals within the cohort study.

A) DNA Extraction

B) Vpr Amplification

4.1.2 Specific Aim #2

Genotyping analysis of Vpr from patient samples.

A) Vpr Cloning and Screening

B) Patient Sequence Analysis

C) Phylogenic Tree Composition

D) Reporter Assay and Protein Quantification

5.0 MATERIALS AND METHODS

The patient population used in this study is part of the Multicenter AIDS study (MACS) group. Using appropriate protocols and IRB approval, samples were received from Baltimore and Northwestern as part of the MACS. Frozen peripheral blood mononuclear cells (PBMCs) were shipped to us in dry ice and the samples were stored at –20˚C before further use.

Table 1: Demographic information and disease status of the MACS patient population used in this study.

PT #	Patient ID #	Patient Location	Cell Isolation	PT Classification
1	20176	North Western, IL	visit 28/11-13-98	LTNP
2	20330	North Western, IL	visit 28/11-04-97	LTNP
3	21109	North Western, IL	visit 28/12-29-97	LTNP
4	23007	North Western, IL	visit 28/02-02-98	LTNP
5	11091-28	Baltimore, MD	visit 1	LTNP
6	10630-28	Baltimore, MD	visit 1	LTNP
7	13098-31	Baltimore, MD	visit 1	LTNP
8	10273-29	Baltimore, MD	visit 1	LTNP
9	10238-27	Baltimore, MD	visit 1	LTNP
10	13116-29	Baltimore, MD	visit 1	LTNP
11	11057-31	Baltimore, MD	visit 1	ND
12	102191H	Pittsburgh, PA		ND
14	102232J	Pittsburgh, PA		ND

5.1 DNA EXTRACTION

Genomic DNA was extracted from peripheral blood mononuclear cells (PBMCs) from each of the eleven patients, courtesy of the Multicenter AIDS Cohort Study (MACS) (Table 1). The PBMCs were obtained from the Northwestern University, IL and John Hopkins University, Baltimore MACS facilities. The DNA extraction was carried out using the GFX Genomic Blood DNA Purification Kit (Amersham Pharmacia Biotech Inc, Piscataway, NJ) for each of the MACS patients. Briefly, PBMC pellets were allowed to thaw at room temperature after being removed from -80°C. Extraction Solution (500 µl) was added to the cells and immediately vortexed before allowing the mixture to incubate for five minutes at room temperature. The cells and extraction solution were placed inside a GFX column, and was centrifuged for one minute at 8,000 rpm. The flow-through was discarded, 400µl additional Extraction Solution was added to the column, and spun for another minute at 8,000 rpm. After discarding the flow-through, 500µl of the Wash Solution was added to the column. The column was spun for three minutes at 8,000 rpm and the flow-through was discarded. The tube was spun a final time to ensure that all of the lysate was removed and only the genomic DNA remained bound to the column. Next the GFX column was placed into a new microcentrifuge tube and 200µl of autoclave water (pre-heated to 70°C) was added into the column. The column was incubated for one minute at room temperature before being spun at 8,000 rpm for one minute. In order to make sure the new tubes now contained the recovered purified genomic DNA to be used throughout the experiments, 10µl from each sample was run on a 1% agarose gel (0.60g Agarose Low EEO heated with 1X TBE solution).

5.2 VPR AMPLIFICATION

The genomic DNA extracted from the PBMCs contains copies of the full length HIV-1 viral genome. In order to clone *vpr*, polymerase chain reaction (PCR) was required for its amplification. The *vpr* primers used for PCR corresponded specifically to nucleotides of the pNL43 HIV-1 proviral genomic sequence and contained both an external *vpr* primer, including approximately an additional one hundred base pairs upstream and downstream of the actual gene, and an internal *vpr* primers, containing approximately ten additional base pairs upstream and downstream of the gene (Table 2). The later is thought to be more conventional for future functional studies.

Table 2: List of the primers used for amplification of vpr during pcr.

Vpr (external)	Forward	5’ GAC GGA CAT AAC AAG GTA GGA 3’
	Reverse	5’ GTC GCT GTC TCC GCT TC 3’
Vpr (internal)	Forward	5’ AGG ACA GAT GGA ACA AGC CCC AGA AGA CCA AG 3’
	Reverse	5’ CTA GGA TCT ACT GGC TCC ATT TCT TGC TCT 3’

The PCR protocol used is as follows, starting with the PCR reaction samples and ending with the thermocycler conditions: 35µl of autoclaved water, 5µl of 10X buffer, 2µl of dNTPs, 5µl of genomic DNA, 1µl of the forward primer (10pmol/µl), 1µl of the reverse primer (10pmol/µl), and, 1µl Taq Polymerase were added sequentially to a PCR grade microcentrifuge tube for a final solution volume of 50µl for each patient. The tubes were then run under the PCR

conditions of 95°C for two minutes, 30 cycles of denaturing at 95°C for one minute, annealing at 54°C for one minute, extension at 72°C for two minutes, and a final elongation time of 72°C for fifteen minutes. 10µl of the finished amplified product was run on a 1% agarose gel for the identification of the *vpr* fragment. The protocol for internal Vpr (Vpr_i) has only one difference; the PCR reaction sample requires the use of 10µl of genomic DNA. The PCR conditions are as follows: 95°C for two minutes, 45 cycles of denaturing at 95°C for one minute, annealing at 60°C for one minute, extension at 72°C for two minutes, and, a final elongation time of 72°C for fifteen minutes. To verify this smaller fragment, 10µl of the finished amplified product was run on a 2% agarose gel (1.20g Agarose Low EEO heated with 1X TBE solution).

5.3 VPR CLONING AND SCREENING

5.3.1 Vpr Cloning

After the PCR products were verified to be that of *vpr* based on the amplified fragment size by electrophoresis, the next step was to incorporate the gene into an expression vector to conduct further gene function analyses. Vpr was cloned in to pcDNA3.1/V5-His TOPO-TA vector using the Cloning/Expression Kit (InVitrogen, Carlsbad, CA) according to the manufacturers' instruction. Each sample was ligated with the vector and transformed into *E. coli* bacteria in order to produce clones for study purposes. The cloning procedures are as follows: 8µl of the PCR product, 1µl of the provided Salt Solution, and, 1µl of the TOPO vector were combined in a microcentrifuge tube, mixed gently, and allowed to incubate at room temperature for thirty minutes. 5µl of the incubated TOPO Cloning Reaction was added to a tube of competent *E. coli* cells that were provided with the cloning kit. The tube was mixed gently and allowed to incubate

on ice for another thirty minutes. Next the tube was heat-shocked in 42°C water for 45 seconds, then returned back to the ice for two minutes. 450µl of media was combined with the contents of the tube into a new inoculation tube and shook at 197 rpm for 2 hrs. Different quantities (100 and 200 µl) of the transformed samples were spread over an ampicillin-containing Luria Bertani (LB) agar plate in order to allow for optimal colony growth. Plasmid DNA was isolated by lysis boiling prep. Briefly, individual colonies were collected and grown overnight in 4mL LB with ampicillin (100mg/mL). Cells were spun down (10,000 rpm for five minutes) and cell pellets were lysed using a combination of 450µl of STET buffer and 25µl of lysozyme. Cell lysate was vortexed and placed in a boiling bath for approximately one minute before being spun down at 13,000 rpm for ten minutes. After the newly formed debris pellet was removed from the supernatant, 25µl of sodium acetate and 250µl of 2-propanol were added and the solution incubated at room temperature for 30 minutes. Following incubation the solution was spun down at 13,000 rpm for ten minutes, washed with 70% cold ethanol, and, spun down using the aforementioned parameters. The supernatant was decanted and the DNA pellet was allowed to dry completely at 37°C before dissolving in 50µl of TE buffer.

5.3.2 Vpr Screening

The screening of individual colonies involved the restriction digestion of the purified plasmid DNA. The enzymes used in the restriction digest were EcoRI, HindIII, and, XhoI (New England Biolabs, Beverly, MA). The first digestion used HindIII and XhoI to release the *vpr* DNA fragment from the plasmid TOPO vector and HindIII and EcoRI were used in the second digestion as a means to verify the orientation of the cloned *vpr* gene. All positive colonies were used to prepare clean DNA for sequencing using the QIAprep Miniprep kit (QIAGEN, Valencia, CA) and quantitated by spectrophotometer. Sequencing of the positive colonies was performed at

the University of Pittsburgh Genomics and Proteomics Core Laboratory. All sequences were verified using forward and reverse primers to confirm any changes.

5.3.3 Nucleotide Sequence Analysis

Based on the nucleotide sequences obtained from the core facility, the sequences were subjected to a number of analyses. First, sequences were loaded into the Edit Sequence program for the purpose of determining the chromatography of the individual sequences. Within this particular program adjustments could be made to any nucleotide residues not properly read by the Genomics and Proteomics Core Laboratory. This procedure usually involved changing N's to one of the corresponding four-nucleotide base pairs: adenine (A), thymine (T), guanine (G), or cytosine (C), as well as deleting any bases that did not contain an actual peak on the chromatogram. From here the sequences were read into the DNA Strider program for *vpr* verification. If DNA Strider could not verify the presence of *vpr*, the sequence was blasted using the public National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/) to search for any sequence homology with the pNL43HIV-1 proviral sequence (Los Almos National Library) *vpr* gene. After gene verification the newly derived *vpr* sequences were loaded into the ReadSeq program (http://bimas.dcrt.nih.gov/molbio/readseq) and converted into the FASTA format for easy reading by other sequence analysis programs. Finally the sequences, along with the HIV-1 pNL43-Vpr sequence, were downloaded into the Clustal W online program (http://www.ebi.ac.uk/clustalw/), which allowed for sequence alignment and phylogenic tree analysis.

5.4 CELL CULTURE

5.4.1 Primary Cell Line

HeLa cells used for transfection were obtained from the NIH AIDS Research and Reference Program (ARRRP). HeLa cells were cultured in Dulbecco's modified Eagle medium (DMEM), supplemented with 10% heat inactivated fetal bovine serum (FBS) (Cambrex, Walkersville, MD) and 1% penicillin-streptomycin (GIBCO, Grand-Island, NY). The cells were then maintained in a 37°C humidified chamber at 5% CO_2.

5.4.2 Transfection

HeLa cells were transfected with 0.5μg of Vpr expression plasmid and the HIV-1 LTR with a luciferase reporter gene using Lipofectamine 2000 reagent (InVitrogen, CA). When applicable mutated Vpr clones were utilized for the transfections, specifically clones from different MACS patients as shown in Table 3. Both the lipofectamine and the HIV-1 LTR with luciferase were diluted into OPTI-MEM (GIBCO) prior to being added to the HeLa cells. After an incubation time of 3 hours at 37°C, 5% CO_2, the cells were washed and DMEM was added for an additional incubation time of 48 hours at 37°C, 5% CO_2. 1X Repoter Lysis Buffer was used to lyse the cells following transfection. Cell lysates were spun at 12,000 rpm at 4˚C to remove cell debris and cell lysates were used to measure the luciferase activity. Protein concentration in cell lysates were measured and normalized with the luciferase activity.

Table 3: HeLa Cell Transfection Data. Clones used from MACS and PittMen Study for transfection analyses.

Plate #	Well #	PT Vpr (0.5ul)	Clone #
1	1	puc19 Vector	control
	2	PT 176	12
	3	PT 330	18
	4	PT 109	4
	5	PT 007	12
	6	PT 9128	3
2	7	PT 3028	41
	8	PT 9831	43
	9	PT 7329	6
	10	PT 3827	11
	11	PT 1629	45
	12	PT 5731	42
3	13	PT 91	15
	14	PT 91	13
	15	PT 32	8

6.0 RESULTS

6.1 SPECIFIC AIM #1: ISOLATION OF *VPR* FROM SAMPLES OF HIV-1 INFECTED INDIVIDUALS WITHIN THE COHORT STUDY

6.1.1 Genomic DNA preparation

The HIV-1 positive patients within the MACS group were infected with the viral strain from clade B, which is found primarily within North America. Genomic DNA was obtained from the PBMC cell pellets using the GFX Genomic Blood DNA Purification kit (Amersham Pharmacia Biotech Inc, Piscataway, NJ). The resulting solutions from each patient were run on an agarose gel to ensure that DNA had been extracted from the PBMCs (Figure 2). A total of eleven patient PBMCs were successfully extracted from the cell pellets. To ensure that patient samples were not contaminated or mislabeled, no more than four samples were used at a given time. The working patient samples of genomic DNA were kept in 4°C for future use with PCR.

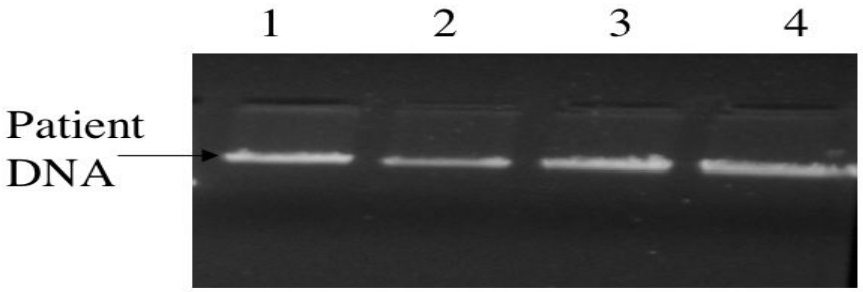


Figure 2: Electrophoretic analysis of genomic DNA. After the extraction from the MACS patient PBMC cell pellets, samples were run on a 1% agarose gel to confirm the extraction of genomic DNA. Lane assignments: 1) PT176 2) PT330 3) PT109 4) PT007.

6.1.2 PCR Amplification

The patient samples contain few copies of the integrated proviral DNA therefore a nested PCR amplification step was necessary to amplify the viral gene products. This includes both single round as well as multiple rounds of PCR amplification steps. For the first round of PCR, external primers were used (primers designed 110 base pairs before the start codon of Vpr as well as 128 base pairs beyond the stop codon of Vpr) to amplify a 446 bp fragment. First, patient samples were processed through a round of PCR to amplify the *vpr* gene and run on an agarose gel (Figure 3A). The presence of a band approximately 446 base pairs verified that *vpr* had been amplified from the patient genomic DNA. After verification, the second round of PCR was performed using internal primers amplified the *vpr* gene, ORF of 299 bp. This second round of amplification used genomic DNA from the first round PCR instead of the working patient samples of genomic DNA as a template. This particular PCR produced a band that was approximately 299 base pairs in length (Fig. 3B).

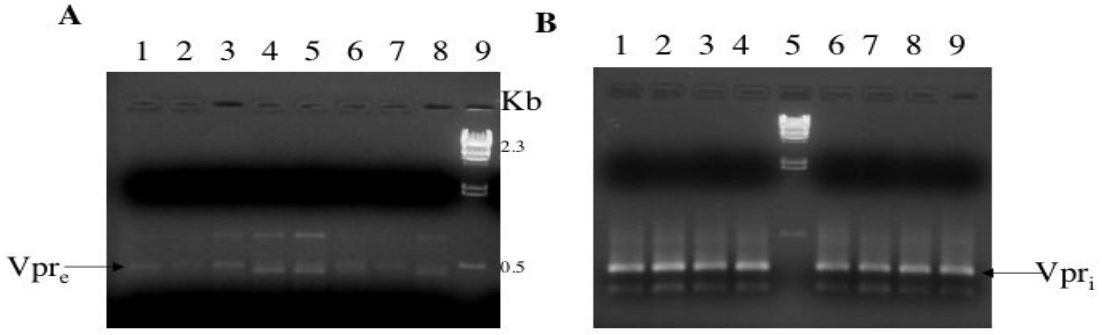


Figure 3: PCR of Vpr_e and Vpr_i. Following the PCR amplification of *vpr*, the PCR products were run on a 1% agarose gel to confirm the size of the fragments. (A) The first round of PCR indicating the external *vpr* fragments approximately 446 bp. Lane assignments: 1) PT 330 2) PT007 3) PT3028 4) PT9831 5) PT3827 6) PT7329 7) PT1629 8) PT5731 9) Marker. (B) The second round of PCR indicating the internal *vpr* fragments approximately 299 bp. Lane assignments: 1) PT 330 2) PT007 3) PT3028 4) PT9831 5) Marker 6) PT3827 7) PT7329 8) PT1629 9) PT5731.

6.2 SPECIFIC AIM #2: GENOTYPING ANALYSIS OF VPR FROM PATIENT SAMPLES

6.2.1 Cloning and screening of the *vpr* gene

In order to further our knowledge on the aspects of the individual gene functions, the amplified *vpr* gene fragments needed to be incorporated into a eukaryotic expression vector. The *vpr* gene fragments were ligated into a pcDNA3.1/V5-His/TOPO TA cloning expression vector (InVitrogen, CA) with the His tag attached to its C-terminus. This particular vector construct also includes a T nucleotide overhang, which is specific to the A nucleotide overhang provided by Taq-amplified PCR products for cloning purposes. The ligation reaction was transformed into the competent *E. coli* cells and plated on an ampicillin containing LB plate and incubated at 37˚C overnight. Colonies were selected and grown in LB media for further screening. Patient

samples were screened using two sets of double digestion incorporating twelve colonies. The first digestion consisted of a HindIII and XhoI reaction, which released the *vpr* gene fragment from the vector. Out of the first screening of twelve colonies I averaged between six to eight positive clones, the second screening of colonies averaged seven positive clones. The sole purpose of the second digestion, which involved a HindIII and EcoRI reaction, was to confirm the orientation of the *vpr* fragment inside the vector. The clones, which released the right sized fragment on the agarose gel, were used to prepare clean DNA for the sequencing analysis (Figure 4).

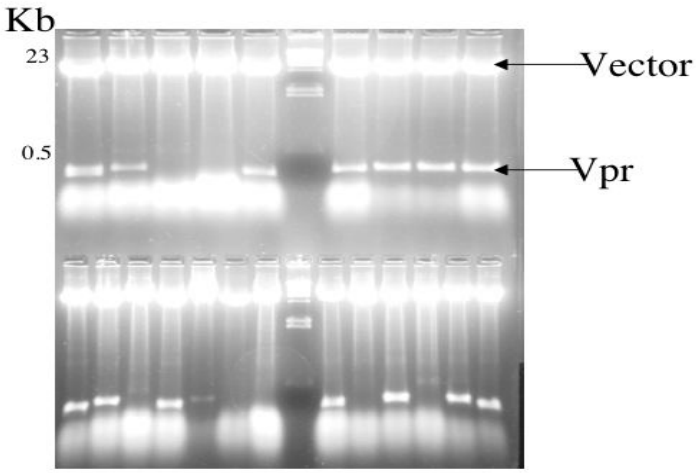


Figure 4: Electrophoretic analysis of *vpr* cloning by restriction digest. Patient clones were screened in order to verify the presence of the ligated *vpr* fragment into the pcDNA3.1 vector through plasmid digestion using HindIII and XhoI.

6.2.2 Patient sequence analysis

All of the positive clones from the eleven MACS patients were sent off to the University of Pittsburgh Genomics and Proteomics Core Laboratory for sequencing. Results from the

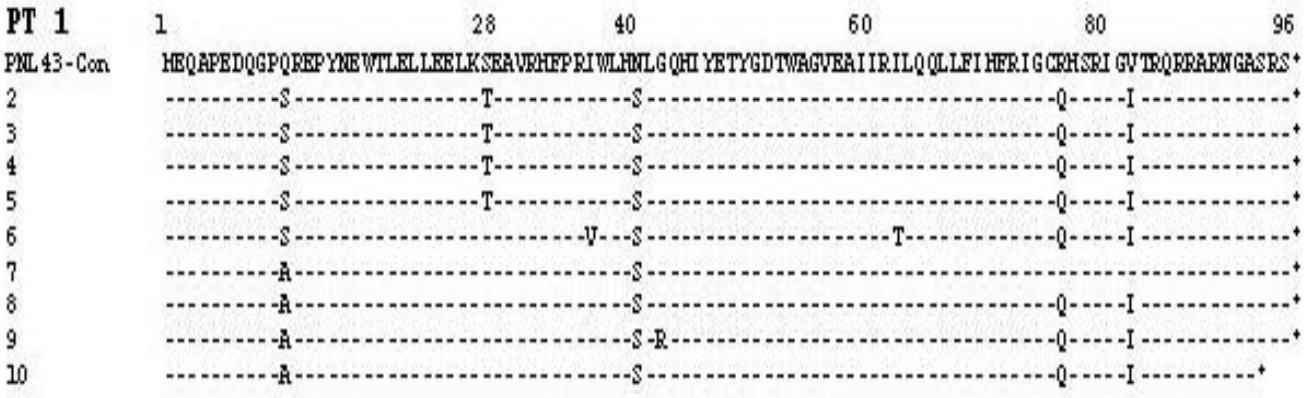
sequence analysis were verified and subjected to several analysis programs. Verification of the sequences was obtained by using the program methods of Edit View, DNA Strider, and, NCBI as described earlier. Briefly, the sequences were loaded into the Edit Sequence program for the purpose of determining the chromatography of the individual sequences. Within the program nucleotide residues could be adjusted for peak positions not previously read by the Core Lab. The sequences were then read into the DNA Strider program for *vpr* verification. If however the program could not identify *vpr*, a gene blast was performed using the NCBI database. The database compared the MACS patient sequences to that of the pNL43 HIV-1 proviral sequence *vpr* gene. After *vpr* confirmation, the sequences were read into the ReadSeq program and converted into the FASTA format before being downloaded into the Clustal W online program. The Clustal W program produced sequence alignments and the formation of phylogenic trees, which allowed for further analysis of the patient sequences.

Since all of the MACS patients are from North America (possible clade B), Vpr from the pNL43 HIV-1 proviral sequence was utilized for the consensus. Within each patient, the individual sequences were analyzed for the different variations observed in order to determine the interpatient variation, while keeping in mind that all of the analyses were based on the deduced amino acid sequences and that any of the change variations observed within a nucleotide could be silent and/or nonsense which would result in no significant changes within the amino acid or the protein composition.

Comparisons of the deduced amino acid sequences (A) and their respective phylogenetic tree analysis (B) for all eleven MACS patients are shown in figures 5–15. In general the sequences within theses patients are conserved. These results are very similar to the published reports indicating that accessory genes are more conserved than the structural proteins Env and Gag (52). Sequence analysis of patient #1 (PT1) indicates that this patient exhibits two major variants within the *vpr* alleles (Figure 5A). Interestingly most of the mutations occur within a particular

residue. For examples the observed mutations in PT1 are at amino acid positions 11, 28, 41, 77, and, 83. At position 11, glutamine (Q) is mutated to either serine (S) or alanine (A), whereas N41 is mutated to serine (S), where S has a role in protein phosphorylation. The most interesting genotype of PT1 is the presence of R77Q. Previous studies have demonstrated that R77Q is involved in LTNPs phenotype (8). Given the fact that many of these patients are LTNP, this observation directly correlates with the functional phenotype of Vpr in disease progression.

A.



B.

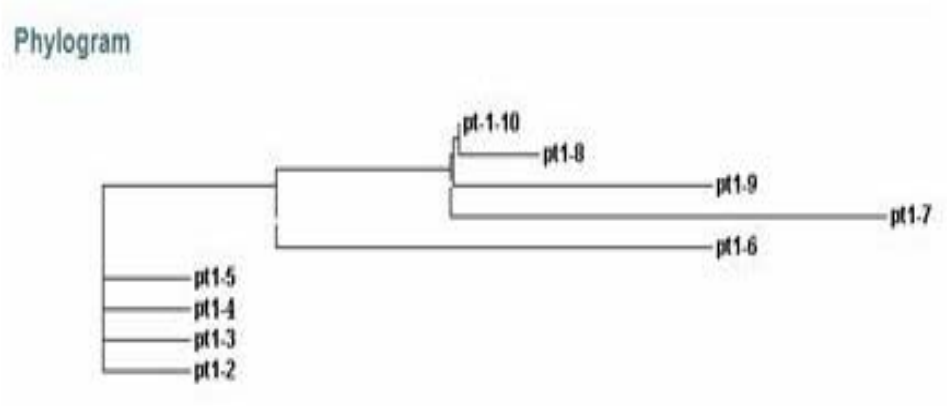


Figure 5: MACS patient #1 analysis. (A) Deduced amino acid variant sequences. (B) Phylogenetic tree of the patient variants.

Using the deduced amino acid sequences, a phylogenetic tree was generated that grouped these sequences into variants (Figure 5B). The phylogram also shows that *vpr* clones 2, 3, 4 and, 5 share similar homology within them, while the other alleles are grouped into two other branches. Similar analyses were performed for all of the MACS patients. Patient #2 (PT2) , showed higher mutation rates as well as multiple quasispecies (Fig. 6A &B). The mutations observed are at amino acid residues 19, 28, 37,41, 45, 64, 83, 85, and, 86. As seen in PT1 S28 and N41 have been mutated, but in PT2 the amino acids are mutated to asparagine (N) and glycine (G), respectively. The mutations observed are highly conserved in the fact that when a mutation does occur, it is the same for all of the alleles. This is evident in the constructed phylogram, which shows three very distinct branched groups. Only three of the *vpr* alleles from patient #3 (PT3) (Figure 7A) were observed to have mutations. For allele #1 the mutation occurs at the end of the sequence starting with the amino acid, which corresponds to position 88. For allele #7 the mutation occurs at amino acid position 80, but instead of the R80A mutation, this one converts to lysine (K). The most unique allele from PT3 is #3, which has mutations located at aspartic acid (D) 7 glutamic acid (E) and tyrosine (Y) 15 histidine (H). But what makes this allele unique is that fact that an isoleucine (I) is inserted into the sequence between amino acid positions 26 and 27. As for the phylogram, Figure 7B shows that all of PT3's alleles are located on different branches. Figure 8A depicts the observed mutations for patient #4 (PT4), which can be seen at amino acid positions 37, 40, 41, 83, 84, and, 85. The corresponding mutations for those mutations are isoleucine (I) to threonine (T) and glutamic acid (E), histidine (H) to glutamine (Q), asparagine (N) to serine (S) and glycine (G), valine (V) to isoleucine (I), threonine (T) to isoleucine (I), and, arginnie (R) to proline (P). The generated phylogenetic tree for PT4 (Figure 8B) is grouped into four distinct branches. Patient #5 (PT5) is the only patient in which all of

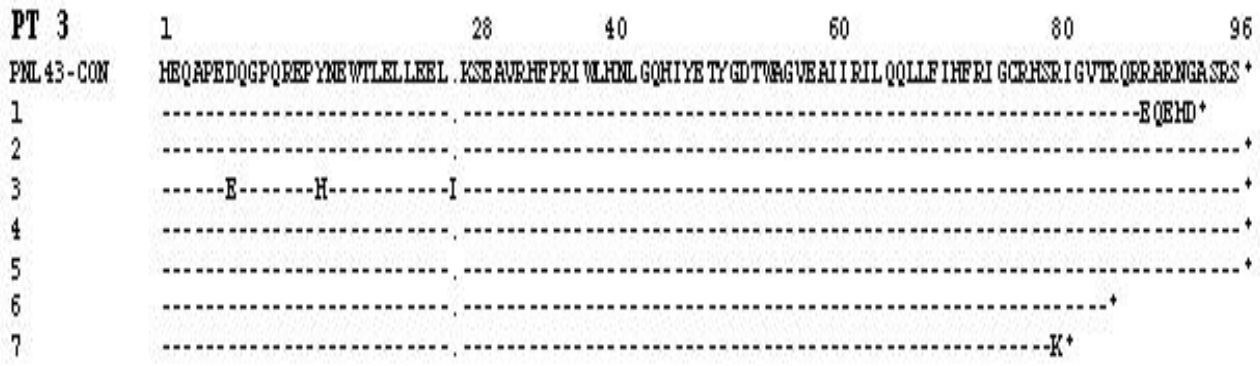
A.

```
PT 2        1                   28        40                  60                  80                  96
PML43-Con   HEQAPEDQGPQREPYNEWTLELLEELKSEAVRHFPRIWLHNLGQHIYETYGDTWAGVEAIIRILQQLLFIHFRIGCRHSRIGVTRQERRARNGASRS*
1           ----------------A--F-----N--------P---G---Y----------------T----------------I-PR----------*
2           ----------------A--------N--------P---G---Y--------R-------T----------------I-PR----------*
3           ----------------A--------N--------P---G---Y----------------T----------------I-PR----------*
5           ----------------A--------N--------P---G---Y----------------T----------------I-PR----------*
5           ----------S---A--------N--------P---G---Y--------------------------------------------------*
6           ----------S----------------------------------------------------------------I-PR----------*
```

B.

Figure 6: MACS patient #2 analysis. (A) Deduced amino acid variant sequences. (B) Phylogenetic tree of the patient variants.

A.



B.

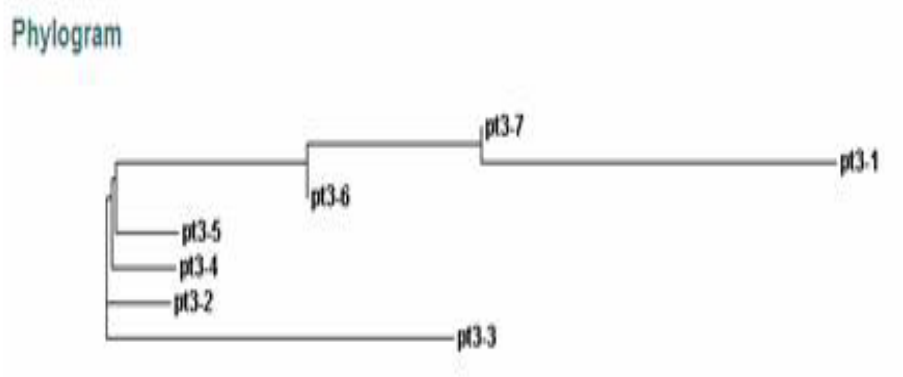


Figure 7: MACS patient #3 analysis. (A) Deduced amino acid variant sequences. (B) Phylogenetic tree of the patient variants.

A.

```
PT 8          1                    28        40                  60                  80                  96
PWL43-CON     MEQAPEDQGPQREPYNEWTLELLEELKSEAVRHFPRIWLHNLGQHIYETYGDTWAGVEAIIRILQQLLFIHFRIGCRHSRIGVTRQRRARNGASRS*
1             ------------------------------------------------------------------------------------R--IIQ----------*
2             ------------------------------------------------------------------------------------R--IIQ----------*
3             -------------------------N--------P--PS---Y----------------T--------L---P-----I-PR----------*
4             ------------------------------------------------------------------------------------------------*
5             ------------------------------------------------------------------------------------------------*
6             ------------------------------------------------------------------------------------P-------C--*
7             ------------------------------------------------------------------------------------ALLD.-ESHKWSQ*
```

B.

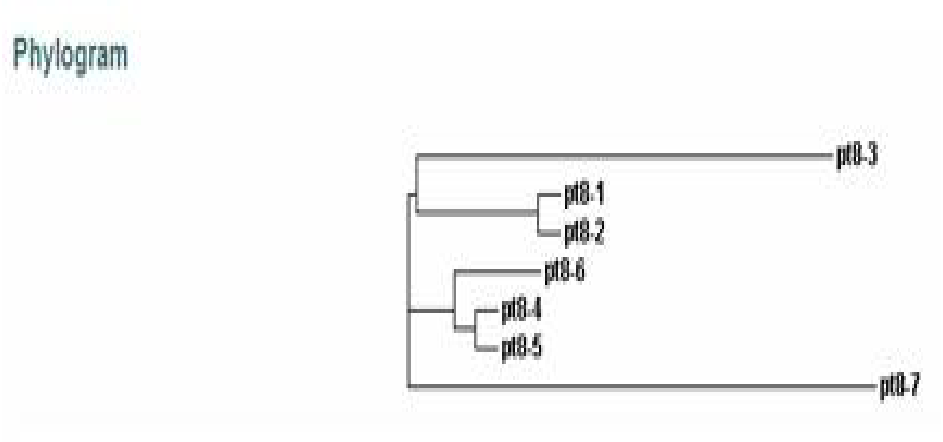


Figure 8: MACS patient #4 analysis. (A) Deduced amino acid variant sequences. (B) Phylogenetic tree of the patient variants.

the deduced *vpr* alleles match up precisely with the pNL43 consensus sequence. This is evident in both figures 9A and B due to the lack of observed mutations and multiple grouped branching. Two of the amino acid sequences from patient #6 (PT6) are exactly identical to the pNL43 consensus sequence, while the other two contain mutations, but in different spots, all of which can be seen in (Figures 10A and B). The mutation observed in allele #4 constitutes a change starting at amino acid position 66. Allele #3 has a mutation at amino acid positions 45, 77, 83, 84, 86, and 87. However the mutation that stands out the most is the R77Q amino acid change, which has been linked to the LTNPs phenotype. The mutations observed in patient #7 (PT7), figure 11A, are seen at amino acid positions 15, 20, 28, 33,37,41, 77, 83, and, 87. S41N and R77Q are the main mutations that seem to be showing up more frequently throughout the previous patient sequences. Figure 11B shows that PT7's sequences are grouped into two main branches, with two subdivisions, which strongly suggest the similarities between alleles 4-6. The amino acid sequences deduced from patient #8 (PT8) are comprised of two alleles that have the R80A mutation, which are shown in figure 12 A. Also shown within the same two alleles are the mutations that have previously been identified as V83I, T84I, and R85Q. The last allele has been mutated at the end of the sequence, starting with amino acid position 82. Figure 12 B shows the groupings of the branches for the individual alleles. For example alleles 4 and 5 are identical to the pNL43 consensus, and thus are grouped together on the same branch, as are alleles 1 and two, which possess the same multiple mutations. Patient #9 (PT9) possess the V83I, T84I, and R85Q mutation set and the 20, 28, 33,37,41 mutation set (Figure 13A). PT9 also has includes four alleles with the R77Q mutation as well as six different alleles, which contain the R80A mutation. Allele #10 (R77Q) also contains and ending mutation that begins at amino acid position 91. Within the phylogenetic tree (figure 13B) only five of the R80A alleles

A.

```
PT 5          1                   28        40                  60                  80                  96
PNL43-CON     MEQAPEDQGPQREPYNEWTLELLEELKSEAVRHFPRIWLHNLGQHIYETYGDTWAGVEAIIRILQQLLFIHFRIGCRHSRIGVTRQERARNGASRS*
1             ------------------------------------------------------------------------------------------------*
2             ------------------------------------------------------------------------------------------------*
3             ------------------------------------------------------------------------------------------------*
4             ------------------------------------------------------------------------------------------------*
5             ------------------------------------------------------------------------------------------------*
6             ------------------------------------------------------------------------------------------------*
```

B.

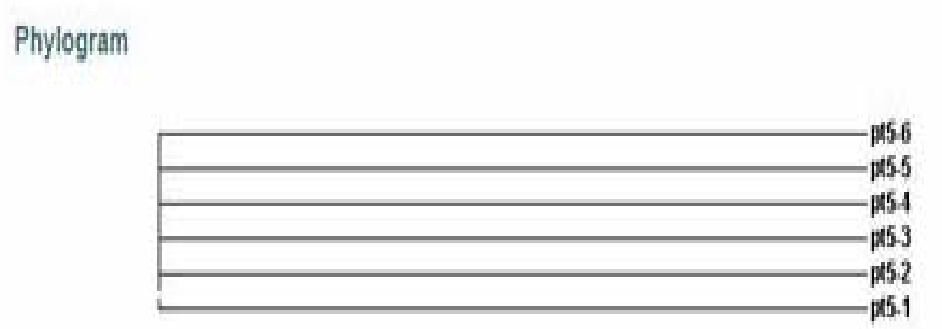
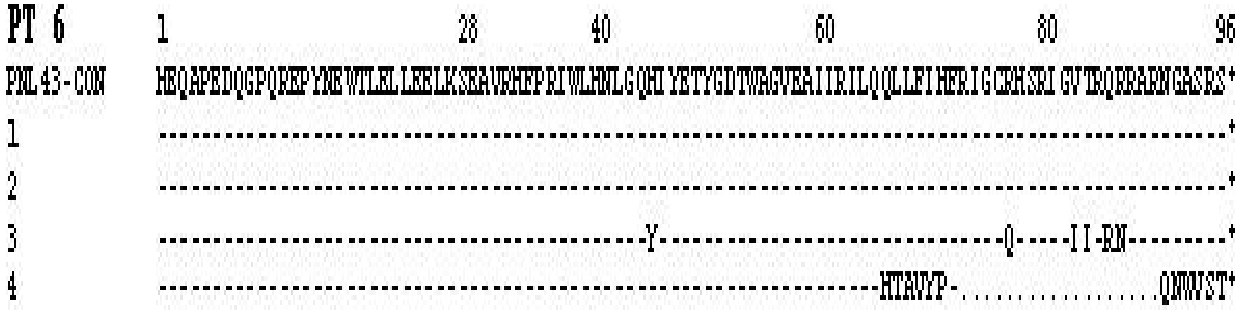


Figure 9: MACS patient #5 analysis. (A) Deduced amino acid variant sequences. (B) Phylogenetic tree of the patient variants.

A.



B.

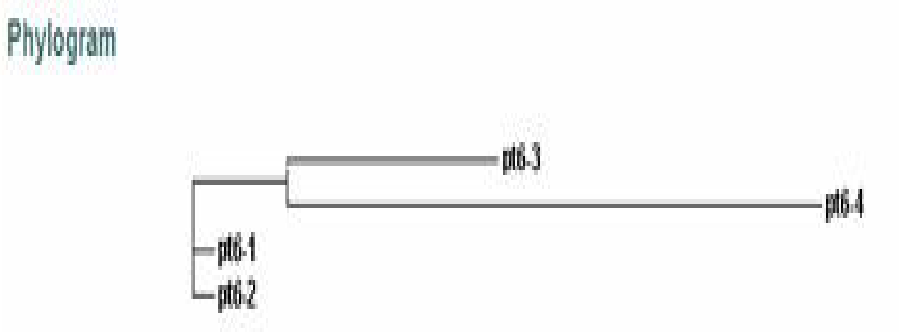
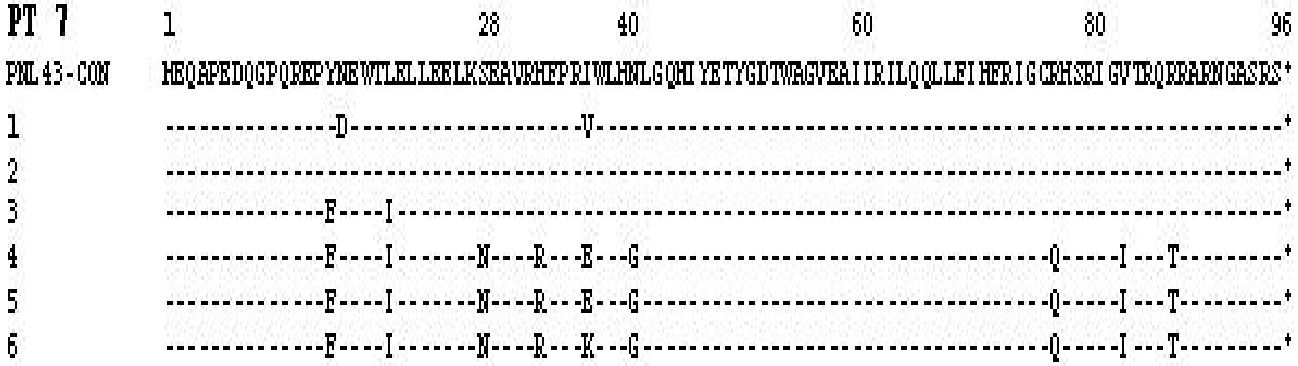


Figure 10: MACS patient #6 analysis. (A) Deduced amino acid variant sequences. (B) Phylogenetic tree of the patient variants.

A.



B.

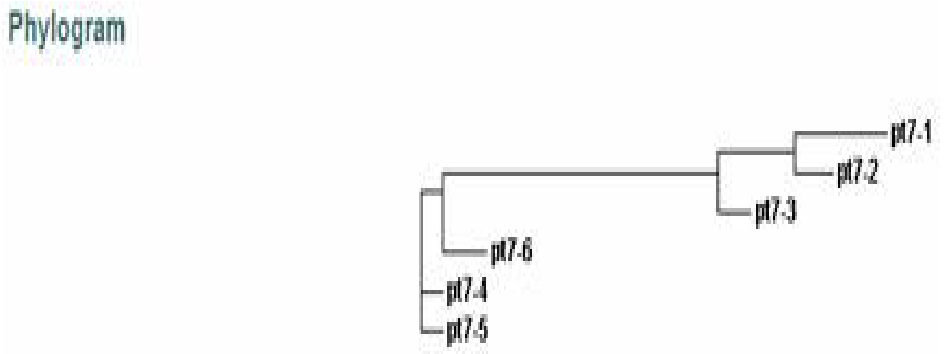


Figure 11: MACS patient #7 analysis. (A) Deduced amino acid variant sequences. (B) Phylogenetic tree of the patient variants.

A.

```
PT 8          1                   28          40                  60                  80                  96
PNL43-CON     HEQAPEDQGPQREPYNEWTLELLEELKSEAVRHFPRIWLHNLGQHIYETYGDTWAGVEAIIRILQQLLFIHFRIGCRHSRIGVTRQRRARNGASRS*
1             ------------------------------------------------------------------------------------A--IIQ----------*
2             ------------------------------------------------------------------------------------A--IIQ----------*
3             -------------------------N--------P--PS---Y----------------T--------L---P-----I-PR----------*
4             ------------------------------------------------------------------------------------------------*
5             ------------------------------------------------------------------------------------------------*
6             ------------------------------------------------------------------------------------P-------C--*
7             ------------------------------------------------------------------------------------ALLD.-ESKKTWSQ*
```

B.

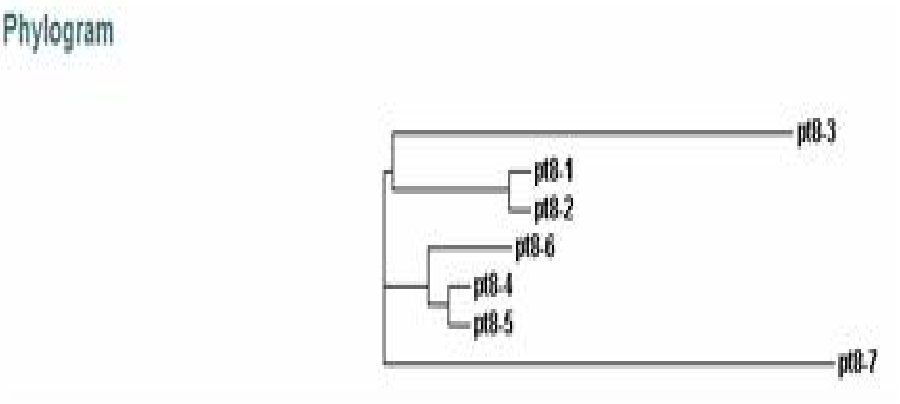


Figure 12: MACS patient #8 analysis. (A) Deduced amino acid variant sequences. (B) Phylogenetic tree of the patient variants.

are grouped together off of the main branch of the tree, suggesting a strong variation between the sixth allele. The mutations observed within patient #10 (PT10) are mainly comprised of V83I, The mutations observed within patient #10 (PT10) are mainly comprised of V83I, T84I, and R85Q mutation set as well as the 20, 28, 33,37,41 mutation set. The allele with the latter mutation set also exhibits the R77Q mutation (Figure 14A). The phylogram in figure14B shows that for the most part the sequences are conserved, due to the lack of multiple grouped branches. The deduced amino acid sequences from patient #11 (PT11) are highly conserved and are identical to the pNL43 consensus sequence, except for the amino acids starting at position 70 (Figure 15A). Alleles #9-11contain the mutation isoleucine (I) to leucine (L) and a truncation of the sequence, while alleles #7 and #8 have a mutated sequence end beginning at amino acid position 82. Alleles #5 and #6 contain the R80A mutation as well as the V83I, T84I, and R85Q mutation set. Figure 15B shows a nice representation of PT11's sequences with grouping and branching corresponding to the various observed mutations.

A.

```
PT 9          1                        28        40                  60                  80                  96
PNL43         MEQAPEDQGPQREPYNEWTLELLEELKSEAVRHFPRIWLHNLGQHIYETYGDTWAGVEAIIRILQQLLFIHFRIGCRHSRIGVTRQRRARNGASRS*
1             ------------------------------------------------------------------------------A--IIQ----------*
2             ------------------------------------------------------------------------------A--IIQ----------*
3             ------------------------------------------------------------------------------A--IIQ----------*
4             ------------------------------------------------------------------------------A--IIQ----------*
5             ------------------------------------------------------------------------------A--IIQ----------*
6             ---------------------------------------------------------------------------Q----I---T---------*
7             -------------F----I-------N----R---E---G-------------------------------------Q----I---T---------*
8             -------------F----I-------N----R---E---G-------------------------------------Q----I---T---------*
9             -------------F----I-------N----R---E---G---------------K-------------------Q----T---T---------*
10            -------------F----I-------N----R---E---G-------------------------------------A--IIQ-T---HEPVDP*
```

B.

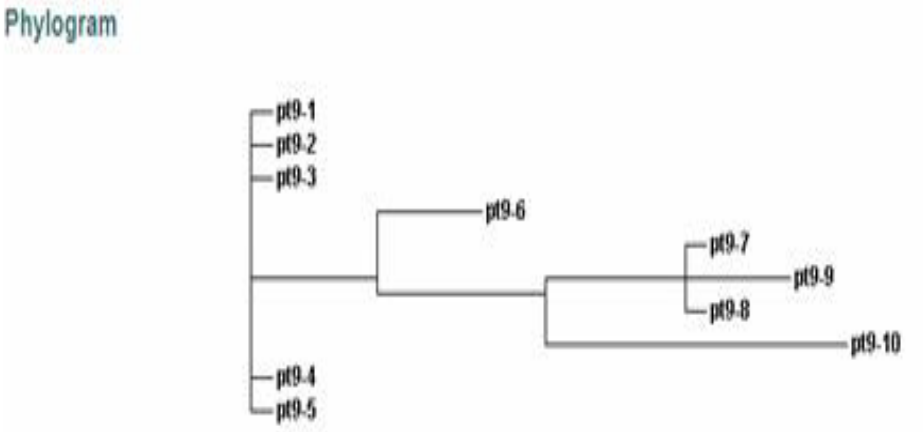


Figure 13: MACS patient #9 analysis. (A) Deduced amino acid variant sequences. (B) Phylogenetic tree of the patient variants.

A.

```
PT 10   1                   28          40                  60                  80                  96
PNL43-CON MEQAPEDQGPQREPYNEWTLELLEELKSEAVRHFPRIWLHNLGQHIYETYGDTWAGVEAIIRILQQLLFIHFRIGCRHSRIGVTRQFRARNG----------ASRS*
1         ------------------------------------------------------------------------------IIQ------EPVDPRRAIPPHWTSGSELGTKLN*
2         ----------------A-------------------------------------------------------------IIQ--------------------*
3         ------------------------------------------------------------------------------IIQ--------------------*
4         ------------------------------------------------------------------------------IIQ--------------------*
5         ------------------------------------------------------------------------------IIQ--------------------*
6         ------------------------------------------------------------------------------------------------------*
7         ------------F----I-------N---R-------G---------------------------------Q-----I----------------------*
```

B.

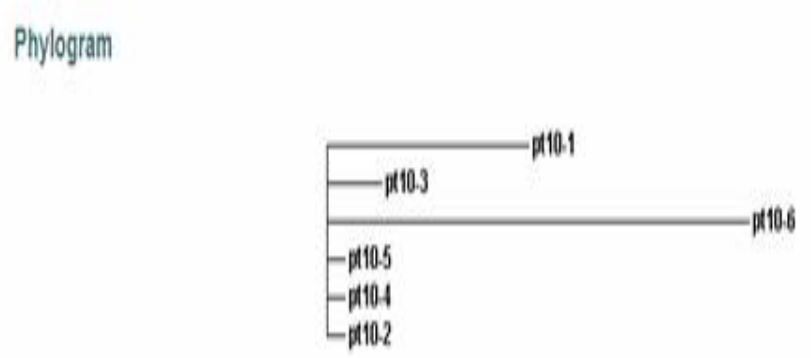


Figure 14: MACS patient #10 analysis. (A) Deduced amino acid variant sequences. (B) Phylogenetic tree of the patient variants.

A.

```
PT 11          1                   28        40                  60                  80                  96
PNL43-CON      HEQAPEDQGPQREPYNEWTLELLEELKSEAVRHFPRIWLHNLGQHIYETYGDTWRGVERIIRILQQLLFIHFRIGCRHSRIGVTRQRRARNGASRS*
1              ------------------------------------------------------------------------------------------------*
2              ------------------------------------------------------------------------------------------------*
3              ------------------------------------------------------------------------------------------------*
4              ------------------------------------------------------------------------------------------------*
5              ----------------------------------------------------------------------A--IIQ----------*
6              ----------------------------------------------------------------------A--IIQ----------*
7              ------------------------------------------------------------------------ALLDRE.SKKWSSRS*
8              ------------------------------------------------------------------------ALLDRE.SKKWSQ*
9              ----------------------------------------------------------------L.....VDIAE*
10             ----------------------------------------------------------------L.....VDIAE*
11             ----------------------------------------------------------------ISEL....VDIAE*
```

B.

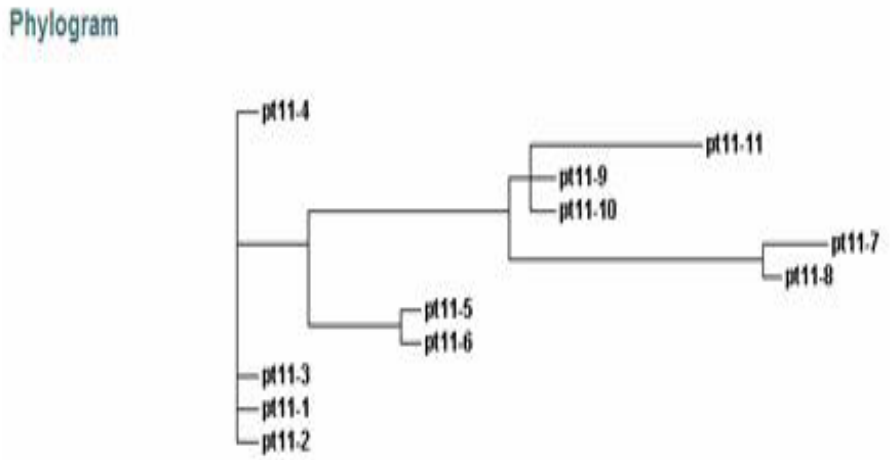


Figure 15: MACS patient #11 analysis. (A) Deduced amino acid variant sequences. (B) Phylogenetic tree of the patient variants.

6.2.3 Phylogenic Tree Composition

Additionally, using the sequences of all the patients used in this study, a Neighbor-Joining tree was generated in order to identify the variation within these patients (Figure 16). Results indicate that certain patient variants are closely related, while the rest of the patients have variants within themselves, which are not similar to each other, which can been seen based on the groupings of the various branches produced. Patients #1,4, and, 9 are grouped in a very similar way that is reflected by their individual trees (Figures 5B, 8B, and, 13B). Patients #5 and 6 are closely related to their earlier trees with the exception of one outlier (Figures 9B and 10B). Patient #7 is grouped in a way that makes the branching almost opposite that of its individual tree (Figure 11B). Patients #2, 3, 8, 10, and 11 show no similarities to their individual trees (Figures 6B, 7B, 12B, 14B, and, 15B). Overall, the Neighbor-Joining tree supports reported studies that indicate *vpr* as having a well-conserved genome (52).

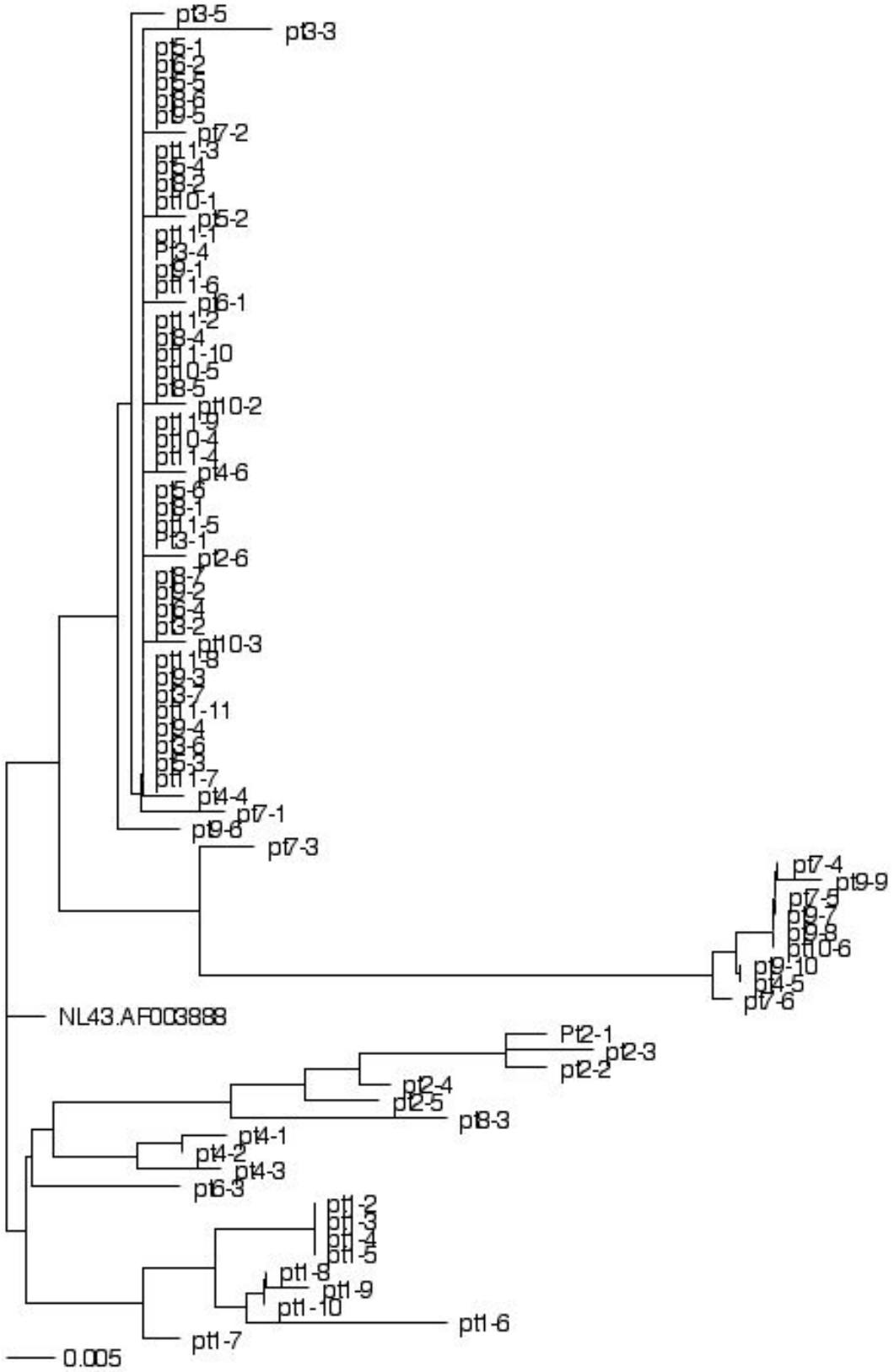


Figure 16: Phylogentic tree comparison. Comparison of all of the deduced amino acid sequences from the MACS patients with the consensus.

6.2.4 Reporter Assay and Protein Quantification: Correlation of biological function with the sequence variation of *vpr* alleles from patients

HIV-1 Vpr is known to transactivate the HIV-1 LTR and increase virus transcription and replication. To correlate whether these sequence variations have any biological functions, HeLa cells were transfected with the HIV-1 LTR luciferase and HIV-1 vpr from patients. Transactivation was measured using the RLU of vector transfected cells as 0 or the basal level activity. Vpr specific transactivation of the HIV-1 LTR was calculated and the increase in log activity is presented in Figure 17. Results indicate that *vpr* alleles, which exhibited the largest increase of transfection were comprised of deduced amino acid sequences similar to that of the *vpr* wild type virus. Patients #5, 8, 10, and, 11 had sequences that were homologous to the consensus sequence, which in turn yielded transfections between 1.5 and 3.0 fold above the basal level. PTs#7 and 11 were observed as having the same fold of approximately 3.0. PTs #3 and 14 were observed as being the only two alleles that had a transfection fold of greater than 10, which is interesting due to the fact that PT7 was homologous to pNL43, whereas PT11 exhibited both the R77Q and the Q3R mutations. Overall, these findings show that the clones derived from each of the MACS patients are in fact potentially infectious and have the capability of expression *in vivo*.

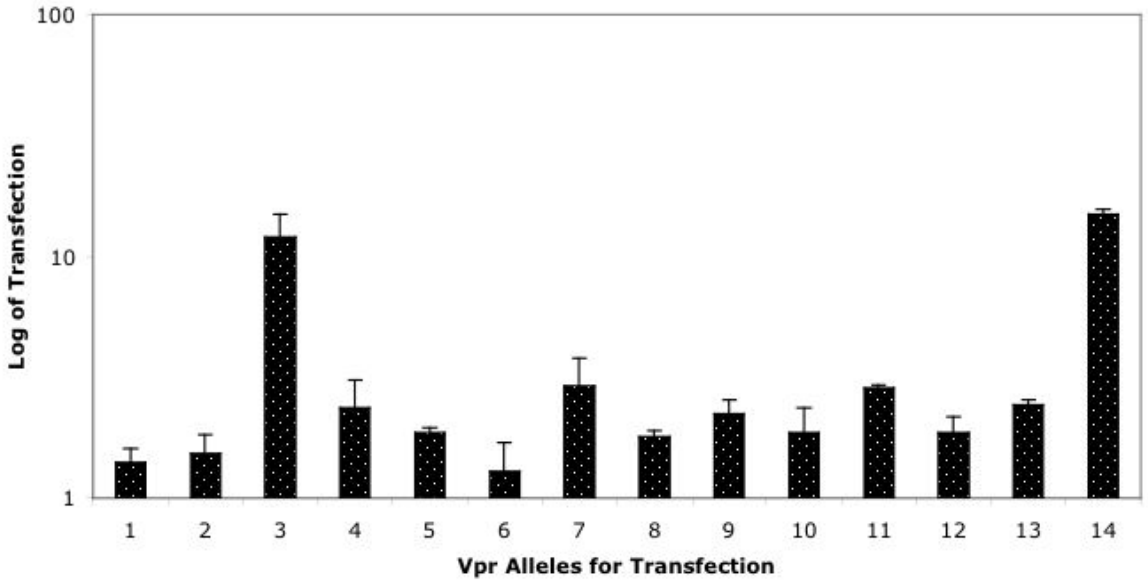


Figure 17: Log graph. Transfection data of the MACS patient and PittMen Study *vpr* alleles.

7.0 DISCUSSION

The HIV-1 *vpr* gene encodes a protein of 96 amino acids with a predicted molecular weight of 15 kDa, and is relatively conserved in HIV and SIV (4, 5, 6, 7, 16, 53, 54). The *vpr* gene is dispensable for viral replication in T-cell lines and activated peripheral blood mononuclear cells (PBMC) *in vitro* (44). Analyses of HIV-1 accessory genes (including *vpr*) in long-term nonprogressors (LTNP) and asymptomatic patients suggest that defects in accessory genes are related to LTNP. The presence of defective or mutated *vpr* quasispecies has been associated with long-term nonprogressive mothers (4, 55). Also, other studies have suggested that maintenance and selection for an intact *vpr* gene occurs *in vivo* (55, 56). To test the role of Vpr *in vivo*, several studies were performed using a simian immunodeficiency virus (SIV) model. Members of HIV-2 and SIV, in addition to *vpr*, also contain *vpx*, a duplication of *vpr* (57). *In vitro* studies have demonstrated that mutations in *vpx* can result in loss of infectivity of macaque macrophages (58). Macaques infected with SIVmac239 containing a mutation in the Vpr initiation codon methionine (M) progressed to AIDS at a slower rate than those infected with wild type virus. Though *vpr* is selected against in tissue culture, selection for Vpr function *in vivo* occurs in both humans and chimpanzees infected with HIV-1, suggesting that *vpr* employs a novel mechanism for maximizing virus production and pathogenesis *in vivo* (58). More recently, several studies have linked the attenuated nature of Vpr *in vivo* and loss of T cell cytopathicity in HIV-1 infected patients (38, 43). Transgenic mice expressing Vpr in the absence of other viral proteins further confirmed these findings, directly linking the cytopathic nature of Vpr and loss

of T cell loss *in vivo* (59, 60). These observations suggest an important role for Vpr in *vivo* viral pathogenesis and disease progression.

HIV-1 Vpr is present in detectable levels in the virus particle, making it one of the first HIV proteins seen by the host cell (38). There are many diverse sources of Vpr available within the infected population. Upon infection of cells by HIV-1, Vpr is synthesized as a late protein along with the structural proteins. The protein is also associated with virus particles, which enables the virus particles to bring Vpr into the cells upon infection. In addition to these infectious particles, there is also an abundance of non-infectious particles (on the order of 1:50,000-1:100,000 infectious versus non-infectious), which also contain Vpr. Hence, non-infectious virions could also transfer Vpr protein into cells through endocytosis (61, 62). It has been suggested that the free Vpr may be the result of Vpr released from infected cells as well as from lysis of virus particles. The intrinsic ability of Vpr to traverse through the cell membrane, as demonstrated recently by several groups (10, 11, 63), provides another avenue by which Vpr released from the infected cells could influence the uninfected, but exposed bystander population. It is widely accepted that Vpr has a role in cell cycle arrest and apoptosis (independent of cell cycle arrest) of primary cells and several cell lines (44, 64), however the role of cellular proteins involved in this are not known. Vpr is known to activate several caspases 3, 8, and, 9 as well as the mitochondrial membrane potential, indicating that several apoptotic pathways could be triggered by Vpr either simultaneously and/or sequentially (44, 65). The important observation from these studies is that Vpr either directly and/or indirectly induces apoptosis in cells that are exposed to this protein, resulting in the possible induction of bystander cell death *in vivo*. The results observed in this study also support this hypothesis.

The capability of HIV-1 Vpr to directly affect the host's cellular functions depends very much on its ability to maintain the highly conserved regions conserved. Vpr, for the most part, is able to preserve itself and still accomplish all of its functions. However, during the replicative cycles

alterations are made and mutations as well as truncations are introduced into the *vpr* geneome. It is these alterations or mistakes that ultimately lead towards the adjustment of otherwise normal functions. The one mutation that was observed the most within this particular population of MACS was the R77Q mutation. This mutation was precisely shown to impair the ability of Vpr to induce cellular apoptosis. And recent studies have also shown this point mutation to occur specifically within the HIV-1 population identified as LTNPs (8, 38, 45). The results presented in this report support this theory as well as another current finding that the correlation between R77Q and LTNPs is not that biologically relevant (15). Of the eleven patient sequences analyzed, Figure 18 illustrates the five patients (PT1, PT6, PT7, PT9, and, PT10) that showed the mutation incorporated into the viral genome. The reason that these results are able to support both of the Vpr studies is by the simple fact that all but one of the MACS patients has been identified as a LTNP. And the one patient (PT11) that still remains undetermined does not exhibit the R77Q mutation in any of its deduced amino acid sequences. The recently deduced amino acid sequences of the patients also reveled the R80A mutation in a total of three patients; PT8, PT9, and, PT11. The virus depends on the suspension of cells in the transition phase for replication. R80A abolishes the ability of this function for the virus, ultimately decreasing the amount of viral replication. Which is very interesting when considering that the only patient not identified as a LTNP possesses this particular mutation. Another interesting observation revolves around the fact that PT9's deduced amino acid sequences posses both R77Q and R80A, but not within the same qusiaspecies Half of the the variants maintained the R77Q change, while the other half has maintained the latter.

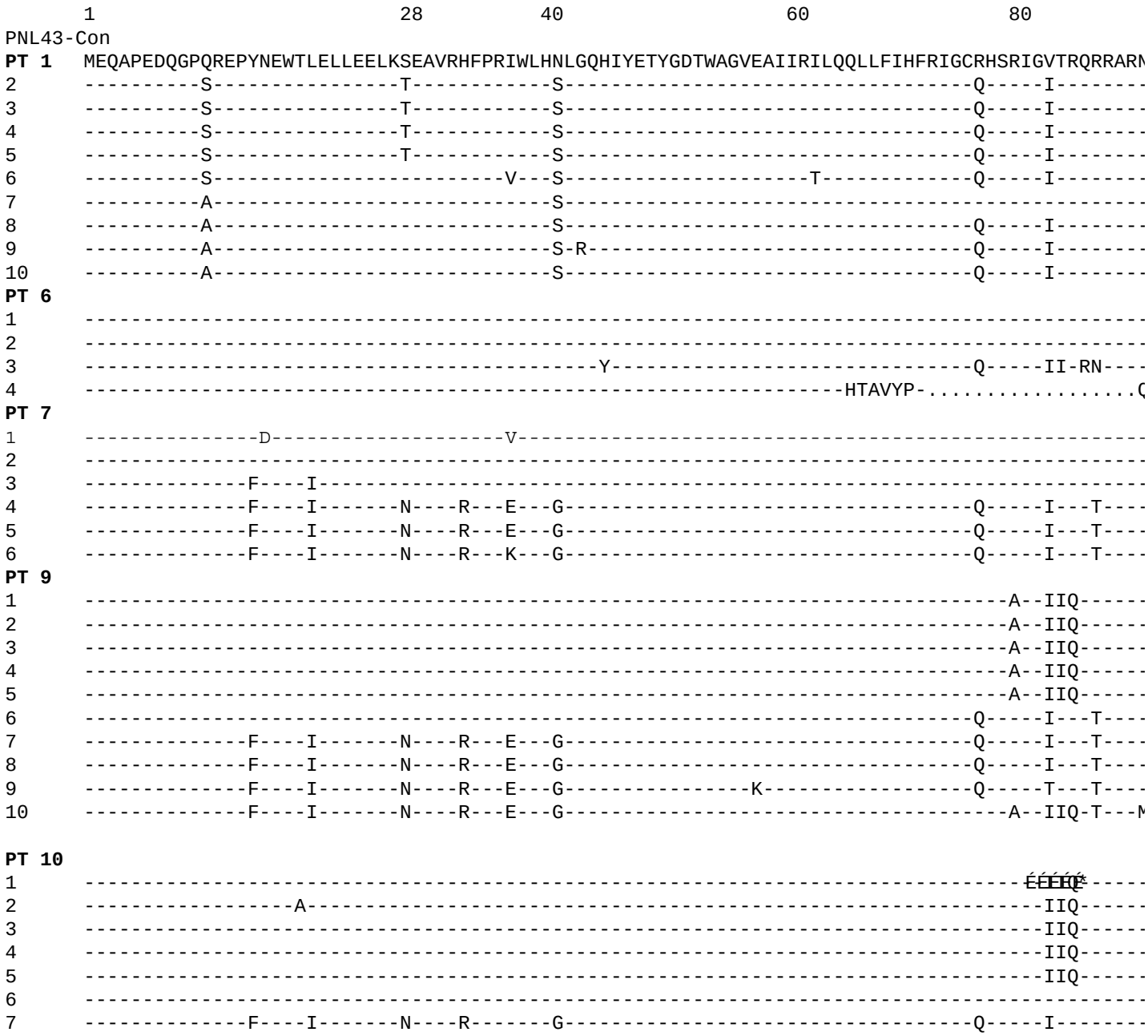


Figure 18: Patient #1, #6, #7, #9, and, #10 deduced amino acid variant sequences exhibiting the R77Q mutation.

Once the amino acid sequences were derived, the next step was to perform relevant functional studies. The functional analysis would in turn be able to identify correlations, if any, between the observed mutations and Vpr functions. The transfection data acquired from this study reveals two sets of outliers when compared to the rest of the patients at basal level. The first set includes the deduced amino acid sequences of PT 7 and PT 11. PT7 who has been identified as a LTNP, has a variant sequence which exhibits an introduced truncation of the virus, while PT11, who has not been identified as either LTNP or RP, has a sequence variant which has incorporated the R80A mutation. The other set includes deduced amino acid sequences of PT3 and PT14. The sequence variant of PT3, also identified as a LTNP, also has a truncation introduced into its viral genome, while PT14 has a sequence variant that has introduced to mutation into its genome-Q3R and R77Q. So even though these sets produced higher folds of transfection, the sequences are very similar in the fact that one from each set is either truncated or has had the introduction of at least one known Vpr mutation. Further analysis of the two sets of outliers should provide a more significant correlation between the deduced amino acid sequences and the virus' functional capabilities.

HIV-1 Vpr is a unique non-structural protein that is associated with the virus particle. A number of functions necessary for viral replication and possibly for pathogenesis have been assigned to Vpr. A greater understanding of the complex interactions between HIV-1 gene product *vpr* and its possible role in HIV-mediated pathogenesis is clearly important. For instance, this study has shown that due to the lack of apoptotic function it is possible that Vpr mediated bystander cell death could in fact be prevented. Thusly in the case of LTNPs, the immune function has the chance of being restored by the $CD4^+$ T-cells. Such observations resulting from this study may lead to the development of new therapeutic interventions for AIDS.

8.0 FUTURE DIRECTIONS

The basis for the research presented here is based upon the deduced amino acid sequences from patients within the MACS population. From these sequences several point mutations as well as truncations have been observed and functional analyses have been carried out in order to better understand the different variants. This study has been carried out for the main purpose of understanding the ***(linkage/interaction)*** between *vpr* gene mutations/polymorphisms and viral pathogenesis as well as the progression towards disease. Future studies involving the MACS patient clones will entail:

(1) Deducing more Vpr amino acid sequences from the MACS patients.

By adding more Vpr clones to the already existing clones per patient will give a better understanding of the quasispecies represented within each patient. This information will give more insight as to which mutation seem to dominate based on the environment of the host, i.e. LTNPs or Rps.

(2) Further characterization of the Vpr clones for their known biological functions.

Additional analysis will be performed on the Vpr clones in order to identify changes from the normal biological functions to that of changes due to *vpr* mutations. The functional analyses to be carried out include virus replication, cell cycle regulation, and immune dysregulation.

(3) Sequential time point deduced amino acid sequences.

It will be of interest to look at patient samples throughout their life, starting from right around the time of infection to well after their CD4+ T-cell counts have dropped. By analyzing the deduced amino acid patient sequences at these time points, the emergence, and possible disappearance, of gene mutations, deletions, and, truncations can be observed and better understood.

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