T.R.N.C

NEAR EAST UNIVERSITY

INSTITUTE OF HEALTH SCIENCES

THE CORRELATION BETWEEN HIVSUSCEPTIBILITY AND RIG-1 R_7C POLYMORPHISM IN DEMOCRATIC REPUBLIC OF CONGO POPULATION

Nanshin DAPAR

MEDICAL MICROBIOLOGY

AND CLINICAL MICROBIOLOGY PROGRAMME

MASTER THESIS

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SUPERVISOR

Assist. Prof. Dr. Umut GAZI

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CORRELATION BETWEEN HIV SUSCEPTIBILITY AND RIG-1 R7C POLYMORPHISM IN THE DEMOCRATIC REPUBLIC OF CONGO POPULATION

ABSTRACT

RIG-1 like receptor is a critical cell receptor detects numerous RNA viruses. HIV carries a protease enzyme which deteriorates RIG-1 in the lysosome thereby making it possible to escape RIG-1 signaling. Single nucleotide polymorphism (SNP) plays a major role on how people react to diseases. Therefore, a mutation on the RIG-1 SNP may affect its function.

Forty-five HIV patients and 29 HIV-patient control groups from the Bandundu Hospital of the Democratic Republic of the Congo were included in this study. The collected blood samples were dried on a whattman-labeled filter paper and sent to BM Laboratories in Ankara for molecular analysis (since the HIV + samples were prohibited from entering the TRNC). By DNA sequencing, samples expressing the *RIG1 R7C* gene were identified, which is then followed by statistical analysis to assess the effect of the polymorphism on HIV.

There was not any significant difference in age and gender between the groups. The study frequency of subjects with wild type genotype GG in HIV+ and HIV- were 61.9% (n=26) and 50% (n=14) respectively. Frequency in heterozygous genotype GA was 33.3% (n=14) in the patient group and it was 42.9% (n=12) in control group. The frequency for homozygous genotype AA was 4.76% (n=2) in patient and 7.14% (n=2) in control group. According to the statistical analysis, all groups shows no significant association between the genotype and allele frequencies of RIG-1 R7C polymorphism in HIV and control group. This result suggests that there is no relationship between RIG-1 R7C polymorphism and HIV susceptibility in Congo population. More studies are recommended to confirm these results.

Keywords: Human immunodeficiency virus, RIG-1, R7C SNP, Democratic Republic of Congo.

KONGO DEMOKRATİK CUMHURİYETİ'NDE HIV DUYARLILIĞI İLE RIG-1 R7CPOLİMORFİZMİ ARASINDAKİ KORELASYON

ÖZET

RIG-1 benzeri reseptör, kritik bir hücre reseptörüdür, ve sayısız RNA virüsünü tespit etmede etkindir. HIV, lizozomda RIG-1'i bozan ve böylece RIG-1 sinyallemesinden kurtulmayı mümkün kılan bir proteaz enzimini taşır. Tek nükleotid polimorfizmi (TNP), insanların hastalıklara nasıl tepki gösterdiği konusunda önemli bir rol oynar. Bu nedenle, RIG-1 SNP'lerireseptör işlevini etkileyebilir.

Kongo Demokratik Cumhuriyeti Bandundu Hastanesi'nden 45 HIV+ hastası ve 29 HIV- hasta kontrol grubu çalışmamıza dahil edilmiştir. Toplanılan kan numuneleri, whattman etiketli filtre kağıdında kurutulmuş ve moleküler analiz için (söz konusu HIV+ numunelerin KKTC'ye girişi yasak olduğundan) Ankara'da bulunan BM Laboratuvarları'na gönderilmiştir. DNA dizi analizi ile *RIG1 R7C* genini ekspres eden numuneler tespit edilmiş ve bunu takiben yapılan istatiksel analiz ile söz konusu polimorfizmin HIV duyarlılığına olan etkisi değerlendirilmiştir.

Gruplar arasında yaş ve cinsiyet açısından anlamlı fark tespit edilmemiştir. HIV+ ve HIV- numunelerinde doğal genotip GG frekans sıklığı sırasıyla %61.9 (n = 26) ve %50 (n = 14) idi. Heterozigot genotip GA sıklığı hasta grubunda %33.3 (n = 14) olup kontrol grubunda %42.9 (n = 12) idi. Homozigot genotip AA sıklığı hasta grubunda% 4.76 (n = 2), kontrol grubunda% 7.14 (n = 2) idi. Yapılan istatistiksel analizler, HIV+ hasta ve kontrol gruplarında tespit edilen RIG-1 R7C polimorfizm genotip ve allel frekansları arasında anlamlı bir ilişki olmadığını göstermektedir. Bu sonuç, Kongo popülasyonunda RIG-1 R7C polimorfizm ve HIV duyarlılığı arasında bir ilişki olmadığını düşündürtmektedir. Başka bakış açıları ve tekniklerin kullanılacağı gelecek çalışmalar hem bu alanın aydınlatılmasına katkıda bulunacak hem de elde eddiğimiz sonuçların doğrulanmasını sağlayacaktır.

Anahtar kelimeler: HIV virüsü, RIG-1, R7C SNP, Kongo Demokratik Cumhuriyeti.

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SYMBOLS AND ABBREVIATIONS

AIDS	Acquired Immune Deficiency Syndrome
APC	Antigen Presenting Cell
ART	Antiretroviral Therapy
CARD	Carboxyterminal Regulatory Domain
CTD	C Terminal Domain
DC-SIGN	Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin
DNA	Deoxyribonucleic Acid
HD	Helicase Domain
HIV	Human immunodeficiency virus
HLA	Human Leukocyte Antigen
IFN	Interferon
IRF-3	Interferon Regulatory Transcription Factor 3
ISG	Interferon Stimulating Gene
LGP2	Laboratory of Genetics and Physiology 2
MAVS	Mitochondrial Antiviral Signaling Protein
MDA5	Melanoma Differentiation-Associated protein 5
MHC	Major Histocompatibility Complex
NCBI	National Center for Biotechnology Information
NF-kB	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells
NLR	Nucleotide Oligomerization Domain Like Receptor
NOD	Nucleotide-binding oligomerization domain-containing protein
PAMP	Pathogen Association Molecular Pattern
PCR	Polymerase Chain Reaction
PEP	Post Exposure Prophylaxis
PrEP	PreExposure Prophylaxis

PRRs	Pattern Recognition Receptors
RANTES	Regulated on Activation, Normal T Cell Expressed and Secreted
RIG-1	Retinoic Acid inducible Gene 1
RLRs	RIG-1 Like Receptors
RNA	Ribonucleic Acid
SNP	Single Nucleotide Polymorphism
TLRs	Toll Like Receptors
VISA	Virus Induced Signaling Adapter

1. INTRODUCTION

1.1The Human Immunodeficiency Virus (HIV) and HIV infections in Democratic Republic of Congo population

HIV is a member of a virus family, called retroviruses. Following HIV binding to a host cell, the viral RNA is inserted into host cell cytoplasm. HIV contains a catalyst known as reverse transcriptase. This permits the single-stranded RNA to be converted to double stranded DNA (dsDNA) which by integrase is integrated into the cell chromosome. Provirus (HIV DNA) is reproduced alongside the chromosome when the cell undergoes cell division (Crandall 1999).

The Democratic Republic of the Congo (DRC) is confronting a vast scale of HIV/AIDS plague; it is estimated that on the average, 1.19million people making 4% of the population live with HIV/AIDS at the end of 2005. The primary method of transmission is heterosexual. The most seriously influenced ages are 20– 29 years for ladies and 30– 39 years for men (WHO 2005). HIV predominance among pregnant ladies is 3.5 percent and the rate of mother-to-kid transmission of HIV is evaluated to be 33 percent (John 2014). The plague has seriously affected kids – an expected 770 000 kids younger than 17 years had lost one or both parents because ofAIDS in 2003 (WHO 2015). The predominance of HIV disease is higher in eastern than in the western partof the country and the outbreak has been worsened by expansive scale populace developments that came about because of the ethnic clash and political insecurity of the mid-1990s, the related financial crisis, abnormal amounts of untreated sexually communicable diseases and weakliving structure of the economy (WHO, 2005).

1.2 Retinoic acid-inducible gene (RIG-1) family

Retinoic acid-inducible gene RIG-1 is a part of the RIG-1 like receptor (RLR) which is a critical cell binding site that is expressed by the gene *DDX58* in humans. Included in this family are the melanoma differentiation-associated gene 5 (MDA5) and lab of genetic qualities and physiology 2 (LGP2).Theyfunction as a pattern recognition

receptor (PRRs) and detects various types of viruses; its inadequacy may firmly weaken the host antiviral immunity(Pothlichet et al., 2009).

Among thesingle viral partsresponsible for the initiation ofantiviral immune response, nucleic acids are seen as the most essential. In warm-blooded animals, there is a minimum of two receptor frameworks set up to distinguish viral pathogens and increase a type I interferon (IFN)-dependent antiviral immune reaction: interaction toll like receptors 3, 7, 8 and 9which are expressed in endosomes; and cytosolic PRRs which are involved in detection of nucleic acids in the virion (Alexopoulou*et al.,* 2001). Examples for the latter include RIG-1 and MDA5 which are cytosolic helicases involved in the detection of intracellular double stranded RNA and or 5'triphosphate single stranded RNA, the two common by products of viral contamination and copying (Schlee et al., 2009).

RIG-1 is an important molecule that identifies various pathogens including influenza virus and hepatitis-C virus (HCV). The carboxyterminal regulatory domain (CARDS) and the central ATP binding helicase domain are the means by which RIG-1 associates with its ligand. RIG-1 homocomplexes sends a signal when it binds to MAVS (also known as CARDIF, IPS1 or VISA). These are adaptor proteins which function to mediate CARD dependent reaction with RIG-1 (Kumar *et al.,* 2006). This signaling occurs through the amino terminal tandem CARDS. There is an activation of transcription factors NF-KB and the interferon regulatory factor IRF-3 when the signaling occurs. This triggers proinflammatory immune response important for defense against viral pathogens (Yoneyama et al.,2008).

Infection of dendritic cells (DCs) by viruses with negative-strand RNA, results in the induction of IFNβ expression, as a result of the activation of RNA helicase RIG-1. In a study carried out by Hu *et al*, IFNB1 expression correlated with *DDX58*expression level which is induced by the Newcastle disease virus triggered by RIG-1 activation .DDX58 has a common single nucleotide polymorphism (SNP), rs10813831 (A/G), encoding Arg7Cys amino acid alteration within CARDs of RIG-1 protein (Hu *et*

al., 2010). The aim of this study is to investigate the correlation between RIG-1 R7C polymorphism and HIV susceptibility in DRC population.

2. GENERAL INFORMATION

HIV is a highly infectious virus which quickly weakens the host immunity and eventually leads to AIDS, by targeting $CD4^+$ T cells (Boasso *et al*., 2008). AIDS is the final stage of HIV infection where opportunistic infections such as pneumonia, tuberculosis, coccidioidomycosis, and a lot of other infections take advantage of the weak immune system. HIV can use several methods to escape from both the natural and adaptive immune reactions (Kirchhoff et al.,2010).

There is a rapid production of IFN (IFNβ/α) when an anti-viral innate immune response is triggered. IFN acts to hinder viral replication (Honda *et al.*, 2006). When the HIV viral products are sensed, antiviral immune response is initiated by either the plasma membrane or cytoplasmic PRRs which triggers various intracellular signaling cascades leading to the production of various mediators. intracellular viral replication and helps the initiation of adaptive immune responses (Nakhaeiet al., 2009).

Plasma membrane and cytoplasmic PRRs have different ways of inducing an anti-viral immune response, mainly due to the differences in their cellular localization. Among the plasma membrane PRRs, Toll-like receptors (TLRs) have a vital role in controlling HIV infections. IFN α and pro inflammatory cytokine production is known to be induced when the HIV is recognized by TLR7 and TLR8 in macrophages and dendritic cells (DCs) (Beignon *et al.*,2005).On the other hand, when the TLRs is continuously activated by HIV, this may lead to chronic immune cells activation and mostly promotes the death of $CD4^+$ T cell (Solis *e tal*., 2011).Lately, it was also observed that TLR8 and dendritic cell specific intercellular molecule 3 grabbing non-integrin(DC-SIGN) has advanced the replication of HIV by improving the translation of full length viral transcript(Solis et al., 2011).

2.1 The Human Immune System

The Human immune system basically functions to fight against pathogens that entered the body. It is composed of two components: innate and adaptive immunity. (Nicholson *et al.*,1995). The main requirement of an immune system is to differentiate the host cells, tissues and organs from foreign invaders and to eliminate them. In some cases, the immune cells can recognize and eliminate some host cells usually changed by diseases such as cancer. The fastest cellular component that reacts to infections is called natural immunity while the acquired immunity is activated when specific molecules sends a signal (Beck et al., 1996).

2.1.1 Innate Immune System

The parts of the innate immune system are numerous, have several pathogen recognition properties. It can detect the presence of DNA and RNA sequences, proteins and sugar motifs through different PRRs like TLRs (Akira et al., 2006). Receptor engagement results in the activation of intracellular signaling that leads to the expression of activation markers as well as release of cytokines and chemokines which are important mediators during an immune response (Douville *et al.*, 2010). Cellular components include “phagocytic cells (e.g., macrophages), antigen presenting cells (e.g., dendritic cells)’, and killer cells (e.g., natural killer cells). Also, epithelial cells act as a physical barrier against the entry of pathogens as well as other foreign particles (Hatoetal2015).

2.1.2 Adaptive Immune System

The development of the adaptive immune reaction is quite slow, unlike in the innate immune response. It can take weeks or a few days to develop after the initiation of an infection. There are 2 main parts of the adaptive immune system, cellular part mediated by T cells and humoral part mediated by B cells. The two cells are derived from hematopoietic stem cells in bone marrow (Gudmundsdotter et al., 2009).

T-cells consist of the CD4 helper cells and the cytotoxic $CD8^{+}$T cells. Among those, in a conventional immune response, CD4+ cells recognize peptide antigens only

when presented with human leukocyte antigen on antigen presenting cells. Antigen presenting cells (APCs) are cells which process antigen, thereby breaking it into peptides and presenting it in conjunction with major histocompatibility complex (MHC) to the T cell receptor for an adaptive response to take place. Classical APCs include macrophages, dendritic cells and B cells, and they are involved in the presentation of exogenous antigen together with MHC class II molecules to CD4+ T-cells. Following T cell activation, differentiate into different CD4+ T-cell subsets depending on the cytokines found in the environment. The four subsets of $CD4^{+}$T cells are:

1) T helper Th1 cells involved mainly in controlling infection with intracellular pathogens.
2) Th2 for extracellular pathogens and evacuating infection seen outside the cell.
3) Th17 also functions in controlling extracellular organisms and causing tissue inflammations.

4) Regulatory T cells involved in the regulation of immune responses.

There are two MHC molecules involved in antigen presentation: while MHC class II molecules are involved in in antigen presentation to CD4+ T-cells, MHC class I molecules are responsible for CD8+ T-cell activation. Cytotoxic cells are able to kill host cells when it recognizes endogenous foreign peptides transported to cell surface with MHC 1 molecules. Cytotoxic T cells kills infected cells by releasing granzymes and perforin which that induce apoptosis in any target cell and induce pore formation on the target cells, respectively. Also produced by CD8 cytotoxic T cell is IFN-γ which inhibits replication of virus and is important for MHC 1 expression as well as elevating intracellular killing activities in macrophage. Cytotoxic cells are precise in killing infected targets without causing damage to normal cells, by this minimizing tissue damage as infected cells are eradicated (Andersen et al., 2006).

2.1.3 Broad Principle of the Antiviral Natural Resistant Structure

Viruses are intracellular parasites and hence depend entirely on the biosynthetic machinery of the host cells for their replication and spread. While mammalian cells have created advanced components for protection against infections, viruses can use several strategies to escape immune detection and reaction (Barbalat et al., 2011).

The detection of pathogens by innate immune cells is mediated by germline-encoded PRRs that can bind to the pathogen-associated molecular pattern (PAMPs). Engagement of antiviral PRRs by their related PAMPs trigger signaling pathways that induce the expression of proinflammatory cytokines, (such as IFN-β and IFN-α), and interferon-stimulated genes (ISGs) (Chui et al., 2009).

PAMPs presented during a viral infection include DNA and dsRNA and ssRNA. dsRNA PAMPs can exist in the form of the viral genome or as a replication intermediate. Viral dsRNA is recognized in the late endosome TLR3, while uridine-rich short ssRNA is recognized by TLR7 and TLR8 in the endosomal compartments of plasmacytoid dendritic cells. Cytoplasmic 5’ triphosphorylated ssRNA and short dsRNA are recognized by the RNAhelicaseRIG-1. Long cytoplasmic dsRNA, such as polyI:C, is recognized by the related helicase MDA5 (Uzri et al., 2009).

PRRs can respond to molecules typical for pathogens which include pathogen carbohydrate, nucleic acids and pathogen peptides. This group includes TLRs which is responsible for recognizing PAMPs in extracellular environs. Those found on the cell surface is limited to proteins and lipid while in endosomes detects nucleic acids. Separating these two is important to allow innate cells react to viral envelope component. Whereas in endosome, nucleic acids are detected which is the point viral genomes are uncoated and enters into the cytoplasm. RLRs alongside Nucleotide oligomerization domain like receptors (NLRs) and cytosolic DNA sensors are found in the cytoplasm where it subjects the viral component to inspection and mount proinflammatory and antiviral response. PRRs regulate transcription factors which is responsible for the production of cytokines and interferons in addition to inducing the

expression levels ofMHC2, CD40, CD80 and CD86 for adaptive immunity to act efficiently (Thompson et al., 2011).

RLRs is an important factor in antiviral defense pathway. Major function of RIG-1 is to recognize 5' triphosphorylated uncapped ssRNA to differentiate for host cell nucleic acids from viral genome (Chui *et al.*, 2009). In addition, RIG-1 is also able to recognize short dsRNA produced during viral replication. RIG-1 is involved in detection of several viruses with ssRNA such as HIV and activates antiviral immune responses (Thompson et al., 2011).

2.2 HIV Structure

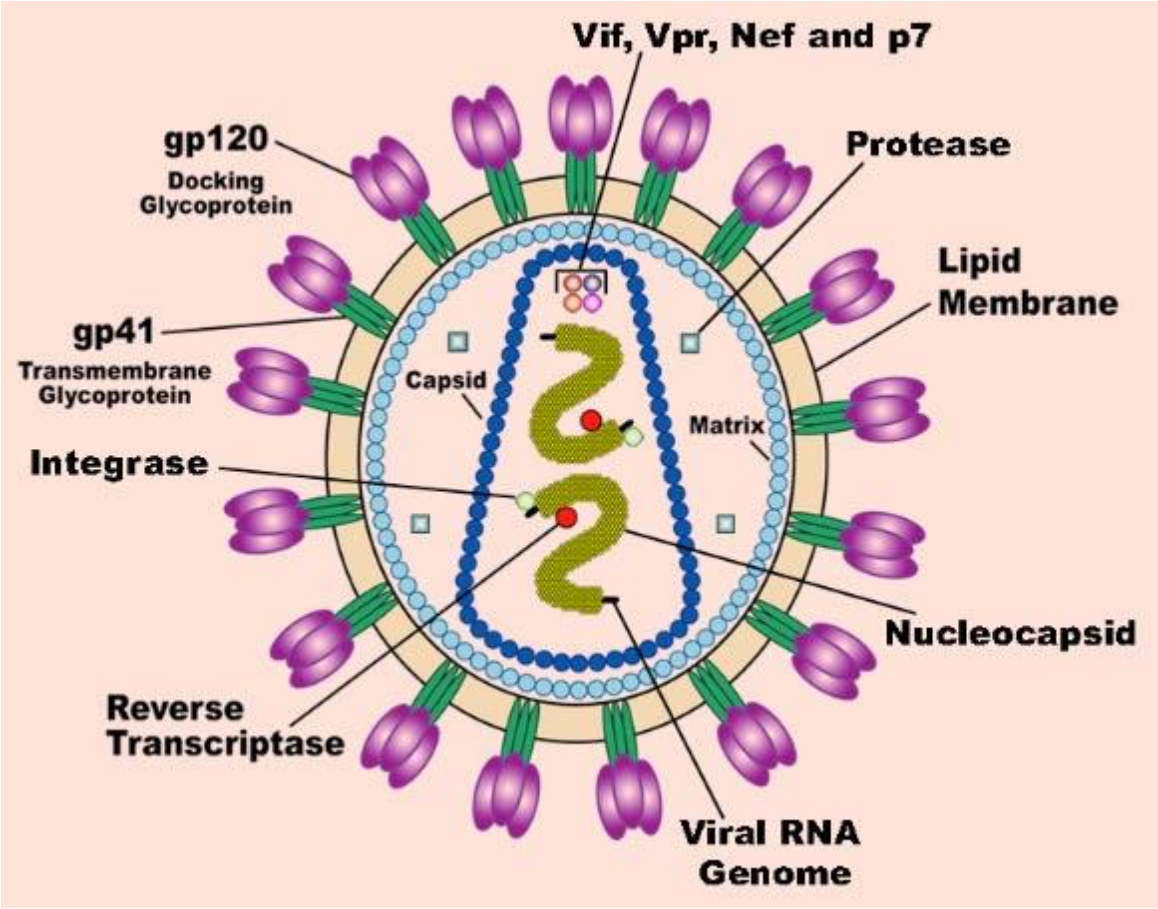

Figure 1: HIV virion; www.afa.org.ng

HIV is an RNA virus, and as it buds out of the host cell, it gains a phospholipid envelope. Projecting from the envelope are peg-like structures that the viral RNA encodes. Each peg comprises of three or four gp41 glycoproteins (the stem), topped with three or four gp120 glycoproteins. Inside the envelope, the bullet-shaped nucleocapsid is made of protein and two single strands of RNA. Besides, reverse transcriptase, integrase, and protease which are the three important enzymes for the life cycle are also found with the nucleocapsid (figure 2) (Crandall 1999).

2.2.1 Immunological Assessment

HIV infection causes a serious reduction in the numbers of CD4+ T cells (Nishman et al., 19998). The resistant status of a child can be surveyed by measuring the number (per mm^3) or level of CD4+ cells, and this is viewed as the standard approach to evaluate and describe the seriousness of HIV-related immunodeficiency. The continuous destruction of CD4+ T cell is an indication of HIV advancement in the body and this creates opportunity for several attacks from other infections which can lead to fatal consequences (Vajpayee et al.,2005).

2.2.2 HIV Time Phase and Host Resistant Response

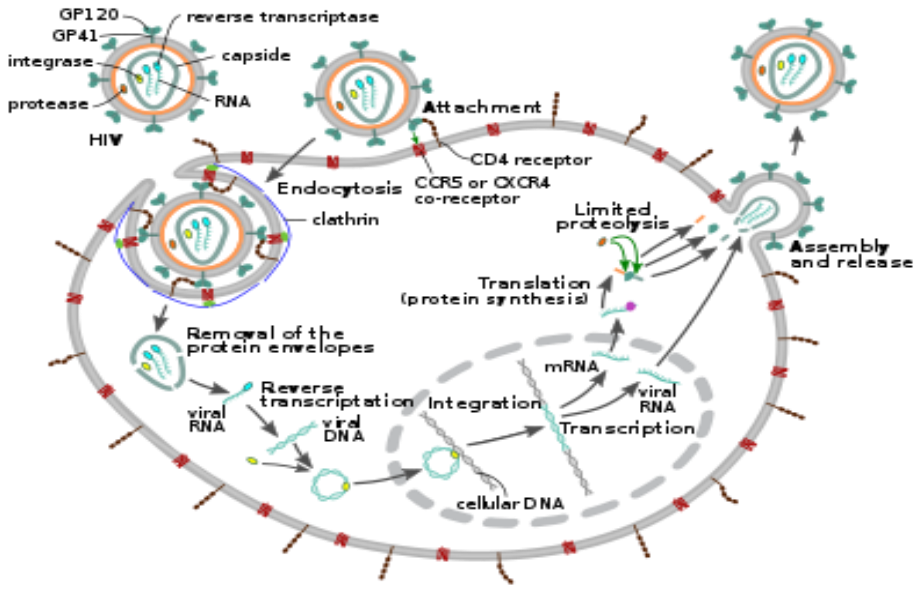

Figure 2: HIV viral replication cycle (www.afa.co.org)

HIV gets attached to the cell by means of the CD4 receptor and other co receptors which are CCR5 or CXCR4. The virus binds to the host CD4 (expressed by T-cells as well as monocytes and macrophages) cell via the gp120 then it is fused into the cell membranes after which it is disintegrated. Within the infected cellreverse transcriptase transcribes the RNA genomic structure into a DNA and then the viral DNA (provirus) will be integrated into the host DNA. HIV provirus is a replication factor for more viruses (Jardine *et al*., 2013). There are a number of host proteins which interact with the HIV DNA or HIV proteins to either stop or help the replication of the virus in infected human cells (Table 1).

Table 1: Host proteins that interact with HIV proteins (Maartens et al. 2014).

	Action	HIV target
APOBEC-3G[39]	RNA editing protein	vif
TRIM-5α[40]	E3 ubiquitin ligase tripartite motif targets viral capsid	Capsid
Tetherin (BST-2)[41]	Immunomodulatory membrane protein that inhibits virus budding	vpu
SAMHD1[42,43]	Hydrolyses dNTPs and restricts efficient reverse transcription of HIV	vpx*
TREX-1[44]	Cytosolic exonuclease that inhibits the immune recognition of viral products such as HIV DNA	HIV DNA
LEDGF/p75[45]	Brings HIV DNA in close proximity to chromatin	integrase

APOBEC-3G=apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like-3G. TRIM=tripartide motif. SAM=sterile α motif. HD1=histidine aspartic acid domain-containing protein 1. LEDGF=lens epithelium-derived growth factor. *Expressed by HIV-2 only.

***Table 1*: Host proteins that interact with HIV proteins or DNA and either restrict or help with HIV infection in human cells**

2.2.3 HIV Tropism

The main target cells of HIV are those with carry the CD4 receptor expression that includes macrophages, T cells and in some cases DCs. The virus interacts with the immune cells by attaching to the CD4 receptor through its virion envelope glycoprotein gp120. Chemokine coreceptors CXCR4 and CCR5 are co factors which function with CD4 receptors on host cell in binding to the virus (Benkirane et al., 1997).

Macrophage tropic(M tropic) strain uses CCR5 as preferred co receptor upon infection in macrophages and CD4+ T cells (philphott *et al.,* 1999).Most individuals

infected harbors the M tropic for a long period of time which may evolve to dual tropism and T cell line tropic(T tropic) after many years. A change in the tropism indicates the progression of the disease and this time the virus replication increase. A single amino acid alteration on gp120 is a factor which causes the switch from M tropic to dual and T tropic. T tropic strains usually evolve in AIDS patientswhereitbindsCXCR4 co receptoronCD4 T lymphocytes, to enter the host cell (Wilkonson et al., 1998).

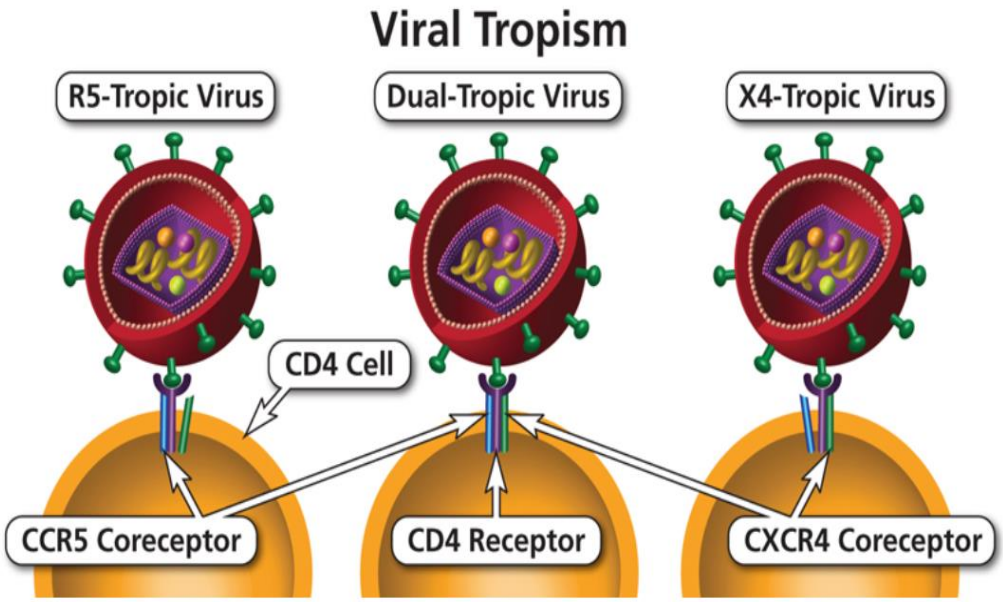

Figure 3: stages of HIV tropism (aidsinfo.nih.gov)

The identification of HIV co receptors has helped the development of antiretroviral therapy ARTs. These drugs aim at blocking the viral envelope protein interaction with the co receptors CXCR4 and CCR5 and thereby aims to prevent the entry of HIV into susceptible cells (Clapham et al., 2013).

2.2.4 Stages of HIV

Typically, when a person is infected with HIV, it may take 7 to 10 years before clinical signs of AIDS is seen. This disease progression differs in individuals with factors such as the difference in genes, and strength of the immune system amongst others. Upon infection, a person remains asymptomatic and is difficult to determine if a person is infected without running a blood test. CD4 level continues to drop and the immune system breaks down (Amborzia *et al.*, 1998). There are 3 stages which show the progression of HIV in humans (Figure 3).

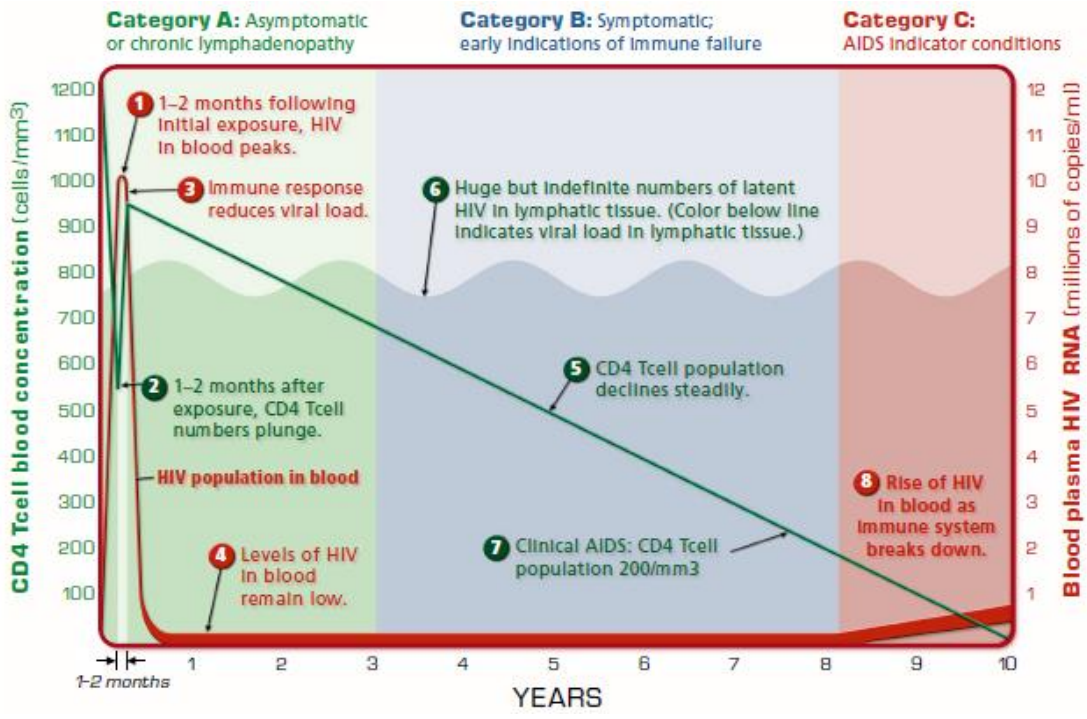

Figure 4: Typical Progression of HIV Infection & AIDS (Cradall et al 1999).

1. Asymptomatic phase:

This is the first phase of infection; it mostly grows after a period of 2 to 4 weeks when an individual first come in contact with the virus, and the levels of the virus in the blood is relatively low. In this period, some people have a headache, rash and general

body fatigue. Viral replication in this phase is generally fast and its risk of transmission is high (US gov. 2017).

2. Symptomatic phase:

This phase is known as the latency phase. At this phase, the virus keeps replicating but at a much slower rate, the CD4 T cell levels continued to decrease steadily. Individuals infected do not easily show symptoms but are capable of transmitting the virus. At this stage, an infected person should be placed on treatment with HIV drugs as this would slow its advancement to AIDS (US gov. 2017).

3. AIDS:

This is the last stage of infection whereby infections or other pathogens take advantage of the weak immune system and cause severe damages. An HIV infected person is concluded to have AIDS when the CD4 count is less than 200cells/mm^3 or if there's a presence of certain opportunistic infection. Without treatment at this stage, patients can barely survive a maximum of 3 years (US gov. 2017).

2.2.5 Few examples of Opportunistic infections

Opportunistic infection refers to infections that occur and are life-threatening in individuals with a weak immune system. These infections caused by virus, parasites, bacteria and fungi are easily controlled in healthy individuals but in cases of people with weak immune system, such as those with HIV infection or cancercan cause severe damage (CDC 2017).

1. Herpes simplex virus

This infection is highly contagious and is transmitted during direct sexual contact. Affected people have lesions on the face lips and mouth. Infection can spread even from patients without a visible lesion or sore on the face. There is not any treatment for complete eradication of HSV1 yet, but antiviral treatment can help to reduce the rate of replication and lessen transmission (CDC 2017)

2. Salmonella Infection

It is a part of normal intestinal microbiota of many individuals and can also cause food borne disease. Salmonella infection is characterized by fever, diarrhea, dysentery and headache (Grant *et al.,* 1997). People infected with HIV are prone to suffer from more complications when compared to a person with a strong immune system (CDC 2017).

3. Candidiasis

The most common species is *Candida albicans.* This fungal infection occurs when there is an excess growth of the fungi in the moist region of the human body. The body parts easily affected is mucous membrane of the mouth, anus and vagina but in HIV infected persons, it affects areas such as the trachea, lungs or esophagus (CDC 2017).

4. Toxoplasmosis

Toxoplasmosis is a parasitic infection caused by the parasite *Toxoplasma gondii* (Maynart *et al.,* 2001*).* Cats are the main reservoir of this parasite and contact with contaminated meat and water can lead to infection. Transmission can also occur from mother to child during pregnancy or birth. Mild symptoms like swelling of lymph nodes, muscle pain last a long period of time (Wiktor *et al.,* 1999. Rana *et al.,* 2000). Complications can harm brain, eyes and other areas. Brain toxoplasmosis is a complication in HIV patients which occurs usually in patients with a CD4 count <100 cells/μL and not on treatment (CDC 2017).

5. Pneumoniae

Bacteria, viruses and fungi can infect the human lungs and thereby cause inflammation. Pneumoniae is typically the inflammation of the lungs, and people with a weak immune system are prone to suffer from this infection. HIV infected patients, children and older people are easily infected, with symptoms such as chest pain, cough,

fever, short breath and body weakness. Pneumonia in HIV patients occurs recurrently (CDC 2017. Corbett et al., 2002).

6. Tuberculosis

Mycobacterium tuberculosis and *Mycobacterium bovis* cause this infection. Tuberculosis is an airborne disease which is transmitted from an infected person to another via aerosols that spread out by respiratory route through sneezing, coughing etc. In an active form of tuberculosis, the bacteria spreads from the lungs to other areas such as brain, kidney, and CNS and can be fatal (Lucas *et al.,* 1993. Rana *et al.,* 2000). Symptoms include chest pain, coughing of blood, fever, chills, weakness and sweating. Brain and CNS are commonly affected in AIDS patient (CDC 2017).

7. Kaposi sarcoma

This is a type of cancer caused by Kaposi sarcoma herpes virus (HHV8) which belongs to the family Herpesviridae. This affects the blood vessels and causes them to grow abnormally. Infection also causes the formation of pink like spots on the body and is dangerous when it affects vital organs like lungs kidney, brain etc (CDC 2017).

8. Invasive Cervical Cancer

The lower part of the uterus which is the top of the vagina is termed the cervix. This type of cancer starts within the cervix and gradually spread to other body parts. It can be prevented if one regularly goes for examination and if detected at an early stage. Immediate actions must be carried, as it is known to be life threatening in HIV infected persons (CDC 2017).

9. Legionnaires Disease

Legionnaires disease is caused by any type of legionella bacteria. It is an atypical pneumonia spread by breathing in mist containing the bacteria or by aspirating contaminated water. Its symptoms include, high fever, muscle pain, nausea, vomiting, cough and shortness of breath. Older individuals, chronic lung disease and poor immune system are risk factors for infection (CDC 2017).

10. Cryptococcal Meningitis

This is caused by the fungi Cryptococcus. Meningitis is an infection of the brain and spinal cord lining, it can be fatal leading to coma and death. The risk of this disease is highest in individuals with CD4 count less than 100. This infection is one major HIV related opportunistic infection. Its early symptoms include fatigue, stiff neck, nausea, vomiting, fever and eye defects (CDC 2017).

11. Cytoisospora belli

This is type of parasite which primarily exist in epithelial cells of small intestine and develop in the cytoplasm. A fully matured Oocyst is spindled shaped and has two sporocyst. This parasite can live in immune competent individuals without causing much harm whereas in immunocompromised persons, symptoms can be fatal including extreme diarrhea, weakness, anorexia, abdominal pain, nausea and vomiting. Transmission is by ingesting contaminated food items (CDC 2017).

12. Coccidioidomycosis

Coccidioidomycosis is a pulmonary or hematogenous spread disseminated disease caused by Coccidioides. It usually occurs as a benign asymptomatic or self limiting respiratory infection. It is acquired by inhaling spore laden dust and. Its epidemics occur during heavy rains which promote growth of mycelia and spread by heavy wind. Progression is uncommon in in healthy people but more likely to occur in immunocompromised people (CDC 2017).

13. Cytomegalovirus disease

Cytomegalovirus is a common virus in same family as herpes virus. It is one of the common cause of mononucleosis like syndrome including fever, fatigue, malaise. It is transmitted by having direct contact with body fluids from infected person. It can cause blurred vision and blindness, painful swallowing, pneumonia, and diarrhea mostly in immunocompromised individuals. Healthy people do not experience any symptoms (CDC 2017).

2.2.6 Prevention and Treatment of HIV

HIV can be prevented by avoiding sexual contact with infected person, and sharp objects such as needles; blades etc should not be shared. Additionally, new prevention medicines such as pre-exposure prophylaxis (PrEP) and post exposure prophylaxis (PEP) have been discovered (William et al.,2017).

There are different drugs given to HIV infected persons and it is prescribed based on the HIV type, person's viral load and CD4 count and some other factors. This HIV drugs do not cure the infection but can help limit the replication of the virus (William et al., 2017).

2.2.7 Types of HIV

HIV-1 and HIV-2 are two different types of viruses. The first known type of virus isHIV-1and it accounts for the largest percentage of individuals affected by HIV-1 worldwide. The two types of HIV have different genetic makeup and this difference is used to identify the type of virus. HIV-2 is less predominant and its rate of disease progression is relatively slower and less infectious as compared to HIV. Its genetic makeup is a little over 50% different from HIV and found predominantly in Africa (Sharp et al 2011).

HIV-1 which is mostly associated with worldwide infection has 4 strains, grouped into M, N, O, P. The strain responsible for HIV epidemic across the globe is M strains, the other groups are less common. Group N and P are a few times seen in Cameroon while group O is mostly in central and West African countries. There are genetically 9 different subtypes with M group which are A, B, C, D, F, G, H, J, K, and circulating recombinant forms (CRF) when 2 types mix (Sharp et al 2011).

Table 2: Geographical distribution of HIV-1 subtypes (aidsinfo.nih.gov)

Box 1.4 Geographical distribution of HIV-1 Group M subtypes.

HIV-1 (Group M) subtype	Geographical location
A	Western and eastern Africa, Russia, Ukraine
B	Europe, the Americas, Japan, Australia, Korea, India, Singapore
C	Mainly southern (Botswana, Zimbabwe, Malawi, Zambia, Namibia, south Africa) and eastern Africa, India, Nepal, Malaysia, China, Scotland.
D	East and central Africa
F	Central Africa, south America, eastern Europe
G	Western and eastem Africa, central Europe
H, J, K	Widespread distribution in Africa (Burkina Faso, Mali, Nigeria, Ivory Coast, Gabon, Democratic Republic of Congo), southern Europe, Asia

2.3 RIG-1 like Receptors (RLRS)

The protein family DExD/H box is composed of closely related RIG-1 and MDA-5.They consist of an N-terminal tandem caspase activation and recruitment domain (CARD) fused to a DExD/H-box helicase domain (composed of Hel1, Hel2 and Hel2i) and the C-terminal domain (CTD) previously called repressor domain (RD). Stimulation of RIG-1 or MDA5 by viral RNA release the associated CARDs, which aggregate with K63 polyubiquitin chains and then activate the adaptor molecule MAVS. MAVS (also known as IPS-1, Cardif or VISA) recruits TBK-1, which phosphorylates IRF3 to induce transcription of typeI IFN genes.CTD carries the most important binding site for RNA ligand. RIG-1 appears to bind 5’ of dsRNA but only if it has 5’triphosphate while MDA5 attaches to long dsRNA. It is known that host RNA polymerase cleaves triphosphate when a new RNA is synthesized at the 5’ and the presence of this is the key

note for the RNA virus to replicate (Cui *et al.*, 2008). The third RLR relative LGP2 does not have CARDs and do not instigate type1 IFN (Schlee et al., 2013).

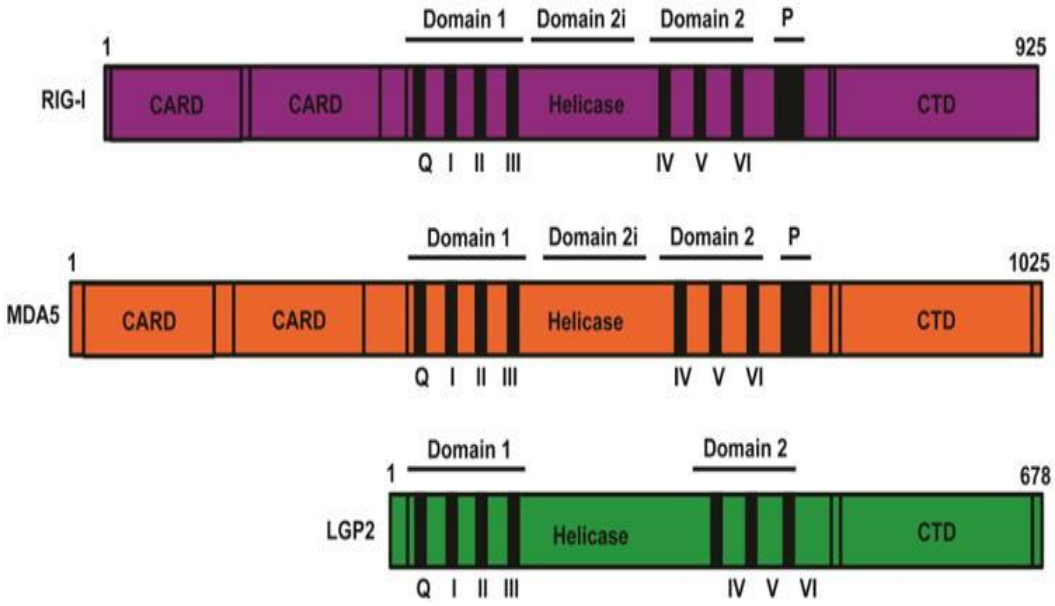

Figure 5: RIG-1 like Receptor Protein area composition. (Bruns et al., 2015)

Mainly, RIG-1 detects 5'triphosphorylated ends of RNA virus which is the point that indicates a non-self RNA. Removing 5'ppp totally from RNA stops signaling while modification of monophosphate or diphosphate on 5' weakens signaling. A study carried out on influenza virus confirmed one phosphate on 5' is enough to start a signaling but for complete RIG-1 signaling, 5'ppp is required (Wang *et al.,* 2015). RIG-1 dependent signaling activation is determined by sequence composition of RNA ligand. RIG-1 induce the expression of IFN in response to poly uridine motifs which carries interspersed C nucleotide (poly-u/uc) (Kurkani *et al.,* 2014). A study carried out by Saito *et al* shows that HCV poly-u/uc directs stable interaction with RIG-1 in a 5'ppp dependent manner that confers signaling activation. HCV RNA is recognized as non-self by RIG-1 through recognition of the poly-U/UC tract located in the viral genomic RNA,

thus defining the poly-U/UC tract as a PAMP motif of HCV. The HCV poly-U/UC tract is approximately 100 nucleotides in length and is essential for virus replication and viability. This explains that 5'ppp is not enough to trigger a RIG-1 signaling but additional motif works with 5'ppp to mark RNA as a RIG-1 ligand (Saito et al., 2008).

2.4. Present Summary of RLR-Interceded Anti-Viral Signaling

When there is a viral infection, the virus-derived RNA interaction leads to a change which exposes the CARDs, allowing it to associate with MAVS protein. MAVS acts as a polymeric signaling scaffold to assemble filamentous structures along the outer membrane when stimulated by RIG-1 CARD arrangement or MDA5. This MAVS scaffold allows the activation and assembly of signaling proteins and serine kinases. The kinase function is to activate the transcription factors which leads to the expression of diverse antiviral genes with the most important being INFβ,the major antiviral cytokine. Jak-stat signaling pathway aids the secreted INFβ act in a paracrine and autocrine way, thereby initiating antiviral effect on the cell infected and nearby cells which provide an effective barrier against the replication of the virus (Au-yeng et al., 2013) (Bruns et al., 2014).

The first recognized RLR protein is the RIG-1 and extensive studies on the RLR is based on findings from RIG-1,after which more discoveries on MDA5 and LGP2 are conducted and the model for RLR signaling is studied (Eisenacher *et al.*, 2012). In the cytoplasm, RIG-1 is available in an auto reserved compliance, in which the CARD attaches to Hel2i, making it inaccessible for downstream signaling interaction. Amid viral contamination, non-self RNA species aggregate in the cytoplasm.

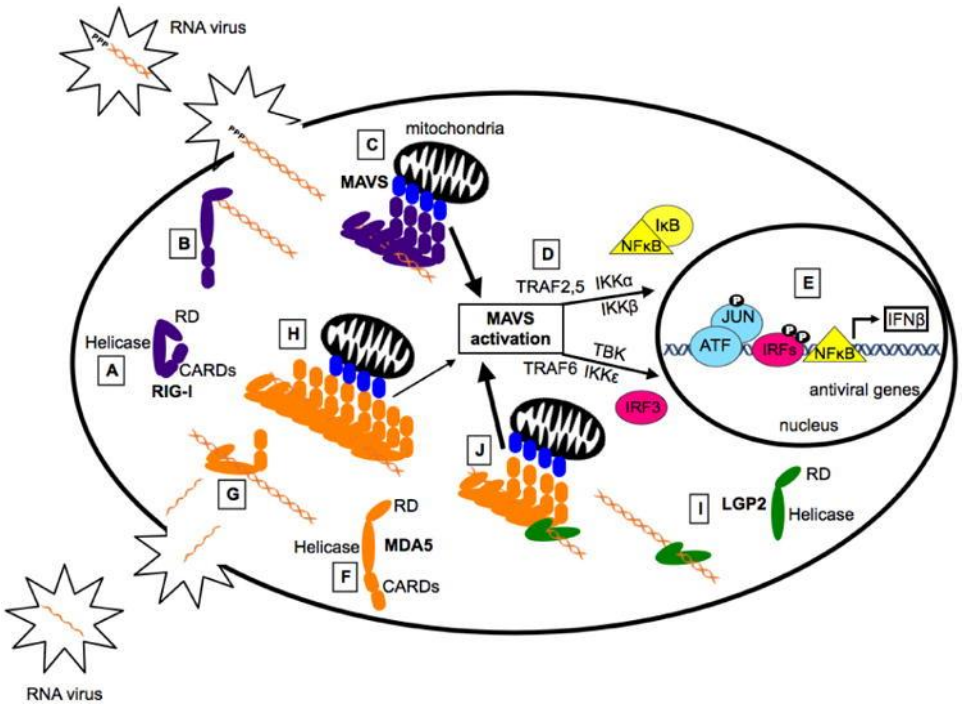

Figure 6: RIG-1 like Receptor Activation and Signaling (Bruns et al., 2015)

RIG-1 is activated as soon as the CTD connects with the suitable non self-5′PPP or 5′PP dsRNA ligand. Helicase domain is transformed into an active ATPase after the structural rearrangement. ATPase allows the forming of filamentous structures by the RNA. The arrangement of numerous CARDs triggers MAVS activation which leads to the assembly of TRAF 2, 5 and 6 signaling scaffold with their associated kinases TBK, IKKα, IKKβ, IKK*E* and leads to latent IRF and NF-kB transcription factors activation. Together with ATF/JUN, transcription factors gather on INFβ gene proximal enhancer, RNA polymerase is recruited to induce INFβ transcription with other antiviral genes. The presence of MDA5 in the cytoplasm in a flexible conformation allows the CARDs not to be inhibited, as it is consistent in several studies which demonstrated that only MDA5 over expression leads to stimulation of INFβ inducer. RNA end modification is not recognized by MDA5 unlike in RIG-1 but attaches slowly, internally along the dsRNA stem with low affinity. Additionally, MDA5 monomers bind the RNA forming

filaments after nucleation, stabilized by protein to protein interaction on adjacent MDA5 monomers. MDA5 CARDs arrangement stimulates MAVS, thereby leads to INFβ transcription and other antiviral mediators. LGP2 lacks CARD which is important for signaling but binds to dsRNA with high affinity not withstanding the end modification, or RNA length. It is demonstrated in in recent studies that LGP2 increases initial MDA5 RNA interaction rate and aids the stabilization of shorter MDA5 filament formation. These shorter stable filaments are formed due to LGP2 increasing the rate of MDA5-RNA interaction leading to greater MAVS activation and achieving antiviral signaling (Bruns, *et al.*,2015).

2.5. RIG-1 Polymorphism

Single nucleotide polymorphisms (SNPs) can majorly affect how people react to diseases, including viral infections. SNPs are DNA arrangement varieties which happen when a single nucleotide is modified. There are more than "4 million SNPs in the human genome", 200,000 of which happen in coding regions. (Misch*et al.*, 2008). SinceRIG-1 receptor is important for type 1 IFN release, and RIG-1CARD assumes a fundamental part within its action, a polymorphism in the CARD may affect RIG-1 work (Pothlichet et al., 2009).

A previous study carried out by collecting information from NCBI database in 2009, showed a minimum of 342 SNPs in human RIG-1 gene, among which 14 are in the coding region and only seven SNPs result in amino acid substitutions: $R_{7}C$, $S_{144}F$, $S_{183}I$, $T_{260}P$, $I_{406}T$, $D_{580}E$, $F_{789}L$ (Fig. 5A). An extra SNP involves thymidine addition at nucleotide position 845 of RIG-1 mRNA, which results in a frame shift and truncated RIG-1 protein. This mutant is thus characterized as P229fs since it includes the initial 229 nucleotides (rather than 925 buildups in the WT RIG-1 protein) followed by 4 additional residues (i.e. FRSV; Fig. 5B) and accordingly, does not contain the helicase and the RD areas (Pothlichet et al., 2009).

RIG-1 SNPs are positioned on different domains of the protein (Figure 6). It is discovered by sequence arrangement that mutations on $T_{260}P$, $S_{183}I$, $I_{406}T$, $D_{580}E$ affects

amino acids in all analyzed species while a mutation in $S_{144}F$, $R_{7}C$, $F_{789}L$ affect amino acids only in higher animals. After random selection in the healthy human population, frequency information on 3 SNPs was provided ($R_{7}C$, $D_{580}E$, $F_{789}L$)(Figure 6c). Frequency values reported were really low and could suggest a negative selection of the SNP due to their role in RIG-1 function. To gain more understanding on viral pathogenesis, host defense mechanism and detailed knowledge of structure and function of RIG-1, the functional role of non-synonymous SNPs in RIG-1 is studied. It was seen that mutations in $F_{789}L$, $R_{7}C$, $S_{144}F$, $S_{183}I$, $P_{229}F$, $T_{260}P$, $I_{406}T$, did not affect or change the expression and stability of RIG-1 protein analyzed by flow western blot and flow cytometry. Molecular weight was also similar except for $P_{229}FS$ which resulted in a truncated protein with its size similar to the 2CARD module (Pothlichetet al.,2009).

This study by Pothlichet *et al* determined if non-synonymous SNPs could alter RIG-1 induced antiviral signaling and proinflammatory pathways by using a functioning cell based assay to evaluate RIG-1 activation of INFβpromoter or IRF-3 or NF-kB dependent promoter. It is determined if the level of RIG-1 activation builds in the absence of HEK 293T of BEAS-2B cell stimulus, a moderate but highly significant INFβ expression and IRF activity but not NF-kB activity in wild type RIG-1 transfected cells was observed. In regard to P_{229}fs RIG-1, a salient constitutive NF-KB and INFβ activities is reported at a level well above that induced by the full length form of wild type RIG-1. Also, P_{229}Fs aids the endogenous inflammatory expression such as IL-8 and antiviral chemokine such as RANTES. $P_{229}FS$ SNP results in truncated constitutively active RIG-1. Therefore, a mutation on this SNP in an individual may continuously produce proinflammatory mediators and antiviral mediators in exaggerated amounts (Pothlichet et al., 2009).

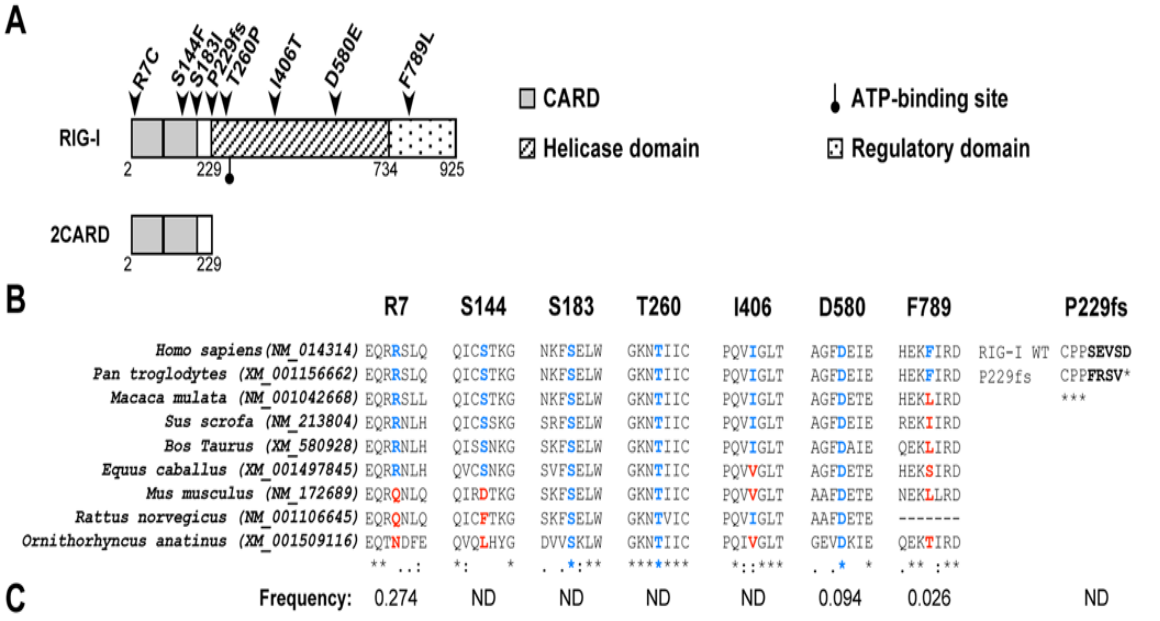

Figure 7: Hereditary changeability profile of human RIG-1 (Pothlichet et al.,2009).

(A) Schematic portrayal of significant spaces of RIG-1 (adjusted from. RIG-1non synonymous SNPs depicted in "NCBI SNP" record are demonstrated as R7C"(rs10813831), S144F(rs55789327), S183I(rs11795404), P229frameshift(fs) (rs36055726), T260P(rs35527044), I406T(rs951618), D580E(rs17217280) and F789L(rs35253851)".

(B) Arrangement of protein succession of RIG-1 SNPs commencing from individual to platypus, utilizing Cluster programming. Amino acids in the color red and blue relate to moderated and nonrationed buildups, separately.

(C) Recurrence of RIG-1 SNPs alleles. This last data was gathered from "NCBI SNP" database and alludes to the total of SNP containing alleles in equally heterozygous and homozygous persons for a given SNP (Pothlichet et al.,2009).

Mutation in $S_{183}I$ is seen to have a deleterious effect on antiviral activity of RIG-1 as this mutation drastically reduce INFβ and NF-kB responses when demonstrated using a replicative, intact Sendai and influenza virus. As R7C inhibits slightly RIG-1 signaling triggered by Sendai virus, D580E inhibits signaling response to dsRNA but not

in Sendai infection. From the effects mentioned, it was concluded that $S_{183}I$ SNP resulted in the strongest RIG-1 signaling inhibition. More priority is put into studying this SNP, and loss of function effect is determined by measuring IL-8 secretion and RANTES in stimulated HEK 293T cells supernatants. $S_{183}I$ inhibits RIG-1 induced signaling despite of its enhancing influence on the RIG-1complexe formation (Cheng *et al.,* 2007. Haussmann *et al.*,2001). Biochemical and structural modeling shows that mutation affects flexibility and hydrophobicity of CARD2 domain but does not affect its ligand binding activity. $S_{183}I$ was rather shown to influence abortive RIG-1 conformation, making it incapable for downstream signaling (Yoneyama et al., 2005, 2004).

2.6. Downbeat Regulators of the RIG-1-Like Receptor Signaling Pathway

In cases where there is a viral replication, negative regulation of antiviral signaling is important to reduce inflammation, prevent immune mediated pathology, and avoid continuous inflammation. Accordingly, RLRs are activated by the positive regulators, which is an important part of the innate antiviral reactions which functions to regulate and kill viral infections. On the other hand, negative regulators in this reaction is important to prevent spontaneous autoimmunity and prevent tissue damage in the situation that the RLR is hyper active and may lead to immune system disorders (Kato et al., 2005).

Negative regulators of RIG-1 and MDA5 act at multiple points in the RLR signaling pathway to inhibit the activation of RIG-1 and MDA5.Additionally, investigation of particular negative controllers is required to decide if each is able to do specifically averting such conditions through their direction of the RLR pathway. There are featured key negative controllers that specifically affect RIG-1, MDA5, and MAVS-mediated pathways. Through hereditary screens, protein–protein cooperation studies, and viral contamination of gene focused on mice and cells (Hipp *et al*., 2005. Nguyen *et al.*, 2016), a few pathways and organic procedures have been connected to controlling RLR signaling, including:

1. ubiquitination/de-ubiquitination,
2. protein trafficking,
3. autophagy,
4. inflammasome-related proteins,
5. Andrivalryfor restricting downstream signaling components inside cells.

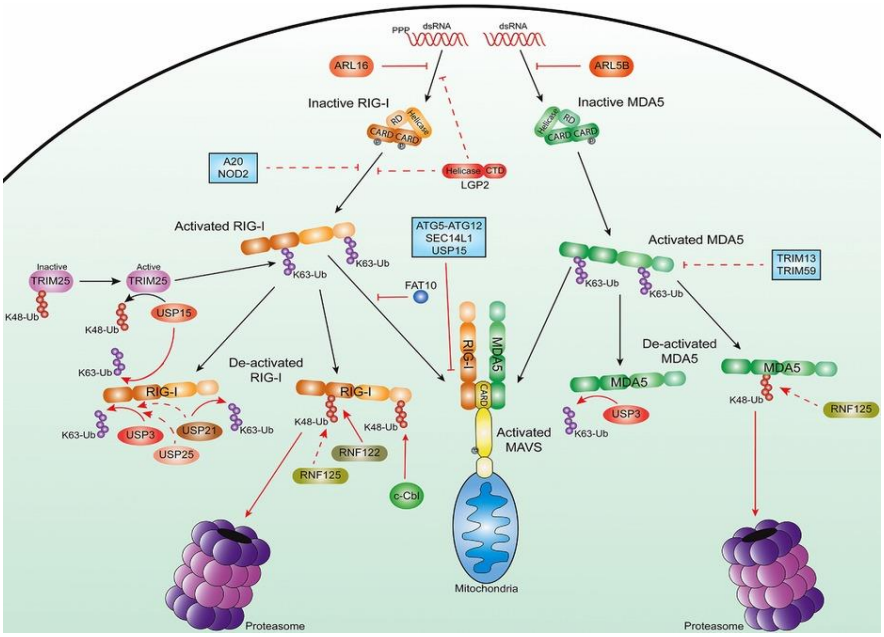

Figure 8: negative regulators of RIG-1 and MDA5 in RLR pathway (Quikie et al.,2017).

Ubiquitination and the opposing process of de-ubiquitination both produce important translational modification that regulate innate immune signaling. E3 ubiquitin ligases recognize and bind a target protein to catalyze the transfer of ubiquitin to a lysine residue on target protein. As opposed to ubiquitination, de-ubiquitination proteins bind target proteins and attaches the bond between ubiquitin and the target protein (Kato *et al.*, 2005). ADP ribosylation factors comprise a family of small GTP binding proteins involved in regulating many aspects of membrane trafficking and cytoskeletal reorganization. Molecular studies show that ARL functions in GTP dependent form and likely inhibits signaling by decreasing RIG-1

binding to stimulatory RNA ligands. Autophagy is a catabolic process which maintain cellular homeostasis through the turnover and recycling of unwanted and damage cellular material in lysosomal dependent manner (Lupfer *et al.*, 2015). In RLR signaling, it is critical for regulating inflammation and maintaining mitochondrial homeostasis (Quikie et al.,2017).

Table 3: negative regulators of RIG-1, MDA5 and MAVs function (Quikie et al 2017).

Protein	Target	Mechanism
LGP2	RIG-I MAVS	Binds and sequesters RIG-I ligands CTD blocks RIG-I activation Binds to an activation region within MAVS
RNF125	RIG-I MDA5 MAVS	Interacts with and ubiquitinates RIG-I, MDA5 and MAVS Proteasome-mediated degradation May lead to K48-linked ubiquitination
RNF122	RIG-I	Interacts with the N-terminal CARD region of RIG-I Promotes K48-ubiquitination of RIG-I K115 and K146 Proteasome-mediated degradation
c-Cbl	RIG-I	Recruited to RIG-I by SIGLEC-G and SHP2 K48-ubiquitinates C-terminal domain of RIG-I at position K813 Proteasome-mediated degradation
A20	RIG-I MAVS	E3 ligase activity dependent/DUB-independent RIG-I and MAVS not degraded Mechanism unknown
USP3	RIG-I MDA5	Binds to RIG-I and MDA5 Removes K63-linked polyubiquitin chains on CARDs
USP21	RIG-I	Associates with RIG-I Removes ubiquitin from RIG-I CARD region

USP25	RIG-I	Removes ubiquitin from RIG-I
USP15	RIG-I	Removes K63-polyubiqutin chain of RIG-I Interacts with RIG-I CARDs to prevent RIG-I-MAVS association
ARL16	RIG-I	Interacts with C-terminal region of RIG-I (aa 792–925) Functions in a GTP-independent manner May reduce RIG-I binding to ligands
ATG5-ATG12	RIG-I MAVS	Intercalates between MAVS and RIG-I CARDs to inhibit signaling Interacts with TUFM to promote autophagy
NOD2	RIG-I	Interacts with RIG-I through the 2nd N-terminal CARD region Mechanism unknown
FAT10	RIG-I	Independent of E3 ligase activity Associates with RIG-I Prevent translocation to mitochondrial membrane Inhibits formation of RIG-I-G3BP-containing antiviral stress granules
SEC14L1	RIG-I	Associates with RIG-I CARDs Competes with MAVS for binding to RIG-I
ARL5B	MDA5	Associates with MDA5 Reduces MDA5 binding to dsRNA
TRIM13	MDA5	Mechanism unknown
TRIM59	MDA5	Mechanism unknown

MARCH5	MAVS	Interacts with MAVS (CARDs and transmembrane domain) Promotes K48-ubiquitination of MAVS K7 K500 Proteasome-mediated degradation
SMURF2	MAVS	Interacts with MAVS Promotes K48-ubiquitination of MAVS Proteasome-mediated degradation
AIP4	MAVS	Associates with MAVS through the adaptor PCPB2 Promotes K48-ubiquitination of MAVS (site unknown) Proteasome-mediated degradation
NLRX1	MAVS	Nucleotide binding region interacts with MAVS CARDs Interacts with TUFM, ATG5-ATG12 and ATG16L1 May promote autophagy
EZH2	MAVS	Associates with MAVS CARDs Prevents RIG-I-MAVS interaction
PLK1	MAVS	Interacts with MAVS N-terminus (aa 180–280) Associates with MAVS CTD (aa 364–470) Prevents interaction of TRAF3 with MAVS
TSPAN6	MAVS	Associates with MAVS Possibly disrupts TRAF3-MAVS interaction (?)
PPMA1	MAVS	Dephosphorylates MAVS
PSAM7	MAVS	Interacts with MAVS (through CARD and transmembrane regions)

3. MATERIALS AND METHOD

3.1 Blood Sample Collection

The samples were collected in General Reference Hospital (GHR) in Bandundu Democratic Republic of Congo (DRC).A rapid test is run for confirmation. Confirmed samples were collected on a dried spot card and transferred to BM laboratory in Turkey for further molecular analysis.

3.2 Method of Collection on DBS Card

The collection card was opened without touching the filter paper surface and gently placed on a table. The lancet device was opened by gently twisting of the small protective tip. The fingertip was wiped with a medical wet wipe. The tip of the lancet device was placed on side of the finger and pressed against it until it clicks and lancet punctures the skin. First blood was swiped with gauze swap and the next blood drop was used to place in all circles on filter paper. Gauze was placed on the punctured area to stop bleeding and covered with plaster. Sample collection card was let dry for 2 hours then it was labeled, dated, placed in a foil bag with a drying agent and kept in a refrigerator. The same procedure was done for 42 HIV positive patients and 28 HIV negative patients in the DRC which will be used for the project. The filter papers were stored in foil bags containing silica gel at 4°C, and then transported from DRC to BM Laboratory Turkey for further molecular analysis.

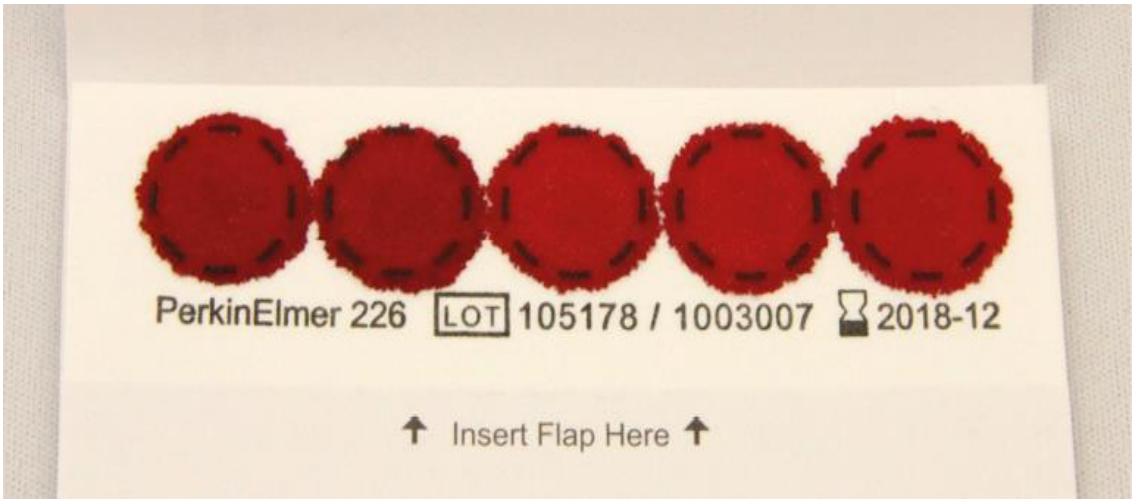

Figure 9: Dried blood spot card

3.3 Rapid Diagnostic Test (RDT)

RDT which is an immune chromatographic technique involving an antigen-antibody test was performed. The reaction of blood antigens with antibodies present in the dipstick in the presence of buffer solution was marked by acridine orange. This specific stain is used for visualization of the antigen-antibody reaction. RDT was performed according to the protocol stated by the manufacturer.

After capillary blood is collected, one drop of the blood was applied to the well on the test strip by a glass stick. One drop of buffer was added to the well. The test strip was left at room temperature for 15 minutes. The blood moved gradually to the reaction sites. A positive test result was obtained when the presence of red bands on both control and examination lines were pictured. Presence of a red band on the control line together with a lack of reaction on the test lines was interpreted as a negative test result. In case of an absence of the red line on the control band, the test was regarded as invalid.

3.4 Molecular Methods

In this study, dried blood samples on Whatman filter papers were used for molecular methods by PCR.

3.4.1 DNA Extraction

The fragments of filter papers having the dried blood samples were cut about 1 cm^2 and the pieces were placed into the 2 ml Eppendorf tubes. DNA extraction was conducted in accordance to the protocol stated by the manufacturer, EURx Gene MATRIX Bio-Trace DNA Purification Kit. Briefly, 350 µl Lyse BT buffer and 10µl of "Proteinase K" was added incubated for 60 minutes (mins) at 56°C. 350 µl of Sol BT buffer was added incubated for 10mins at 70°C. 180µl of 96-100 % ethanol was added and centrifuged for 2 mins at 14000 rpm. 600 µl of supernatant was transferred into the DNA binding spın column in the collection tube. The tube was then centrifuged for 1min at 12000rpm and the spin-column was removed. This spin column was placed back in the tube used for collection. DNA binding spin-column was removed and 500 µl of Wash BTXl buffer was then added into the spin column. The tube was centrifuged at 12000 rpm for 1min. DNA binding spin-column was removed and 500 µl of Wash BTX2 buffer was put in to the spin column. The tube was centrifuged for 2 mins at 12000 rpm. Then spin-column was placed in a new collection tube (2 ml) and 50µl of Elution buffer was added. The tube was left for 5 mins at room temperature. The turn segment was centrifuged for 1 min at 12000 rpm. DNA was collected and stored either at -2 or -8°C for short period and at -20°C for a longer period.

3.4.2 Nested Polymerase chain reaction (PCR) for RIG 1

Preparation of primers and PCR master mix:

PCR was used to amplify target points of R7C in the nucleotide position with forward primers of 5-ACCACCGAGCAGCGACGCA-3 and 5-GAGGACAACTTTGTACAAAAAGTTGGCATG-3and reverse primers of 5-TTTGGACATTTCTGCTCCATCAAATGG-3 AND 5-

GGGGAAACTTTGTACAAAGTTAGTTA-3. First distilled 250µl water was added to the dried primer to obtain 100pMol, 10pMol was taken out and used for nested PCR reactions. The rest was kept in stock for long term storage.

When primers were ready, preparation of the PCR master mix was started by labeling all microtubes for different samples, and the following was added together to make the master mix: 45,45µl PCR buffer, 45,45 µl magnesium chloride solution, 4,55µl dNTPs, 22,73µl forward primer, 22,73µl reverse primer, 7,27µl polymerase, 233,61µl PCR grade water to get a total of 381,78µl of solution. For each reaction 21 µl of master mix and 4µl of the sample temple was added into the tubes. This procedure was done in batches for 18 samples at a time for a total of 70 samples.

The following cycling conditions were applied for Nest 1 PCR reaction: 4mins for 94^0C, 1min at 55^0C, 72^0C for 1min and 72^0C for 4 minutes. The process is repeated to make a total for 40 cycles. A measured 3µl of the Nest 1 PCR product is filled into 20 µl of Nest 2 prepared master mix.

Nest 2 PCR conditions were indistinguishable from those of Nest 1 reaction except for the cycling conditions which is 20 cycles against the 40 cycles in Nest1. The PCR results of the Nest 2 reaction were monitored by agarose gel electrophoresis. The groups were pictured by recoloring with ethidium bromide and observed under UV light.

3.4.3 Nested Polymerase Chain Reaction (PCR) for HIV

The Same procedure is carried out just as in the nested PCR for RIG-1. The only difference is in the primer type used which is a primer used for the nested PCR for HIV. The HIV gag protein was amplified by nested PCR in all 70 samples. Outer primers pairs were 5-GAGAACCAAAGGGGAAAGTGACATAGCAGG-3 and 5-TAGAACCGGTATACATAGTCTCTAAAGGG-3and inner primers 5-ATAACCACCTATCCCAGTAGGAGAAAT-3 and 5-TTTGGTCCTTGTCTTATGTCCAGAATG-3 reverse primers (Sutthent et al., 2002).

3.4.4 Gene Sequencing

Following the copying of RIG-1 and HIV gene, the PCR items were sent to Macrogen Laboratory Netherlands for sequencing. Changes in the gene were analyzed by Sanger's method. DNA polymerases duplicate single-stranded DNA layouts by adding nucleotides to a replicating chain (expansion item). Chain expansion happens at the 3' end of an opening. Chain extension occurs at the 3'end of a primer. Deoxynucleotides added to the extension product are selected as pairs corresponding to the model. The extension product is developed by the formation of a phosphodiester bond between the 3'-hydroxyl of the primer and the 5'phosphate group of the incoming deoxynucleotide, and growth occurs in the 5'-3'direction. In the DNA sequencing method developed by Sanger, dideoxynucleotides are used as substrates. When dideoxynucleotides are incorporated to the 3' end of the growing chain, further extension is prevented, and the elongation is completed with adenine, guanine, cytosine, or thymine base (Sanger *et al.,* 1977). At theRIG-1 R7C position, homozygous sequence GG or AA is shown by the same nucleotide while heterozygous sequence is shown by 2 different nucleotide GA.

3.5 Statistical Analysis

The Finch TV and NCBI Blast hereditary programming were utilized for investigating the sequencing information. Diverse nucleotides on DNA chains were decoded by the Finch TV programming. Mutations related with HIV and RIG-1 were recognized by NCBI Blast program. SPSS Version 22 program was used for statistical analysis. Any significant association was supposed as p value<0.05 at 2-sided values. Genotyping and allele frequencies from the comparison between controls and patients' samples were performed by using Binary logistic regression. Odds ratios with 95% confidence intervals were calculated. With respect to demographic characteristics, the chi-square and student t test were applied.

4. RESULTS

4.1 Subjects Demographic Characteristics

A total number of 70 samples from individuals were used in this study; 42 were HIV positive patients and 28 HIV negative. Samples were obtained from the General Reference Hospital (GRH) Bandudu (DRC). The mean age for subjects in the patient group was 40.45 ± 12.5 (± standard deviation), while mean age for subjects in control group was 37.42 ± 15.97. There were 17(40.5%) male, and 25(59.5%) female subjects in HIV+ group while the control group composed of 9(32.14%) male and 19(67.9%) female subjects (Table 4.1). There was not any significant difference in age and gender of subjects between the two groups (p= 0.378 for age, p=0.480 for gender comparisons).

Table 4: Demographic characteristics of subjects included in the study.

characteristics	Patients n=42	Control n=28	P value
Age (mean ±SD)	40.45 ± 12.5 years	37.42 ± 15.97 years	0.378
Gender Male female	 17(40.5%) 25(59.52%)	 9(32.14%) 19(69.9%)	0.480

SD: standard deviation.

4.2 Relationship between RIG-1 R7C polymorphism and HIV Susceptibility.

In this study, the frequency of subjects with wild-type genotype GG in HIV+ and HIV- patient groups were 61.9% (n=26) and 50% (n=14), respectively. The frequency for heterozygous genotype GA in patients group was 33.3% (n=14), and control group was 42.9% (n=12). There was not any statistical difference in GA genotype frequency between the two groups (p=0.557, OR=1.86, 95% CI=0.23-14.31). The frequency of subjects with genotype AA in patient group was 4.76% (n=2) while that in control group

was 7.14% (n=2). There was not any significant difference in genotype AA frequencies between the two groups (p=0.886, OR=1.67%, 95% CI=0.142-9.59). In addition, there was not any significant difference observed both in dominant model (p=0.453, OR=2.272, 95% CI=0.266-19.405) recessive model (p=0.275, OR=0.373, 95% CI=0.064-2.190) and allele model (p=0.192, OR= 1.933, 95% CI=0.719-5.202). The genotype distributions conformed to Hardy Weinberg equilibrium test in patient and control group (p= 0.948 and p=0.789, respectively) (Table 4.2).

Table 4.2. Genotype and allele frequencies of RIG-1 R7C polymorphism in HIV patients and control groups.

Genotypes	Patients n=42 (%)	Controls n=28 (%)	Odd ratio(95% CI)	P value
GG	26 (61.9%)	14 (50%)	1.00[ref]	-
GA	14 (33.3%)	12 (42.9%)	1.856 (0.236-14.642)	0.557
AA	2 (4.76%)	2 (7.14%)	1.167 (0.142-9.586)	0.886
Dominant model	16 (38.1%)	14 (50%)	2.272 (0.266-19.405)	0.453
Recessive model	2 (4.76%)	2 (7.14%)	0.373 (0.064-2.190)	0.275
G allele	66 (78.57%)	40 (71.4%)	1.00[ref]	-
A allele	18 (21.4%)	16 (28.57%)	1.933(0.719-5.202)	0.192

Dominant model compares GG + GA versus AA.

Recessive model compares AA versus GA + GG.

5. DISCUSSION

This study is to determine the correlation of RIG-1 R_7C and HIV-1 susceptibility in a Congolese population. The Human RIG-1 gene is a pivotal cell receptor which identifies various RNA viruses. A mutation in the gene may deteriorate its function and thereby allowing the progression of an infection. The human RIG-1 gene contains a minimum of 324 SNPs among which 14 are found in coding regions but only 7 of these (that is R_7C, $S_{144}F$, $S_{183}I$, $D_{580}E$, F789L, $T2_{60}P$, $I_{406}T$) result in amino acid substitution. Understanding the impact of SNP on RIG-1 function may grant us better understanding on disease progression and host defense mechanisms(Pothlichet *et al.,* 2009). In our experiment, we investigated if human RIG-1 R_7C SNP is associated with HIV susceptibility.

This study to our knowledge is the first to be conducted in a Congolese population on determining the correlation between HIV and RIG-1 R_7C polymorphism. According to our results, there was no significant correlation between RIG-1 R_7C SNP and individuals' susceptibility to HIV, and therefore, the polymorphism may not be a contributing factor to the susceptibility to HIV infection at least in a Congolese population.

In this study, RIG-1 R_7C (rs10813831) gene mutation was investigated by sequencing. The result obtained shows the genotype present in each individual SNP. The individuals were characterized based on their demographic properties. Age and gender were evaluated for both control and patient samples, and no significant difference was reported between the two groups. The frequency obtained of patients with wild type genotype (G/G), heterozygous genotype (G/A) and homozygous genotype (A/A) was 61.9 %, 33.3% had 4.76%, respectively, while for the control group, it was 50%, 42.9% 7.14%, respectively. Statistical analysis revealed no significant difference between the two groups. Furthermore, allele, dominant and recessive models also revealed no significant difference. Therefore, our study shows that there was no relationship between the SNP investigated and HIV susceptibility.

Our study was however limited as some factors hindered our ability to get more samples from population in DRC, therefore a low sample size of 70 from individuals from Bandudu Reference Hospital was obtained for both patient and control subjects. Therefore, our study was not able to reach a precise conclusion that is applicable for the whole Congolese population. However, due to this weakness, more study is recommended in the same area to reach a precise conclusion in the population, by using especially large cohorts and putting into consideration different demographic characteristics, antiretroviral drugs administered to each individual, patients not on any form of treatment, ethnic background and genetic linkage disequilibrium.

The human innate immune immunity has variation in genes, which is the reason individuals have different ways they react to infections. Even though, our study was not able to show any association between RIG-1 R_7C polymorphism and HIV susceptibility, this may be because of other genetic polymorphisms that have stronger effect on HIV acquisition (such examples include HLA-B57 and HLA-B27). Plus, according to a study carried out by Misch *et al* 2008, a mutation in TLRs 4, 5 and 2 does correlate with the development of infections. A study by Bochud *et al* observed the two TLR9 SNPs to be more frequent in HIV-1 rapid progressors than in other patients, therefore the rapid progression of HIV-1 infection was associated with TLR9 polymorphism (Bochud *et al.*, 2007). Future studies can be conducted in order to examine the association of other TLR SNPs with HIV susceptibility. Our data is in correlation with a previous study that showed HIV protease deteriorates RIG-1 in the lysosome and makes it possible for HIV to escape from the RIG-1 suppose signaling (Papon *et al.,* 2009). Protease enzyme is encoded within pol gene and is essential for virus replication, it cleaves to newly synthesized proteins Gag and Gag pol to develop mature components of the HIV which can be infectious. A mutation in the protease active site disrupts the virions ability to replicate and infect more cells (Solis *et al.,* 2010). Several virus (such as influenza virus, Ebola, vaccinia virus and HCV) escape the innate antiviral response by using the viral protein to antagonize the RIG-1 pathway at different levels (Bowie *et al.,* 2008). HIV protease is particularly known to disrupt RIG-1 signaling by directly degrading RIG-1 or

by disrupting the cleaving of adapter protein MAVS (Meylan *et al.*, 2005). Despite the fact RIG-1 is triggered by the presence of viral RNA, the antiviral gene expression is inhibited by HIV protease enzyme. Protease causes a depletion of endogenous cytoplasmic fraction of RIG-1 and further distributes to membrane lysosomal compartments (Papon *et al.,* 2009). In support of this conclusion, Solis *et al* demonstrated a serial transfection of RIG-1 using two proviral clone,one with a defective protease and a wild type proviral clone. The intracellular function of RIG-1 was decreased during the experiment for wild type provirus but not by the protease deficient provirus. In relation to this study, RIG-1 signaling may have been deteriorated by the action of protease enzyme, thereby masking the effect of RIG-1 in HIV susceptibility.

The susceptibility of HIV and R_7C polymorphism in our study was not correlated and the polymorphism on otherRIG-1 component might be a factor. The RIG-1 CARD plays an important role in sending signal down-stream. Shigemoto *et al* studies proved that mutation on non synonymous SNPs leads to the inactivation tandem CARD. Mutation of RIG-1 and the deletion of CARD reduced response to 5'ppp (Shigemoto et al., 2009). A study by Hu *et al* shows the association between RIG-1 signaling and INFβ expression. The response of human DCs to infection is strongly related to the level of RIG-1 expression and the CARD modifies the common functional polymorphism. The change in RIG-1 CARD is a factor which affects the response of innate immunity in the case of an infection. RIG-1 function is altered in the CARD (Hu *et al.,* 2010). Study by Holm and Sander showed that mutation on C terminal domain leads to inhibition of RIG-1 signaling. Also, viral components confer an ATP dependent conformational change prevents C terminal CARD from interacting with MAVS. For further studies, it is recommended to analyze the functional state of the RIG-1 gene in individual HIV samples and to ascertain that the effect of other polymorphism does not interfere with the finding.

A study carried out by Pothlichet *et al* identified the impact of RIG-1 polymorphism on two variant which affect the signaling pathway for RIG-1. The identified polymorphisms, $P_{229}F$ and $S_{183}I$, showed that there was an interruption in RIG-1 activity. It was confirmed that $S_{183}I$ has an inhibitory effect on RIG-1 MAVS adaptor and this is backed up in recent studies which confirms MAVS association with RIG-1 is not enough to induce gene expression (Lad *et al.,* 2008). It was determined that serine 183 is an important component that helps in communicating CARD modules with MAVS, therefore a polymorphism on this region can deteriorate its effect. Furthermore, more research is recommended on RIG-1 polymorphism and host factors which modulate the innate immune reaction against HIV infection.

In our study, RIG-1 R_7C polymorphism shows no association to susceptibility of HIV. Factors such as viral load of individual or the stage of virus infection and genetic background of the Congolese population, the virus subtype, also the advancement of the host immune deficiency may be a reason. As mentioned earlier, the sample size could be a factor, as the number of samples partially determine the strength of association in disease and gene mutation (Zupin et al., 2018).

This study also aimed to determineHIV-1 subtype, however due to technical problems, we could not differentiate between HIV-1 and HIV-2 infections (data not shown). There was a problem with viral genome isolation which was to be sent for sequencing and this might be because the samples were too old. Another reason could be primers not being so effective. Other primers can be used for further studies.

6. CONCLUSION

In this study, the correlation between RIG-1 R_7C and HIV susceptibility was investigated in a total number of 70 individuals; 42 patients and 28 controls from DRC. Samples were collected from the General Reference hospital Bandudu.

Comparison of patients and control group subjects regarding age and gender, revealed no significant difference. Polymorphism in individual sample was determined by Nested PCR and subsequently DNA sequencing. According to the results obtained from statistical analysis there was no significant difference in all genotype frequencies between the two groups. This suggests that there was no relationship between HIV susceptibility and RIG-1 R_7C polymorphism in DRC population.

This study was however limited with low sample size, and therefore more studies are recommended to determine the effects of RIG-1 R_7C polymorphism in persons infected with HIV in DRC. For further studies to have precise results, it is recommended to distinguish between HIV-1 and HIV-2 infection from the samples collected.

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