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Association of Human Herpes Virus 6 (A , B) with IL_17 and P14 genes polymorphism in Patients with Acute Myeloid Leukemia

A Thesis

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بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

(وَقَالَ الَّذِينَ أُوتُوا الْعِلْمَ وَالْإِيمَانَ لَقَدْ لَبِثْتُمْ فِي كِتَابِ اللَّه الى يوم البعث فهذا يوم البعث ولكنكم كنتم لاتعلمونِ)

صدق الله العلي العظيم

سورة الروم- الآية56

Dedication

I dedicated this study Report

To the purest heart to my role model, and my ideal in life; He is the one who taught me how to live with dignity and loftiness… My dear father

To the heaven of God on earth, to the bridge that ascends me to heaven, to my ideal… My mother

To my dear companion, my second half, who encourages me to innovate and excel…My husband

To the eyes and heartbeat You were and still my support, lean and fame in all stages of life. ...to … My brother & sisters

To the endless love and the secret behind my existence… My children

And special thanks to my sister, my friend and my companion, and she was my biggest supporter

Dr.maryam sabah

To anyone who helped me and was by my side throughout the study period, to everyone who was happy for my joy and prayed for me with all his heart, especially my husband's mother

To myself thank you.you are ahero

Worood 2023

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Last but not least thanks to all those who helped me by every way, even if it was a good word

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SUMMARY

Summary

Human herpes virus-6 (HHV-6) was first isolated from peripheral blood leucocytes of patients with lymphoproliferative disorders, including lymphoma and leukemia. Th17 cells are blamed for being accused in the pathogenesis of acute myeloid leukaemia. Th17 cells are CD4+ cell subtype. They produce IL-17A and IL-17F. Genetic polymorphisms leading to defects in the IL-17-axis may alter the ability to elicit effective immune responses.

This study was aimed to determine the percentage of Human Herpes Virus-6A&B (HHV-6A&B) and whether polymorphisms at ***IL-17 rs2275913 as well as P14*** **rs3731249** locus modulate the risk of developing Acute Myeloid Leukemia (AML) of a group of Iraqi population sample.

A case control study including 200 blood specimens (100 cases Acute Myeloid Leukemia and 100 apparently healthy control(AHC)); (57 males and 43 females with AML ;While AHC 45 males and 55 females) who were recruited from different Teaching Hospitals in Mid-Euphrates and Baghdad Governorates of Iraq. Studied groups were had different ages that range from 2-80 years. The study was carried out at the department of Microbiology, College of Science, University of Babylon during the period between October 2022 and february 2023.

Five ml of blood sample (2ml EDTA Blood and 3ml in gel tube) were collected and then viral genome and total DNA extraction were obtained and stored at -20°C/-80°C till used.

HHV-6A&B detection using PCR. Other routine investigations were done for ***IL-17 rs2275913and P14*** **rs3731249** gene polymorphism by sequencing; serological tests for ***IL-17A*** marker in AHC and patients suffering from AML.

• The obtained results are summarized as follows:

The mean age of the patients with Acute Myeloid Leukemia (**39.3 ±12.41** years).While, the mean age of apparently healthy control (AHC) was (**37.9± 11.17**) years.

•In Acute Myeloid Leukemia group, the most affected age group were from 21-40 years were constituting (48%), followed by the age group of 41 – 60 years (25%) and the lowest group of Acute myeloid leukemia was the age group of 2-20 and of 61 - 80 years were constituted 12%and 15%, respectively.

• Fifty –seven percent of AML were males, while the rest 43% were Females. Regarding the apparently healthy control group, the percentage of females was also higher (55%:55) than the percentage of males (45%: 45).

•According to PCR, only 14% (14 out of 100) of the AML specimens results are positive for HHV-6A genome detection, while 28 out of 100 (28%) are negative for HHV-6A gene detection. In addition, 16% (16 out of 100) of the AML specimens have HHV-6B gene, while 26 %(26 out of 100) specimens showed negative results for HHV-6B genome detection.

•The highly percentage of sex of patients with AML that have positive HHV-6 A-DNA PCR results was males 64.3% (9 out of 14 cases) followed by females 35.7% (5 out of 14 cases).While, the highly percentage of sex of patients with AML that have positive HHV-6 B-DNA PCR results was males 62.5% (10 out of 16 cases) followed by females 37.5% (6 out of 16 cases).

•The results of ***IL-17 rs2275913*** *polymorphism* showed that DNA polymorphism distribution were DNA polymorphism distributions according to A\A; A\G; A\C ; G/C andC/C were 23.3% ; 26.7% ;33.3% ; 16.7% and 0% respectively in patients

with AML and 35% ; 40%;15%; 10% and 0% respectively in AHC group.. The difference in frequency of genotype distribution of the polymorphism between patients and controls was statistically significant**.**

•New recording for ***IL-17 rs2275913*** *polymorphism* in gene bank NCBI and American bank ACCESSION NUMBERS was recorded LC770961 ; LC770962

•The results of ***P14 rs3731249 polymorphism*** showed that DNA polymorphism distribution were DNA polymorphism distributions according to A\A; C/G ;A/C; C/T ;T/G;T/A and C/Cwere 20%% ; 30 % ;36.7% ; 6.7%;5%;5% and 0% respectively in patients with AML and 45% ; 35%;10% ;10%;0% and 0% respectively in AHC group. The difference in frequency of genotype distribution of the polymorphism between patients and controls groups was statistically significant**.**

•New recording for ***P14 rs3731249*** in gene bank NCBI and American bank ACCESSION NUMBERS was recorded LC770963 ; LC770964

•The mean of serum IL-17 concentration for AHC and patients with AML groups were 8.00 ±0.31pg./ml and 14.00 ±0.59 pg./ml .respectively.

• A strong positive relationship (with highly significant correlation) was found between HHV-6A&B and SNP ***IL-17 rs2275913*** in AML (r = 0.968, P = 0.007). Similarly, there is a strong positive relationship (with highly significant correlation) between HHV-6A&B and IL-17 in AML (r = 0.984 , P = 0.008).

•Also A strong positive relationship (with highly significant correlation) was found between HHV-6A&B and SNPs of ***IL-17 rs2275913*** as well as IL-17 concentration according to ages patients who AML (r=0.855, P= 0.001) ; (r=0.788, P= 0.009) and (r=0.739, P= 0.004), respectively. However, there are no significant correlations

among HHV-6A&B and SNPs of ***IL-17 rs2275913*** according to the sex of study population**.**

• In view of the relatively numbers included in study we concluded , the present results indicate the possibility that HHV-6A&B and ***IL-17 rs2275913*** as well as ***P14 rs3731249*** **genes** polymorphism may play a roles in the tumor biology of Leukemia especially (AML) and may contributed to their development.

Chapter Five: Discussion

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List of Abbreviations

Symbol	Description
AML	Acute myeloid leukemia
AHC	Apparently healthy control
CD4	Cluster of differentiation 4
CD8	Cluster of differentiation 8
DNA	Deoxy ribose nucleic acid
ELISA	Enzyme linked immunosorbent assay
HHV-6	Human Herpes Virus 6
HLA	Human leukocyte antigen
ICTV	International committee on taxonomy of viruses
IgG	Immunoglobulin G
IgM	Immunoglobulin M

IL-17	Interleukin 1
INFs	Interferons
MHC	Major histocompatibility complex
mRNA	Messenger Ribose nucleic acid
NK cell	Natural killer cell
OBP	Origin binding protein
ORF	Open reading frame
PCR	Polymerase chain reaction
RNA	Ribose nucleic acid
RT PCR/ qPCR	Real Time Polymerase chain reaction/quantitative
Th2	T-helper 2
TNF-α	Tumor necrosis factor alpha
VTM	Viral transport media
WHO	World Health Organization
NCBI	National Center for Biotechnology Information
IL1RN	interleukin -1 receptor antagonist
EDTA	Ethylenediaminetetraacetic acid

HIV	Human Immunodeficiency Virus
AIDS	Acquired Immunodeficiency Syndrome
EBV	Epstein Barr Virus
GI	Gastroenteritis
CNS	Central Nervous System

CHAPTER ONE

Introduction

1. Introduction

Acute myeloid leukemia (AML) is the most common leukemia among the adult population and accounts for about 80% of all cases. It is characterized by clonal expansion of immature "blast cells" in the peripheral blood and bone marrow resulting in ineffective erythropoiesis and bone marrow failure. With recent advancements in the management guidelines, the cure rates have increased up to 15% in patients older than 60 years and about 40% in patients below 60 years of age. Despite advancements in therapeutic regimens, the prognosis remains very poor in the elderly population (Naymagon *et al*.,2019).

Depending upon the etiology, genetics, immune-phenotype, and morphology, there are different classification systems for AML.The most common risk factor for AML is myelodysplastic syndrome. Other hematological disorders that increase the risk of AML include myelofibrosis and aplastic anemia.Several congenital disorders like Down syndrome and Bloom syndrome also increase the risk of AML, which tends to present in the early 20s. Environmental exposures like radiation, tobacco smoke and benzene are also risk factors for AML .Finally, previous exposure to chemotherapeutic agents as well as oncogene viruses are also a risk factor for AML (Liu *et al*.,2019).

Human herpesvirus 6 (HHV-6) was the sixth human herpesvirus discovered. It belongs to the β-Herpesvirinae subfamily. Although horizontal transmission is considered to be the main route of HHV-6 infection, it can be genetically transmitted from parent to child as inherited chromosomally integrated HHV-6 (iciHHV-6) (Miura *et al*.,2018).

The complete HHV-6 genome is integrated into every nucleated cell of an individual with iciHHV-6. Extremely high copy numbers of HHV-6 DNA can be

detected in clinical specimens containing nucleated cells, which can lead to a misdiagnosis of active viral infection. Theoretically, a parent with iciHHV-6 has a 50% chance of transmitting the integrated HHV-6 genome to the next generation (Gaccioli *et al*.,2020)

HHV-6 can integrate into human chromosomes, resulting in genetic transmission from parent to child. Individuals of either sex with inherited chromosomally integrated human herpesvirus 6 (iciHHV-6) harbor the virus in every cell. Viral reactivation from the integrated HHV-6 genome can occur in pregnancy (Miura *et al*.,2020).

Unfortunately, serological testing and PCR analysis of blood are inadequate in determining the role of HHV-6 in leukemia, and these methods fall short in differentiating between pathological and background HHV-6 infections. (Eliassen *et al*.,2018; Voigt *et al*.,2021). As blood is a relevant sample used in evaluating leukemia, this presents another hurdle; latent HHV-6 or HHV-6 undergoing low-level reactivation is occasionally present in healthy individuals. For these reasons, although many studies have made preliminary investigations into HHV-6 in several types of leukemia such as AML, forward progress of our understanding of the virus in these cancers has been stunted by the reliance upon blood PCR and serological analysis (Eliassen *et al*.,2018; Voigt *et al*.,2021).

Single nucleotide polymorphisms (SNPs) are the most common type of genetic variants and their functions have been gradually uncovered in many fields of biology, especially in human diseases. The roles of inflammatory or immune-related SNPs have been investigated in AML patients and susceptibility, prognosis and survival-related SNPs were determined (Nefzi *et al*., 2016).

IL-17 stimulates expansion of myeloid progenitors and proliferation of mature neutrophils. IL17 is an indirect antigenic factor. It enhances tumor micro-vessel e (SNPs) can alter gene functions and protein expression or function thus influencing cell proliferation increasing cancer risk.The IL-17A rs2275913; G-197A and IL-17F rs763780; A7488G polymorphisms are the most common loci associated with IL-17 activity and cancer. IL-17A rs2275913; G-197A polymorphism in the promoter region of IL-17 cytokine is related to higher expression of the IL-17A and production of high levels of IL-17, which in turn upregulates IL-17- mediated immune responses, while IL-17F rs763780; A7488G polymorphism affects the coding region of the gene leading to up-regulation of IL-17F.Thus dysregulated IL-17A and IL-17F production can result in excessive pro-inflammatory cytokine expression and chronic inflammation with subsequent tissue damage and pro-tumorigenic effects *(Rania et al.,2020).*

Tumor suppressor called P14ARF is activated by mitogenic arousal, where in enhanced MYS activity is mediated by RAS activates P14ARF, which in turn prevents MDM2 from performing its role. Additionally, a P53/MDM2 feedback loop exists in which P53 can activate the MDM2 protein, while MDM2 can also deactivate P53.The essential p16INK4A and p14ARF which control the cell cycle are a protein that the CDKN2A encodes; 9p21.3 (cyclin-dependent kinase inhibitor 2A) gene, and they also play a crucial part in programmed by the tumor-suppressive mechanisms,cell die functions. Therefore, p16INK4A and p14ARF tumor suppressor expression and activity are diminished with deletion, mutation, or genetic polymorphisms of CDKN2A, which results in a dysregulation of cell proliferation and the emergence of cancer(Mahla *et al*.,2022).

1.2. Aim of Study:-

Considering all these points, this research study was designed to detection of Human Herpes Virus-6 A&B **(HHV-6 A& B), IL-17 A rs2275913** and ***P14* rs3731249** genes polymorphism from patients with Acute Myeloid Leukemia (AML) through achieving the following Objectives:

1- Determine the percentage of HHV-6 A& B in blood specimens that range from apparently healthy persons to patients with AML by PCR.

2- Estimation of IL17A rs2275913 and *P14* rs3731249 genes polymorphism in patients With AML and AHC groups by sequencing.

3- Compared levels serum concentration of IL-17 A in healthy individuals to patients with AML by ELISA.

4- Find the association between these of IL-17 A rs2275913 ; *P14* rs3731249 gene polymorphism and HHV-6 A& B among study population .

CHAPTER TWO

Literature RevieW

2. Literature Review:

2.1. Acute Myeloid Leukemia (AML)

2.1.1. Introduction

Acute myeloid leukemia (**AML**) is a cancer of the myeloid line of blood cells, characterized by the rapid growth of abnormal cells that build up in the bone marrow and blood and interfere with normal blood cell production. Symptoms may include feeling tired, shortness of breath, easy bruising and bleeding, and increased risk of infection. Occasionally, spread may occur to the brain, skin, or gums. As an acute leukemia, AML progresses rapidly, and is typically fatal within weeks or months if left untreated **(**Bain *et al*.,2019**).**

2.1.2. Risk Factors

Most cases of AML do not have exposure to any identified risk factors. However, a number of risk factors for developing AML have been identified. These include other blood disorders, chemical exposures, ionizing radiation, and genetic risk factors. Where a defined exposure to past chemotherapy, radiotherapy, toxin or hematologic malignancy is known, this is termed secondary *AML* (Maleki *et al*.,2021).

I. **Other blood disorders**

Other blood disorders, particularly myelodysplastic syndrome (MDS) and less commonly myeloproliferative neoplasms (MPN), can evolve into AML; the exact risk depends on the type of MDS/MPN. The presence of asymptomatic clonal hematopoiesis also raises the risk of transformation into AML (Khoury *et al*.,2022).

II. Chemical exposure

Exposure to anticancer chemotherapy, in particular alkylating agents, can increase the risk of subsequently developing AML. Other chemotherapy agents, including fludarabine, and topoisomerase II inhibitors are also associated with the development of AML; most commonly after 4–6 years and 1–3 years respectively. These are often associated with specific chromosomal abnormalities in the leukemic cells. Other chemical exposures associated with the development of AML include benzene, chloramphenicol and phenylbutazone (Sachiko, 2012).

III. Radiation

High amounts of ionizing radiation exposure, such as that used for radiotherapy used to treat some forms of cancer, can increase the risk of AML. Historically, survivors of the atomic bombings of Hiroshima and Nagasaki had an increased rate of AML, as did radiologists exposed to high levels of X-rays prior to the adoption of modern radiation safety practices (Allodji *et al.*,2021).

IV. Genetics factor

Most cases of AML arise spontaneously, however there are some genetic mutations associated with an increased risk. Several congenital conditions increase the risk of leukemia; the most common is Down syndrome, with other more rare conditions including Fanconi anemia, Bloom syndrome and ataxia-telangiectasia (all characterised by problems with DNA repair), and Kostmann syndrome (Shahrabi, *et al*.,2020).

2.1.3. Pathophysiology

Acute myeloid leukemia is characterized by mutations of the genes involved in hematopoiesis. These mutations result in a clonal expansion of undifferentiated myeloid precursors (blasts) in the peripheral blood and bone marrow resulting in ineffective erythropoiesis and bone marrow failure. Recent studies also revealed that it could arise from a series of recurrent hematopoietic stem cell genetic alterations which get accumulated with age . In most cases, AML appears as *de novo* in a previously healthy person. The exact cause of genetic mutations is unclear, but few risk factors include exposure to radiation, chemotherapeutic agents, and smoking. AML can also evolve from myeloproliferative disorders (MPD), myelodysplastic syndrome (MDS), paroxysmal nocturnal hemoglobinuria, and aplastic anemia. Familial causes of genetic mutations should also be considered (Schmid *et al*.,2019).

Acute myeloid leukemia is a highly heterogeneous disease with a variable prognosis. It can result from genetic mutations, chromosomal translocations, or changes in molecular levels. About 97% of the cases have been studied to have genetic mutations. Despite its heterogeneity, it can be categorized into favorable, intermediate, or adverse-risk groups based on cytogenetics. The prognosis within these categories varies widely .The chromosomal translocations t, t, or inv have a favorable prognosis with 3-year overall survival (OS) rate of about 66% and 33% in patients younger than 60 and older than 60 years of age respectively. People with t, monosomy 5 or 7, and normal cytogenetics (CN-AML) have an intermediate risk. A high risk of treatment failure and death was noted in people with t, inv (Medeiros *et al*.,2019).

Acute myeloid leukemia patients About 25% to 30% of have Nucleophosmin 1 (*NPM1*) mutations. This is the most common mutation found in AML and has a female predominance. Clinically, the mutation has monocytic morphology and in the absence of FMS-like tyrosine kinase 3 or *FLT3-ITD,* predicts favorable OS. *NPM1* mutations are chemosensitive to intensive chemotherapy in both young and old patients. It is associated with other recurrent genetic abnormalities such as *FLT3-ITD* (40%), *FLT3-TKD* (10% to 15%), and *IDH* mutations (25%) (Niu *et al*.,2019).

FLT3 is strongly expressed in hematopoietic stem cells with important roles in cell survival and proliferation. Mutations involving the Internal tandem duplications (ITD) and the tyrosine kinase domain (TKD) of the FLT3 gene have been found in 20% of AML cases and 30% to 45% of CN-AML patients. Both the mutations activate FLT3 signaling, promoting blast proliferation . Patients with FLT3 mutations can have severe leukocytosis. FLT3-ITD mutations have been associated with an increased risk of relapse. Tyrosine kinase inhibitors (TKI) are being tested in FLT3 mutated AML patients. Unfortunately, when used alone, TKIs showed only a transient reduction of blasts, and even if initially effective, the subsequent acquisition of secondary mutations induces resistance over time (Medeiros *et al*.,2019).

Runt-related transcription factor (RUNX1) is an essential component of hematopoiesis. It is also known as AML1 protein or core-binding factor subunit alpha-2 (CBFA2). RUNX1 is located at chromosome 21 and is frequently translocated with the ETO (Eight Two One)/RUNX1T1 gene located on chromosome 8q22, creating an AML-ETO or t (8;21)(q22;q22) AML which is seen in about 12% of AML cases . They are commonly associated with trisomy 13, trisomy 21 and show resistance to standard induction therapy. Mutations in is

citrate dehydrogenase (IDH) are oncogenic. They are seen in 15% to 20% of all AML cases and 25% to 30% of patients with CN-AML. More commonly seen in older individuals.TP53 mutations are associated with very poor prognosis and are resistant to chemotherapy (Fujiwara *et al*.,2019).

.2.1.4. Viral Infection and AML

The most common viruses to cause trouble in AML patients belong to the herpes family of viruses, mostly Human herpes virus 1 (HHV1), chicken pox virus (or varicella zoster virus (VZV), cytomegalovirus (CMV) and Human herpes virus 6 (HHV-6) (Innao *et al.,* 2020; Voigt *et al.,* 2021).

2.2. HUMAN HERPES VIRUS-6 (HHV-6)

2.2.1. Historical Preview:

Herpesvirus was established as a genus in 1971 in the first report of the International Committee on Taxonomy of Viruses (ICTV).

During 1986, Syed Kaki Salah Uddin, Dharma Ablashi, and Robert Gallo cultivated peripheral blood mononuclear cells from patients with AIDS and lymphoproliferative illnesses. Short-lived, large, refractile cells that frequently contained intranuclear and/or intracytoplasmic inclusion bodies were documented. Electron microscopy revealed a novel virus that they named Human B-Lymphotrophic Virus (HBLV) (Kawabata etal.,2011).

HHV-6 was divided into subtypes. Early research (1992) described two very similar, yet unique variants: HHV-6A and HHV-6B. The distinction was warranted due to unique restriction endonuclease cleavages, monoclonal antibody reactions, and growth patter ns (Kawabata *et al.*,2011).

Shortly after its discovery, Ablashi ***et al.,(2006)*** described five cell lines that can be infected by the newly discovered HBLV. They published that HSB-2, a particular T-cell line, is highly susceptible to infection. Ablashi's pioneering research concluded by suggesting that the virus name be changed from HBLV to HHV-6, in accord with the published provisional classification of herpes viruses.

HHV-6A includes several adult-derived strains and its disease spectrum is not well defined, although it is thought by some to be more neurovirulent (Kofman ***et al.***,2011***).*** HHV-6B is commonly detected in children with roseola infantum, as it is the etiologic agent for this condition. Within these two viruses is a sequence homology of 95%(Arbuckle *et al.*,2010*;* Harberts *et al.*,2011 ***)***.In 2012, HHV-6A and HHV-6B were officially recognized as distinct species (Marci *et al.*,2016***).***

2.2.2. Taxonomy and Classification of HHV6

The family Herpesviridae was divided into 3 subfamilies (alphaherpesvirinae , betaherpesvirinae and gammaherpesvirinae) and 5 unnamed genera; 21 viruses were recognized as members of the family as the following (Rizzo *et al.*,2017;Yaara *et al.*,2020).

I.α herpesviruses: Herpes simplex virus types 1 and 2, and varicella-zoster virus, which have a short replicative cycle, induce cytopathology in monolayer cell cultures, and have a broad host range.

II.β herpesviruses: Cytomegalovirus, and human herpesviruses 6 and 7, with a long replicative cycle and restricted host range.

III.γ herpesviruses: Epstein-Barr virus and human herpesvirus 8, with a very restricted host range.

Now, there are more than 130 herpesviruses are known, some of them from mammals, birds, fish, reptiles, amphibians, and mollusks. Nine herpesvirus types are known to infect humans (Yaara *et al*.,2020).

Human herpes virus 6 HHV-6A, HHV-6B belongs to the β Herpesvirinae subfamily. HHV-6A and HHV-6B were recognized by the ICTV as distinct species in 2012.This genus consisted of 23 viruses among 4 groups (Rizzo *et al*.,2017).

Human herpes virus 6 HHV-6A and HHV-6B, which share high genome similarity but differ for some biologic properties, epidemiology, and disease association. Although they are classified as lymphotropic viruses, their in vivo tropism is considerably broader, including T-lymphocytes, macrophages, endothelial cells (ECs), salivary glands, and brain , thyroid epithelial cells, natural killer (NK) cells , and endometrial cells(Caselli *et al*.,2017).

2.2.3. Morphology and Structure of HHV-6

2.2.3.1 HHV-6 Particles:

The diameter of an HHV-6 virion is about 2000 nanometer. The size of the HHV-6 virion increases from 120 nm to approximately 300 nm after the inclusion of the tegument and envelope (Roizman *et al.*,2001).

The virion's outer portion consists of a lipid bilayer membrane that contains viral glycoproteins and is derived from that of the host. Below this membrane envelope is a tegument which surrounds an icosahedral capsid, composed of 162 capsomeres. The protective capsid of HHV-6 contains double stranded linear DNA (Kawabata et al ;2009)

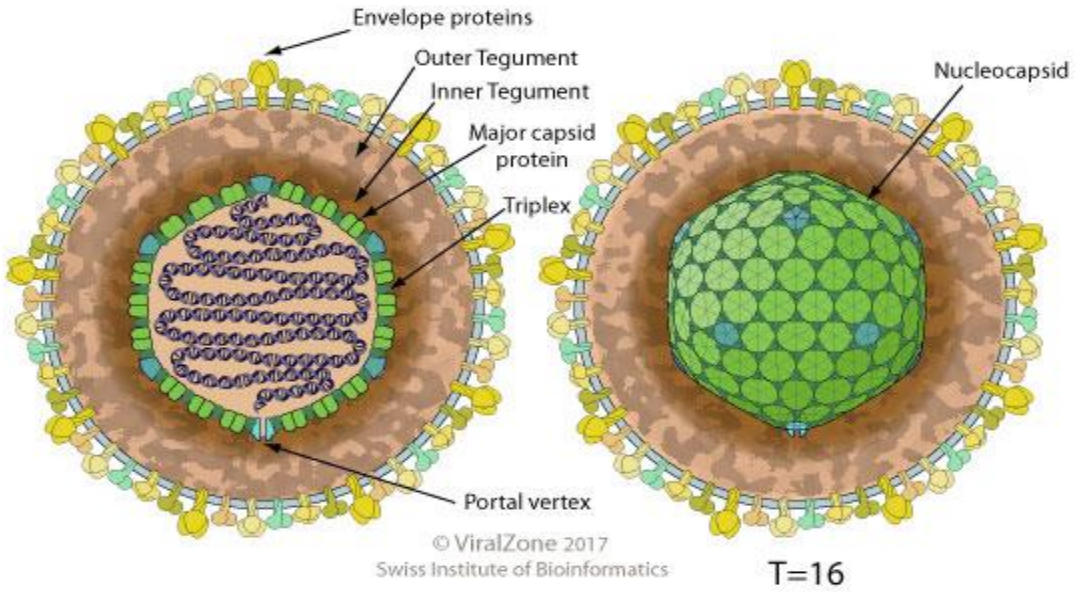

Figure (2.1): HHV-6 structure (Julia *et al*.,2014).

2.2.3.2. HHV-6 Genome Organization

The genomes of HHV-6A and HHV-6B, similar to those of other herpesviruses, consist of large linear double stranded DNA molecules, 160 kb in length, containing a unique segment flanked by direct repeats (Finkel Y. *et al*.,2020).

The genetic material of HHV-6 is composed of linear (circular during an active infection), double stranded DNA which contains an origin of replication, two 8–10 kb left and right direct repeat termini, and a unique segment that is 143–145kb (Tang *et al*.,2010).

The direct repeat termini (DR_L and DR_R) possess a repeated TTAGGG sequence, identical to that of human telomeres. Variability in the number of telomeric repeats is observed in the range of 15–180. These termini also contain

pac-1 and pac-2 cleavage and packing signals that are conserved among herpesviruses (*Arbuckle et al*.,2011).

The unique segment contains seven major core gene blocks (U27–U37, U38–U40, U41–U46, U48–U53, U56–U57, U66EX2–U77, and U81–U82), which is also characteristic of herpesviruses. These conserved genes code for proteins that are involved in replication, cleavage, and packing of the viral genome into a mature virion. Additionally, they code for a number of immunomodulatory proteins. The unique segment also possesses a block of genes (U2–U19) that are conserved among HHV-6, HHV-7, and Cytomegaloviruses (the betaherpesviruses) (Tang *et al.*,2010; Arbuckle *et al.*,2011).

The annotation of HHV-6 coding capacity has traditionally relied on open reading frame (ORF)-based analyses using canonical translational start and stop sequences and arbitrary size restriction to demarcate putative protein coding genes, resulting in a list of around 100 ORFs for each virus (Depledge *et al*., 2019).

In recent years, genome wide-analysis of herpesviruses using short RNA sequencing (RNA-seq) reads, and recently also direct and long-read RNA-seq revealed very complex transcriptomes (Kara et al., 2019; O'Grady et al., 2019), and combined with genome-wide mapping of translation, revealed hundreds of new viral ORFs (Bencun *et al*., 2018; Whisnant *et al*., 2019).

Specifically for HHV-6, recent work using proteomics, transcriptomics and comparative genomics on HHV-6B enabled re-annotation of several viral gene products .Taken together, this unforeseen complexity of herpesviruses suggests the current annotations of HHV-6 genomes are likely incomplete (Finkel *et al*.,2020).

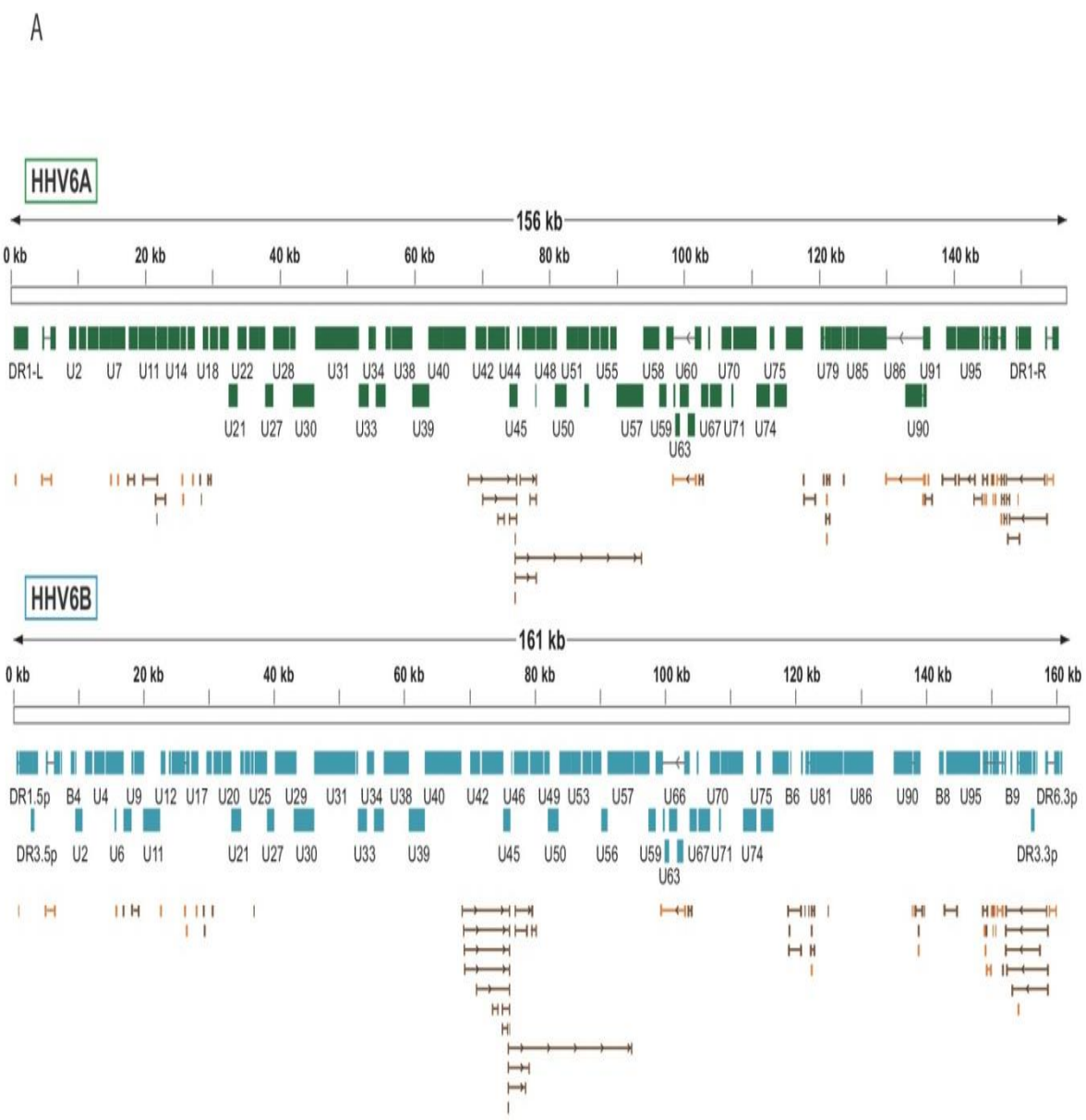

Figure 2.2: Schematic organization of HHV6 A&B genome. Splice junctions mapped using RNA-seq reads are shown throughout the genomes of HHV-6A and HHV-6B. Previously annotated splice junctions are marked in orange and novel splice junctions are marked in brown (Finkel *et al*.,2020).

2.2.3.3: HHV6 Proteins

The homotrimeric fusogenic protein gB mediates the viral-host membrane fusion that is required for putting the nucleocapsid into the host cytosol. The gH/gL complex of HHV-6B and HHV-6A are associated with the unique glycoproteins gQ1 and gQ2 encoded in the open reading frame (ORF) U100, resulting in a tetrameric complex gH/gL/gQ1/gQ2, while a trimeric complex gH/gL/gO is also known including another glycoprotein gO encoded in the ORF U47 (Tang *et al*.,2015; Nishimura *et al*.,2020).

The gH/gL/gQ1/gQ2 complex (hereafter referred to as the 'tetramer') is especially important because gQ1 and the associated gQ2 play critical roles in the interaction with the host receptors (Tang *et al*.,2014 ; Tang and Mori,2015).

U20 is glycoprotein (specific to Roseolovirus) predicted immunoglobulin structure. U20 binds to MHC-1 molecules and prevents antigen presenting cells from presenting HHV-6 peptides — glycoprotein, downregulates HLA I (specific to Roseolovirus).U24 Inhibits proper T cell activation, reducing secretion of cytokines at infection site — phosphorylation target for kinases — glycoprotein M (gM).U94 Involved in transcriptional repression of lytic genes – aids in the specific integration of HHV-6A/HHV-6B into the telomeres — highly expressed during latency (Arbuckle et al.,2011).

. 2.2.3.4: Replication Cycle of HHV6

The HHV6 replication cycle takes 72 hours to complete and consists of four general steps as shown in figure (2-5)

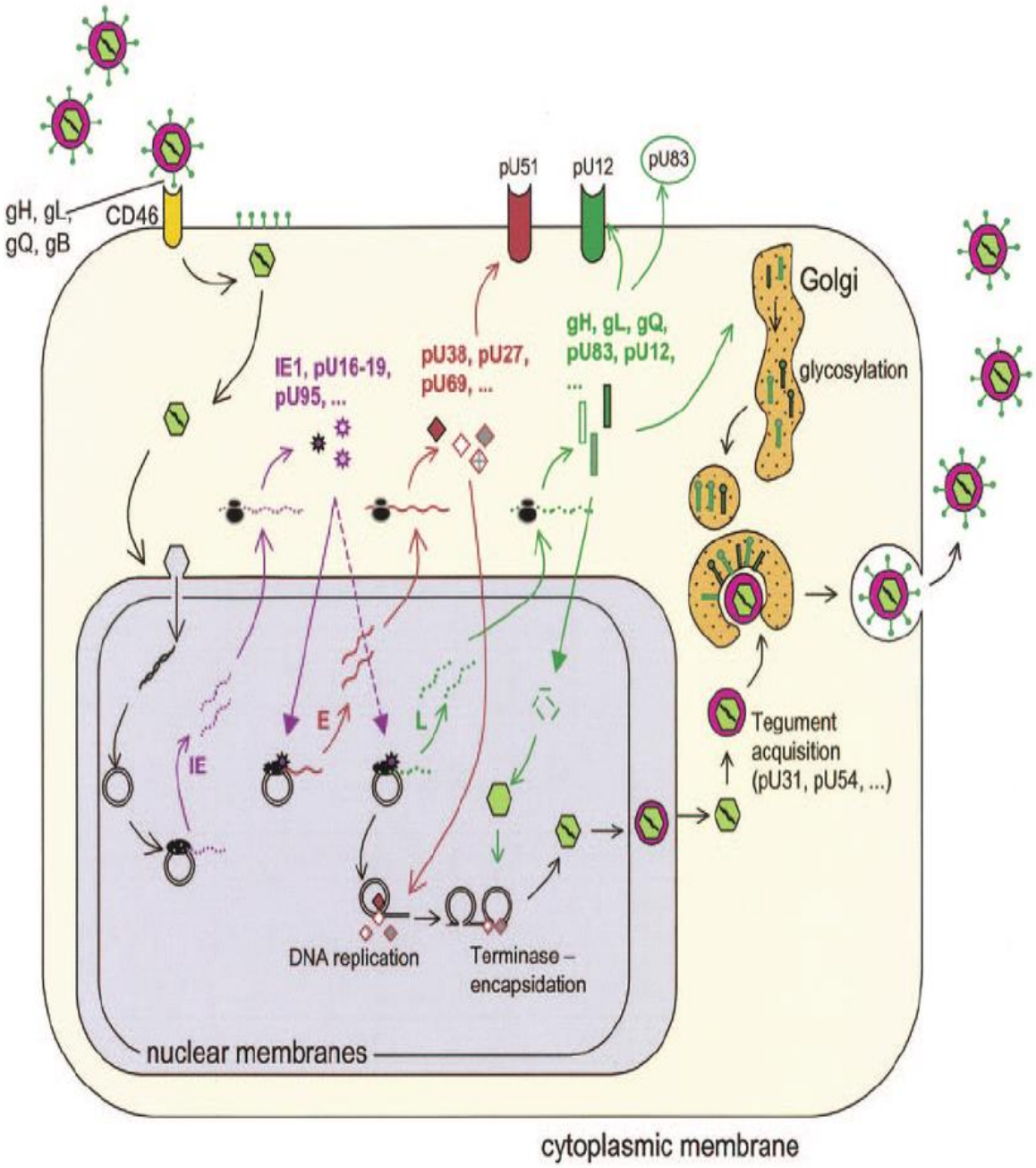

Figure 2.5: Schematic representation of rhinovirus replication cycle (Leen *et al*.,2005).

I.HHV-6 receptor and Host Tropism

HHV-6 infects a wide range of human cells in vitro, but it preferentially replicates in activated $CD4^+$ T lymphocytes .HHV-6A and HHV-6B recognize their specific receptors by differentiating gQ1 and gQ2; the HHV-6A tetramer binds to CD46, which is widely expressed on human cells, whereas the HHV-6B tetramer recognizes human CD134 (hCD134, also known as OX40) which is specifically expressed on activated T cells (Tang *et al.*,2014).

A member of the tumor necrosis factor (TNF) receptor superfamily present only on activated T lymphocytes, functions as a specific entry receptor for HHV-6B .The responsible residues in both HHV-6B gQ1 and hCD134 were identified in previous studies, but the interaction is not simple as it depends on the conformation of gQ1 in cooperation with gQ2, and in addition with gH/gL (Nishimura *et al.*,2020).

In addition to $CD4^+$ T lymphocytes, HHV-6 can infect in vitro $CD8^+$ T lymphocytes (only with HHV-6A), human fibroblasts, natural killer cells, liver cells, epithelial cells, endothelial cells, astrocytes, oligodendrocytes, and microglial cells. However, its capacity to infect continuous T cell lines is limited, and in many cases, it can be obtained only through an adaptation process consisting of serial blind passages of a primary isolate on the target cells(Henri *et al.*,2015).

The capability to infect different cell lines is generally higher for HHV-6A than for HHV-6B and appears to be a phenotypic character for discriminating both species. As a whole, no continuous cell line can be recommended for isolation of the virus. The primary isolation of HHV-6 from a human specimen usually requires cocultivation with primary highly susceptible cells consisting of peripheral blood mononuclear cells (PBMCs) or umbilical cord blood lymphocytes (Henri *et al.*,2015).

III. HHV6 Attachment

HHV-6 attaches to its cell receptor by means of a tetrameric viral ligand complex made up of the glycoproteins H (gH), L (gL), Q1 (gQ1), and Q2 (gQ2) (Yamanishi *et al*.,2013).

IV. Entry and Un-coating

Following attachment, HHV-6 entry into cell occurs through a fusion between the viral envelope and the cell membrane by a mechanism which involves gB and gH functions but remains poorly understood. The nucleocapsid is then transported through the cytoplasm to the nucleus, likely using the pathway of the microtubule network. HHV-6 DNA is released into the nucleoplasm (Henri *et al*.,2015).

V. Impacts of HHV6 Gene Expression on Cell Functions

Viral genes are expressed in a temporally ordered manner, starting with immediate early (IE) genes from the IE-A locus, which is constituted of two genetic units, IE1 and IE2 . Those genes are transcribed in the absence of de novo protein synthesis, and this step is followed by the transcription/expression of early (E) and late (L) genes. The replication of the genome occurs after the synthesis of E proteins, which have enzymatic activities dedicated to nucleotide metabolism and DNA synthesis, i.e., phosphotransferase, ribonucleotide reductase, uracil-DNA glycosylase, origin-binding protein, DNA polymerase, polymerase processivity factor, major DNA-binding protein, and helicase-primase complex activities (Tsao *et al*.,2009) .

Viral DNA is assumed to be replicated through a rolling circle process. Progeny DNA is yielded in the form of concatemeric strands, which are cleaved and

packaged into capsid precursors thanks to specific cleavage-packaging signals present in the DR_L and DR_R regions (Yamanishi *et al*.,2013).

In addition, independently of any complete virus-producing process, the expression of certain HHV-6 genes might occur from persisting episomal or ciHHV-6 forms of viral DNA. Many publications have reviewed the formally demonstrated or putative effects of virally encoded gene products on the regulation and modification of cell functions (Flamand *et al*.,2010;Yamanishi *et al*.,2013).

As an example, considering the gene products of the IE-A region, IE2 might behave as a general transcriptional activator of many viral and cellular genes, while IE1 interacts with PML bodies.The products of the DR7 gene appear to demonstrate a cell-transforming activity, presumably through an interaction with p53 . Regarding the U94 gene, which is analogous to the AAV rep gene, it can bind to the human TATA-binding protein, and its expression in endothelial cells decreases cell migration and angiogenesis (Caruso *et al*.,2009).

The U95 gene product interacts with the mitochondrial GRIM-19 protein, a component of the oxidative phosphorylation system involved in apoptotic processes As indicated below, several proteins encoded by the HHV-6 genome have immunomodulatory functions. Taken together, all these features provide molecular bases for understanding the pathological processes associated with acute and chronic HHV-6 infections (Caruso *et al*.,2009).

VI. HHV6 Releasing

The capsids exit the nucleus, acquiring an intermediate envelope by budding through the inner part of the nuclear membrane, are de enveloped by fusion with the external part of this membrane, and appear as tegumentary forms in the cytoplasm. The acquisition of the final envelope carrying viral glycoproteins

occurs in the trans-Golgi network, and mature visions are released by exocytosis. (Henri *et al*.,2015; Nishimura *et al*.,2020).

The occurrence of a complete replication cycle, which lasts about 3 days, has a major impact on host cell functions and morphology. Infected cells engaged in this virus-producing process ultimately die by apoptosis and/or necrosis (Henri *et al*.,2015; Nishimura *et al*.,2020).

2.2.3.5 Integrated HHV-6A/B

The extreme end of the viral genome is flanked by two identical directly repeated (DRL and DRR) regions of 8–9 kbp. The 5′ end of the DR contains a pac1 sequence (5 6 bp) while the 3′ end contains pac2 sequence (80 bp), responsible for the cleavage and packaging of the viral genome. Adjacent to pac2 are 15–180 reiterations of TTAGGG telomeric repeats (TMR), identical to human telomeric sequences. Adjacent to pac1 are imperfect TMR (impTMR) Figure (2.3A) (Collin and Flamand, 2017).

HHV-6A/B integrated genomes remain largely intact with their ORFs conserved. Analysis of cells with ciHHV-6A/B indicates that these viruses are mostly integrated into telomeres with DRR fused to the chromosome Figure (2.3B) (Tweedy et al.,2016).

In fact, HHV-6-A/B DRR are adjacent to the subtelomeric portion of the human telomere with loss of the pac2 sequence at DRR and loss of the pac1 sequence at DRL. Such a structure is compatible with integration occurring by HR events. In addition, at the DRL end of the integrated genome are impTMR that appear to serve as a template for telomere elongation by the telomerase complex or alternative lengthening mechanisms (Ohye et al.,2014).

In occasional cases, integrated HHV-6A/B consists of a single DR fused to the chromosome, a structure that is compatible with integration occurring by HR events initiated at DRL (2.3C).

Alternatively, individuals containing multiple contiguous HHV-6A/B copies also exist. Such structure can be explained by integration of a viral concatemer Figure (3D).By assessing the number of DR present, one can determine the number complete HHV-6A/B's genomes that is/are integrated(Collin and Flamand, 2017).

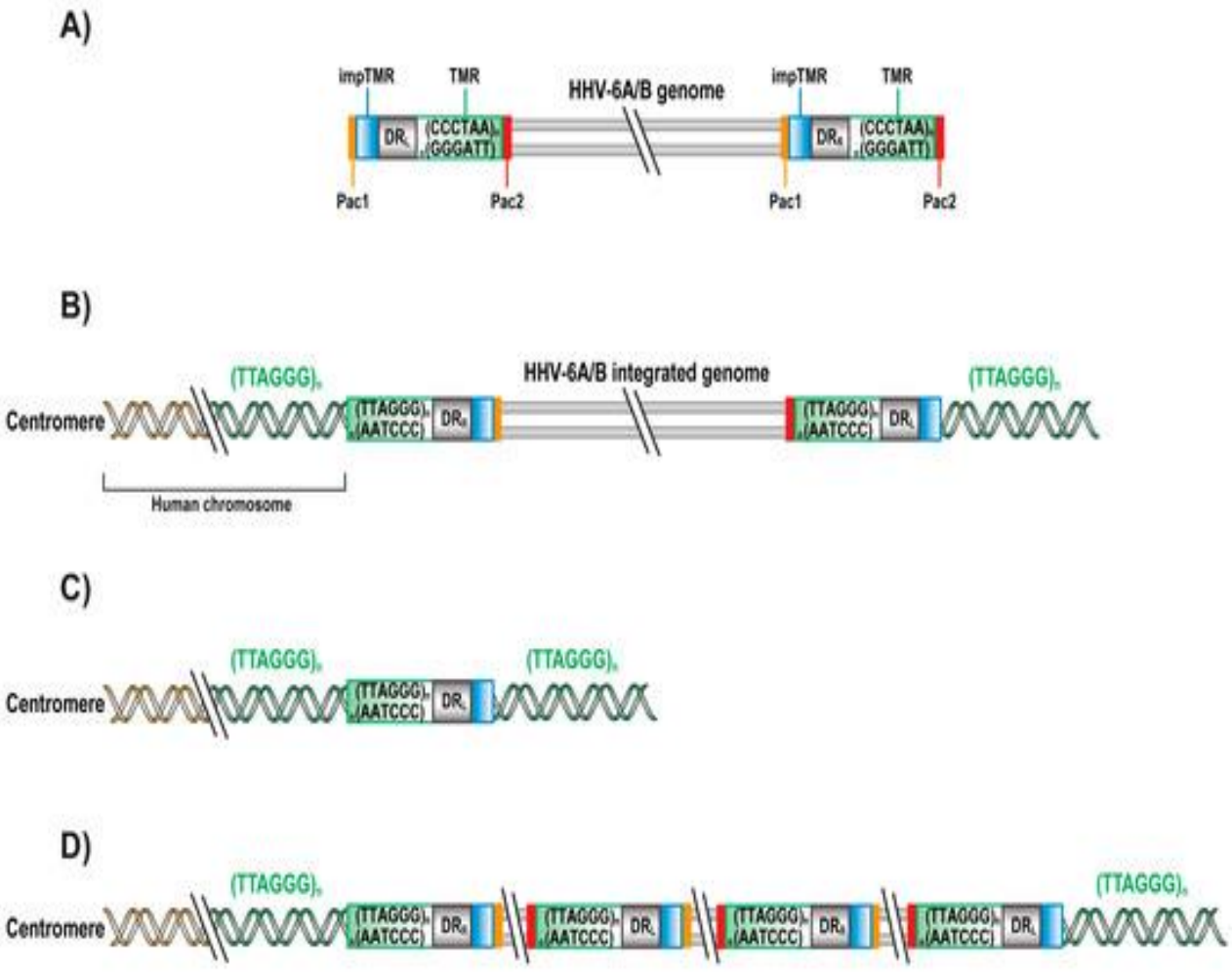

Figure 2.3: HHV-6A/B genomes and their integrated forms. Schematic representation of human herpesvirus 6A and 6B (HHV-6A/B) genomes and the reported integrated forms. (A) The unique region (U) of the 160 kbp HHV-6A/B genomes is flanked by identical direct repeats (DR_L and DR_R) of 8–9 kbp. The DRs possess a pac1 (yellow) and pac2 (red) sequences, adjacent to imperfect telomeric repeats impTMR (blue) and TMR (green) sequences, respectively. The genome is not drawn to scale; (B) Chromosomally integrated HHV-6A/B (ciHHV-6A/B) genome (with loss of pac2 in DR_R and pac1 in DR_L) with elongated telomeres at the DR_L; (C) Single integrated DR_L with elongated telomere; (D) Integrated HHV-6A/B concatemers. Genomes are not drawn to scale (Collin and Flamand, 2017).

2.2.3.6. Excision of Integrated HHV-6

To ensure their long-term maintenance in any given population, integrated viruses must be able to re-initiate a lytic cycle and generate progeny virions. A previous study has reported the presence of an extrachromosomal circular HHV-6 genome with a single DR in cells from an iciHHV-6 subject.This led to the conclusion that the viral genome can be excised from telomeres by one or two t-loop formation and recombination. To explain the presence of a single DR in the excised viral genome, one hypothesis is that excision occurs by a two-step t-loop formation Figure (2.4A). At each cell cycle, telomeres are shortened and reform a t-loop to protect the chromosomes. First, the telomere sequence at the end of the DR_L could form a t-loop by invasion of the TMR within DR_L itself. This t-loop formation would result in a t-loop excision, forming a telomeric circle with a single DR and an intermediate form of the HHV-6A/B integrated chromosome Figure (2.4B) (Huang *et al*.,2014).

The intermediate form would lack the DR_L but still possess the TMR repeats capable of forming a second t-loop, recombine and excise in the TMR of the DR_R. This first possibility could also explain the presence of a single integrated DR if only one t-loop excision is made. Another possible excision mechanism could be a t-loop formation, recombination and t-loop excision of the DR_L in the DR_R directly(Huang *et al*.,2014).

The two possible mechanisms result into a circular viral genome with a single DR that has one pac1 and one pac2 sequence Figure (2.4 C). Because ciHHV-6A/B can be excised from chromosomes and form viral episomes, this suggests that integration is possibly a mode of latency for these viruses (Collin and Flamand, 2017).

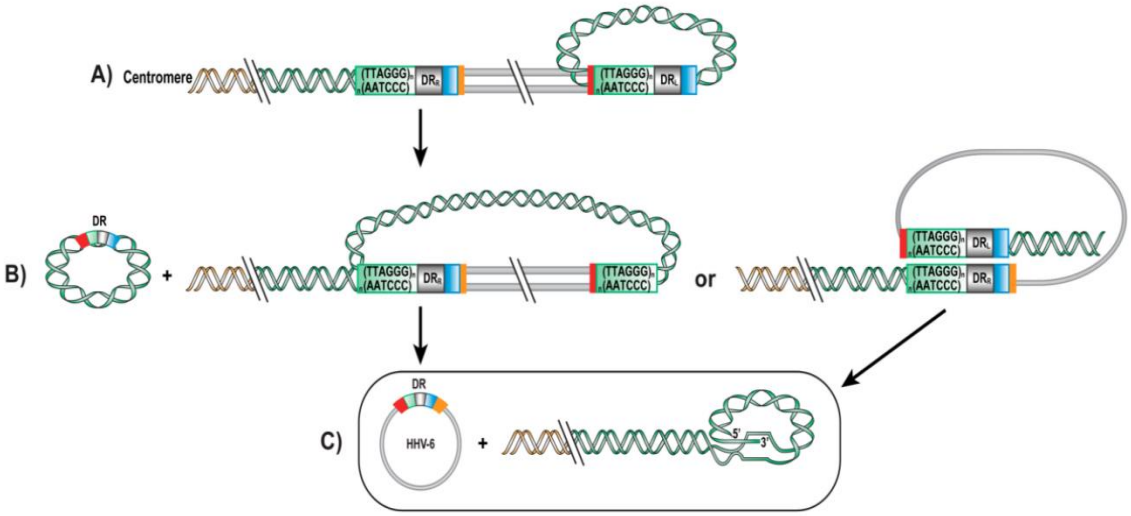

Figure2.4. Possible mechanisms of HHV-6A/B genome excision from telomeres. Schematic representation of hypothetic processes of HHV-6A/B genome excision from telomeres. (A) Telomeric repeats form a t-loop in the TMR of HHV-6A/B DR_L, followed by recombination and excision, resulting into a first t-loop excision: a telomeric circle and a chromosomally integrated

HHV-6A/B lacking a DR but still possessing TMR sequences. (B) A second t-loop formation is made by recombination of the TMR at the end of the genome into HHV-6A/B DR_R, resulting in a fully excised and circular HHV-6A/B genome containing a single DR with a single pac1, pac2, impTMR and TMR sequence. (C) Invasion of the telomeric repeats into the TMR of the DR_R, resulting into a HHV-6A/B free chromosome and a full viral genome with a complete DR(Huang *et al*.,2014).

2.2.3.7: Latency and Reactivation

Like other human herpesviruses, HHV-6 persists indefinitely in its host and is capable of reactivation, meaning the active production of detectable mature virions in some body compartments following a phase of apparently complete clearance. These properties rely on the putative capacity of its genome to be maintained in a nuclear latent form or to drive a low-level productive infection in some cells while inducing a fully lytic infection in other cells. For other human herpesviruses, such as herpes simplex virus, the latent DNA genome has the form of a covalently closed circular episome associated with cellular nuclear proteins. The existence of such a latent nuclear form has not been demonstrated formally for HHV-6, although an episomal state was shown after experimental infection of cervical carcinoma cell lines (Tweedy *et al*.,2015).

The viral gene U94, which is expressed during latent infection, is assumed to play a major role in the establishment and maintenance of intracellular latency (Tweedy *et al*.,2015). Other latency-associated transcripts have also been described.Reactivation occurs through the transcription of IE genes in the IE1 and IE2 regions following the likely transactivation effect of cellular and/or viral factors whose nature is still unknown. This reactivation process results in the

induction of a replication cycle and the possible appearance of a cytopathic effect (Reynaud *et al*.,2014).

2.2.4 Transmission of HHV6

Transmission is believed to occur most frequently through the shedding of viral particles into saliva. Both HHV-6B and HHV-7 are found in human saliva, the former being at a lower frequency. Studies report varying rates of prevalence of HHV-6 in saliva (between 3–90%), and have also described the salivary glands as an in vivo reservoir for HHV-6. The virus infects the salivary glands, establishes latency, and periodically reactivates to spread infection to other hosts (Arbuckle *et al*.,2011; Araujo *et al*.,2011).

Vertical transmission has also been described, and occurs in approximately 1% of births in the United States. This form is easily identifiable as the viral genome is contained within every cell of an infected individual(Araujo *et al*.,2011).

2.2.5 HHV-6 and Disease Association

Several diseases have been associated with HHV-6A/B reactivation in adults, although the causal correlations are still unproven. Most studies on pathogenic association do not specify the HHV-6 virus species, which, however, can be inferred by the reference strains used in the methods(Elisabetta *et al*.,2020)

I.HHV-6A/6B-associated with neurological diseases

In immunocompromised subjects, such as encephalitis, seizures, ataxia, and mild dementia, with a higher prevalence of HHV-6A in cognitive dysfunctions and of HHV-6B in encephalitis and seizures (Pantry *et al*.,2017).

II.HHV-6A/6B correlated to multiple sclerosis

Both HHV-6A and HHV-6B; is predominantly found in peripheral blood mononuclear cells (PBMCs) of patients , systemic sclerosis (HHV-6A) and connective tissue diseases (both HHV-6A/6B) (Caselli *et al*.,2019).

III.HHV-6A correlated with Hashimoto's thyroiditis

HHV-6A was associated with Hashimoto's thyroiditis (Caselli *et al*.,2017).

III.H HV-6A and infertility

HHV-6A was associated with female and male infertility (HHV-6A) (Caselli *et al*.,2017).

IV. HHV-6A and fulminant hepatic failure (HHV-6A/6B) (Caselli et al.,2017).

V. HHV-6A and chronic fatigue syndrome (HHV-6A) (Miyagawa *et al*.,2016).

VI. HHV-6A and neoplasia(Miyagawa *et al*.,2016).

VII. HHV-6A and myocarditis, drug reaction with eosinophilia, and systemic symptoms (HHV-6A/6B) (Miyagawa *et al*.,2016).

VIII. HHV-6A and Alzheimer's disease

A possible role of HHV-6A infection has also been suggested in Alzheimer's disease (Rizzo *et al*.,2019).

IX. HHV-6B and COVID-19 patient

Reactivation of HHV-6B has been recently documented in a COVID-19 patient , which may be correlated with the increased expression of the CD134 HHV-6B receptor and the inflammatory cytokine IL-6 . Consistent with this observation,

HHV-6-associated diseases such as Pityriasis rosea and Kawasaki's disease increased around ten-fold during the COVID-19 pandemic, compared to previous periods (Elisabetta *et al*.,2020).

X.HHV6 and Spontaneous Abortion

HHV-6 has been implicated in cases of poor pregnancy outcome due to ability of virus to disrupt endometrium epithelial cells function that lead to inhibit the creation of appropriate uterine environment for implantation and fetal growth that lead to infertility and miscarriage (Eliassen *et al* ., 2017).

2.2.6. *HHV-6A & B and Leukemia*

The preponderance of data suggests no association between HHV-6 and leukemia. Contrasting findings, and differences in HHV-6 species predominating in bone marrow of leukemia patients, may stem from the use of different probes for HHV-6A and/or divergence in HHV-6A across geographical areas. Only one group has investigated HHV-6 antigen expression in leukemia, with intriguing results: HHV-6 early antigen p41 was detected in bone marrow cells—blasts and megakaryocytes (Eliassen *et al*.,2018).

Voigt *et al*.,(2021) who report a case of a clinically manifest human herpesvirus-6 (HHV-6) encephalitis in a neutropenic patient with acute myeloid leukemia (AML) in a non-transplant setting while on antimicrobial prophylaxis including aciclovir.

2.2.7. Immune Response to HHV6

2.2.7.1. Innate Immune Responses To HHV6 Infection

Both HHV-6A and -6B establish a latent infection in the host following resolution of primary infection. Reactivations in the adult have been associated to

the development of multiple symptomatic diseases often characterized by immune dysregulation (multiple sclerosis, Sjögren's syndrome, autoimmune thyroiditis, and others). Both viruses are considered lymphotropic, showing an elective tropism for CD4+ T-lymphocytes and being able to infect several different cell types of the immune system, including NK cells (Rizzo *et al*., 2017).

Interestingly, in vivo and in vitro evidences indicate that HHV-6A/6B interfere with the immune system of the infected host in several ways . They can modulate surface antigens important for T-cell activation, such as human leukocyte antigen (HLA) class I molecule expression in dendritic cells; they also can affect cytokine and chemokine productions, including selective suppression of IL-12, affecting the generation of effective cellular immune responses (Schmiedel *et al.*, 2016).

Furthermore, they recently observed that HHV-6A infection induces the expression of the tolerogenic non-classical class I HLA-G molecule in primary human mesothelial cells, leading to impairment of NK cell recognition and killing of infected cells . With reference to the NK cell component of the immune response, HHV-6A was reported to establish a productive infection in CD3-negative NK cell clones, leading to the de novo expression of CD4 on the NK cell surface , and HHV-6B was recently shown to induce down-modulation of the activating NKG2D ligand in infected cells (Schmiedel *et al.*, 2016).

Notably, it has been recently reported that NK cells may be directly involved in the onset and progression of autoimmune diseases, through their potential autoreactivity or through their interaction with the other immune cells, thus supporting the hypothesis of a correlation between HHV-6A/6B infection, NK cell function and autoimmunity (Rizzo *et al*., 2017).

2.2.7.2. Adaptive Immune Responses To HHV6 Infection

I. Cellular Response to HHV-6

Information on HHV-6-specific T cell responses is still limited, in particular regarding CD8 T cells . It was shown early that healthy virus carriers have CD4 T cells that respond to HHV-6 lysate or infected cells (Becerra *et al*.,2014).

Target antigens and epitopes of the specific CD4 T cell response were identified first in a study on six selected structural proteins , and more recently by a proteomic approach that has identified ten viral antigens targeted by CD4 T cells (Becerra-Artiles *et al*.,2015).

Responses to five HHV-6B proteins have been investigated so far, and a number of epitopes from these proteins that are presented by infected cells were identified Both CD4+ and CD8+ HHV-6 specific T-cells have been isolated from the PBMCs of healthy donors. In the case of both CD4+ and CD8+ the frequency of HHV-6 specific T-cells is low, but the responding population could be expanded in vitro . Expanded CD4+ and CD8+ populations characteristically secrete IFNγ and TNFα, while also performing cytolytic functions mediated by the secretion of perforin (CD4+) or granzyme B (CD8+) (Martin *et al*.,2018).

Additionally, CD8+ clones were found to also secrete granulocyte macrophage stimulating factor. Studies on T-cell response to HHV6 have identified 8 HLA-DR restricted CD4+epitopes and 5 HLA-A restricted CD8+ epitopes (Becerra-Artiles *et al*.,2015).

II. Antibody Response to HHV-6

During primary infection, anti-IgG and anti-IgM antibodies are produced, with IgM antibodies being the first to be detected . IgG titer begins to increase about

one week post infection and peaks a week later. Additionally, there is an increase in IgG avidity over the course of infection (Ward ,2013).

The antibody response to HHV-6 in CIHHV-6 harboring individuals has not been clearly defined; thus, studies in the future should focus on this topic. However, one study by Tanaka-Taya *et al*.,(2004) measured antibody titer to two HHV-6 antigens (IE-A and glycoprotein gB) in the PBMCs of CIHHV-6 individuals and compared the response to that of healthy non-CIHHV-6 individuals . Interestingly, 57% of individuals with CIHHV-6 had antibodies to the IE-A antigen present in the serum, while IE-A antibodies were undetectable in non-CIHHV-6 individuals (Agut *et al*.,2015).

On the other hand, 14% of CIHHV-6 versus 60% of the healthy individuals had an antibody response to gB . Glycoprotein B is considered one of the major neutralizing epitopes and variant specific gB antibodies have been detected . The absence of gB antibodies or reduced antigB titers may reduce the ability of CIHHV-6 individuals to mount an immune response to an exogenous HHV6 infection(Agut *et al*.,2015).

2.2.8. Laboratory Diagnostic of HHV6

2.2.8.1. Indirect (Serology)

Assays for IgG and IgM detection by immunofluorescence assay (IFA) ; enzyme-linked immunosorbent assay (ELISA) and avidity assays (Ana Lia *et al* ., 2020).

2.2.8.2. Tissue Culture and Cell lines

I. Cell lines and HHV6

The HHV6 detection by culture was performed using different culture systems including JJHan, and HSB-2 cells were cultured in RPMI 1640 al amino acids (Corning Cellgro), HEPES . HCT-116, GM847, HEK293T, MCF-7, and NIH 3T3 cells were cultured in the same medium but supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, Waltham, MA, USA) instead of Nu serum **(**Gravel *et al* ., 2017).

II. Single-cell cloning integration assay.

Ten thousand cells/well (U2OS, HCT-116, HeLa, GM847, MCF-7, NIH 3T3, and HEK293T) were seeded in 48-well plates **(**Gravel *et al* ., 2017).

2.2.8.3. Antigen detection

Uses conventional equipment, gives evidence of virus gene expression, discrimination between HHV-6A and HHV-6B **(**Agut *et al* ., 2015).

2.2.8.4. Qualitative viral DNA PCR

High sensitivity and specificity, discrimination between HHV-6A and HHV-6B(Agut *et al* ., 2015).

2.2.8.5. Quantitative viral DNA real-time PCR

High sensitivity and specificity, discrimination between HHV-6A and HHV-6B, longitudinal follow-up studies, comparison of viral loads in blood versus organs**(**Agut *et al* ., 2015).

2.2.8.6. Detection of viral transcripts by RT-PCR

Distinction between active and latent infections, recognition of active infection in ciHHV-6 subjects (Agut *et al* ., 2015).

2.2.8.7. Droplet digital PCR

Precise method for measuring nucleic acid amounts, identification of ciHHV-6 **(**Agut *et al* ., 2015).

2.3. Interleukin- 17 A (IL -17A)

2.3.1.Defention

Interleukin-17A is a protein that in humans is encoded by the *IL17A* gene. In rodents, IL-17A used to be referred to as CTLA8, after the similarity with a viral gene (O40633) (Chen *et al*.,2017).

2.3.*2.* Structure

IL-17(A) is a 155-amino acid protein that is a disulfide-linked, homodimeric, secreted glycoprotein with a molecular mass of 35 kDa. Each subunit of the homodimer is approximately 15-20 KDa. The structure of IL-17 consists of a signal peptide of 23 amino acids (aa) followed by a 123-aa chain region characteristic of the IL-17 family. An N-linked glycosylation site on the protein was first identified after purification of the protein revealed two bands, one at 15 KDa and another at 20 KDa (Chen *et al.*,2017).

Comparison of different members of the IL-17 family revealed four conserved cysteines that form two disulfide bonds. IL-17 is unique in that it bears no resemblance to other known interleukins. Furthermore, IL-17 bears no resemblance to any other known proteins or structural domains (Mohammed & Al-Janabi , 2021).

2.3.*3.* Gene expression

The gene for human IL-17A is 1874 base pairs long and was cloned from CD4+ T cells. Each member of the IL-17 family has a distinct pattern of cellular expression. The expression of IL-17A and IL-17F appear to be restricted to a small group of activated T cells, and upregulated during inflammation (Eileen and Kepeng ,2022).

Consistent with this finding, Chen *et al.*,(2017) showed that another molecule, SOCS3, plays an important role in IL-17 production. In the absence of SOCS3, IL-23-induced STAT3 phosphorylation is enhanced, and phosphorylated STAT3 binds to the promoter regions of both IL-17A and IL-17F increasing their gene activity. In contrast, some scientists believe IL-17 induction is independent of IL-23.

2.3.*4. Function*

The protein of IL-17 A encoded by this gene is a proinflammatory cytokine produced by activated T cells. This cytokine regulates the activities of NF-kappaB and mitogen-activated protein kinases. This cytokine can stimulate the expression of IL6 and cyclooxygenase-2 (PTGS2/COX-2), as well as enhance the production of nitric oxide *(Mandy et al.,2019).*

2.3.5. IL-17 A Polymorphism and AML

IL-17 is a proinflammatory cytokine family produced by Th-17 cells and has been found to be implicated in the pathophysiology of many cancers including acute myeloid leukemia (AML). Since single nucleotide polymorphism (SNP) alters the genetic functions and cancer susceptibility, we studied SNPs in two members of IL-17 family, IL-17A (rs2275913; G-197A) and IL-17F (rs763780; A7488G) which are the most common loci associated with IL-17 activity and

cancer risk, and correlated the results to AML susceptibility and response to therapy (*Rania et al.,2020)*.

IL-17 increases in the bone marrow and blood in AML patients more than in healthy controls . IL-17 can activate MAPK, PI3K/Akt, NF-κB, and STAT3 downstream signaling pathways to regulate AML progression. IL-17A (rs2275913) polymorphisms might be responsible for chronic inflammatory diseases, autoimmune diseases and malignancies as an inducer of inflammatory chemokines secretion also release cytokines stored in neutrophils and macrophages (Ahmed *et al.,2020)*.

2.4. P14

2.4.1.Defention

$P14^{ARF}$ is a tumor suppressor encoded by the CDKN2a locus that is frequently inactivated in human tumors. $P14^{ARF}$ protein quenches oncogene stimuli by inhibiting cell cycle progression and inducing apoptosis (Cilluffo *et al*.,2020).

2.4.2. Location

The p16(INK4a) and p14(ARF) tumor suppressor genes (TSGs) are encoded within the CDKN2A locus on chromosome 9p21 and function as cell cycle regulatory proteins in the p53 and RB pathways(Inoue *et al*.,2018).

2.4.3.Structure

The structure for the human *p15INK4b-p14ARF-p16INK4a* locus. The genomic structure is well-conserved between human and mice, and thus gene knockout studies have been extensively conducted in mice. The distance between exon 1β and exon 1α is 19.4 kbp in humans and 12.4 kbp in mice. The exon 1α is 3.8 kbp upstream of exon 2 in humans; 5.2 kbp in mice (from 5’ of exon 1α to 5’ of exon

2). The *ARF-INK4a* (*CDKN2a*) locus is located 11.5 kbp apart from the genomic locus for *CDKN2b* that encodes for p15INK4b in humans (from 3' of exon 2 for *p15INK4b* to 5' of exon 1β). All of *p15Ink4b*, *p19Arf*, and *p16Ink4a* genes act as tumor suppressors genes (Inoue et al.,2018). The DMP1 consensus is located −2.3 kb and −0.31 kb of *ARF* (shown in red reverse triangles) and −4.04 kb and −1.40 kb of *INK4a* (pink reverse triangles) in humans. Both of these are Dmp1 target genes although the mode of regulation is different Figure (2-6) (Zhu et al.,2013).

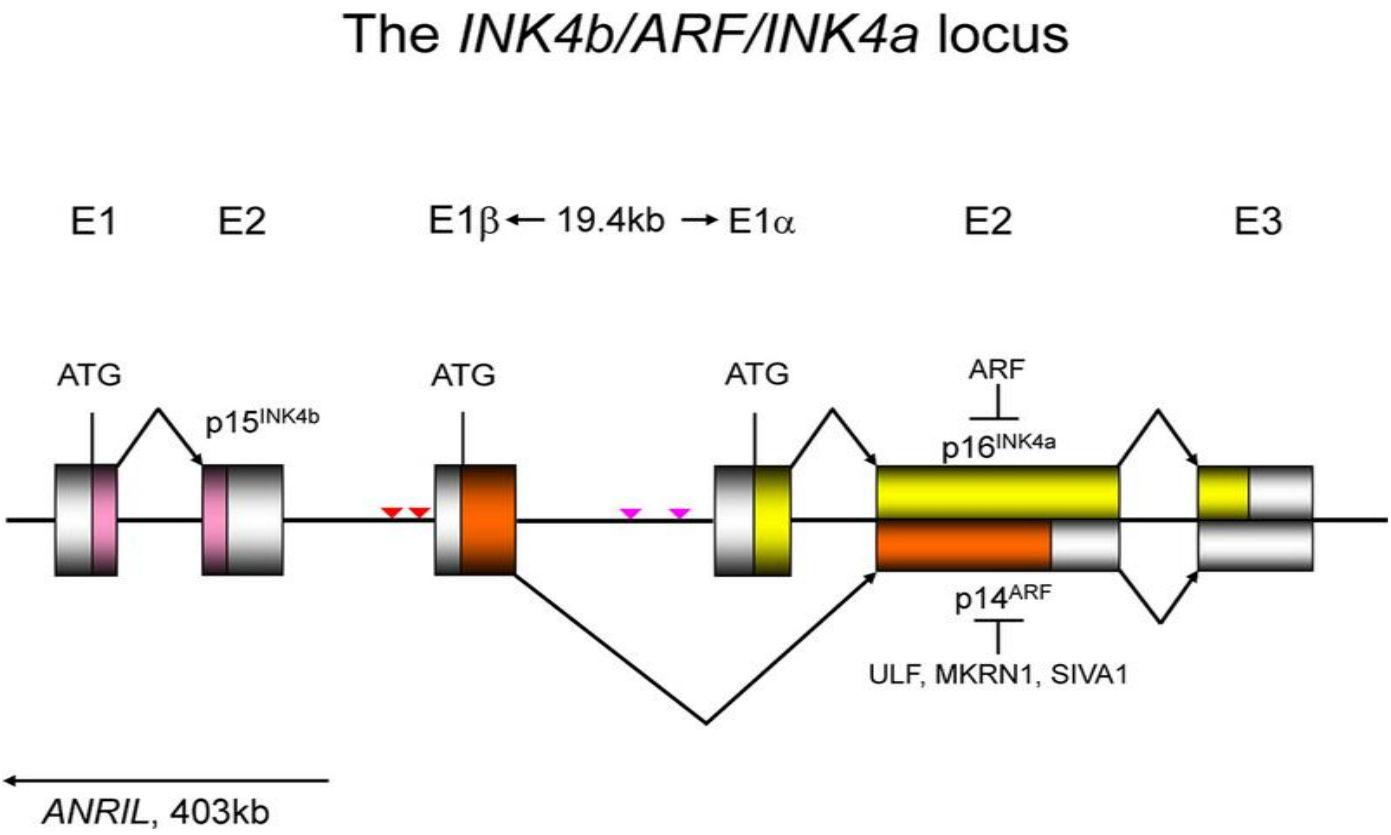

Figure 2.6: The structure for the human *p15INK4b-p14ARF-p16INK4a* locus (Inoue et al.,2018).

2.4.*4.* Gene expression

Expression of *ANRIL* was simultaneously found with p14ARF both in physiologic and pathologic conditions. p14ARF regulates the stability of the

p16INK4a protein in human and mouse cells . Importantly, ARF promoted rapid degradation of p16INK4a protein, which was mediated by the proteasome and, more specifically, by interaction of ARF with one of its subunits, regenerating islet-derived protein 3γ. Thus there is a significant crosstalk between ARF and INK4a at the protein level (Kobayashi et al.,2013). ULF, MKRN1, and Siva1 are E3 ligases for ARF that accelerates its degradation (Wang et al.,2013).

2.4.5. Function

P14^{ARF} functions can be played through interactions with several proteins. However, the majority of its activities are notoriously mediated by the p53 protein. Increased c-MYC activity results in the activation of P14^{ARF} which in turn inhibits the function of MDM2. Also, there is a P53/MDM2 feedback loop in which P53 is capable of activating the MDM2 protein, but MDM2 inactivates the P53 (Allahbakhshian *et al*.,2020).

2.4.6. P14 Polymorphism and AML

P14ARF, a pivotal CDK inhibitor, plays a role in cell cycle arrest in both G1 and G2/M through stabilization both P53 and MDM2 by directly binding to MDM2 (Spirin KS;et al 2004).(Heidari-Soreshjani S,etal 2017)

. In fact, the direct interaction between P14 and mdm2 inhibits degradation of p53 by mdm2 leading to the activation of p53 and its tumor suppressor activities. P14 expression alteration has been demonstrated in some leukemia (Akinfenwa AT:2018).

Therefore, P21, P27 and P14 are three essential cell cycle regulators with altered expression in different kinds of malignancies such as AML.

The aberration may give rise to a novel fusion protein, alternatively abrogation of an otherwise normal gene product, and thus define a new genetic subgroup in such malignancies (Panagopoulos I;2015).

An example is the cryptic t(7;21)(p22;q22)/*RUNX1-USP42* chromosomal translocation which was first described in an AML patient and currently is considered a rare but nonrandom feature of myeloid malignancies where it is frequently found together with del(5q)(Foster N; 2010).

CHAPTER three

Material and Method

3. Materials and Methods

3.1 Subjects

3.1.1 Patients population

This cross sectional case control study was done for a two hundred (200) specimens collected 100 from patients subjected to Acute Myeloid Leukemia (AML) and 100 apparently healthy persons (AHC) as a control group (normal persons) from general hospitals as well as many private clinical in Middle Euphrates – Iraq. The age range of the study population was 2 years to 80 years. The specimens were collected during period from October 2022 to February 2023.

Blood from each study group of patients suffering from Acute Myeloid .

All these specimens were submitted for genetic part for screening human tients and apparently healthy person control A&B) in pa 6-(HHV 6-Herpes virus groups by polymerase chain reaction (PCR). However, the second part for detection SNPs of *IL-17* gene polymorphism by sequencing.

3.1.2 Specimens collection

Blood specimens were .healthy persons collected from patients and Five ml venous blood were collected aseptically from all patients by using gel tubes and EDTA tubes for gating blood serum and buffy coat, respectively; then stored at (-20°C).

3.2 Study Design

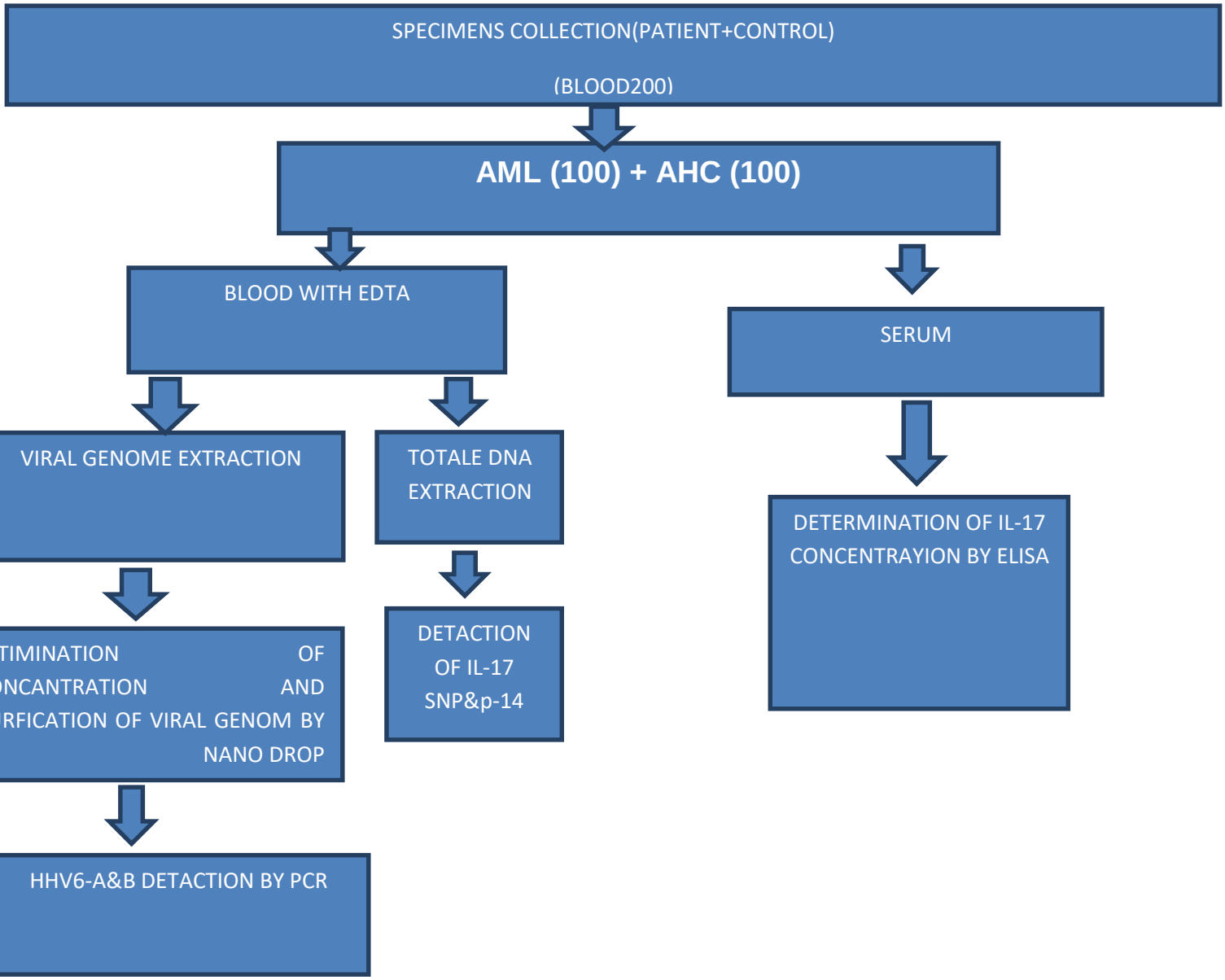

Figure 3.1: Study Design

3.3 Materials

3.3.1 Instruments and Equipment

Instruments and equipment used in this study are listed in table (3-1 A and B).

Table 3.1A: Instruments used in this study with their Manufacturer Company and origin

Instruments	Manufactured Company (Origin)
Centrifuge	MSE / England
Centrifuge Vortex; Eppendorf;	Eppendorf / Germany
Micro centrifuge	Hettich centrifuge, Sigma / Germany
Deep freeze (-70 C); Electric microwave	Sanio electric / Japan
Electrophoresis system	Fisher Scientific / USA
ELISA reader	Bio Tech / USA
Hood	Bio Lab / Korea
Electrical Oven; Incubator	Memmert / Germany
Nanodrop	Optizen / Korea
PCR device	Biometra / Germany
Refrigerator	Arcelik / Turkey
Sensitive balance	Sartoris / Germany
Timer with alarm	Junghans / Germany
Gel documentation system	Cleaver Scientific / UK

Table 3.1.B: Equipment used in this study with their Manufacturer Company and origin.

Equipment	Manufactured Company (Origin)
Eppendorf tubes with different size (2ml, 1.5ml); Disposable tips; PCR tubes	Extra gene / Taiwan
Baker; Inserted cylinder; Disposable (Gloves, Syringes, Gel tubes, EDTA tubes)	China
Micropipettes various sizes (0.5-10, 20-200, 100-1000) µl	Extra gene/Taiwan
Ice-box	Germany

3.3.2. Kits and Markers

Kits and marker used in this study with their Manufacturer Company and origin are listed in table (3-2).

Table 3.2: Kits and markers used in this study with their manufacturer company and origin

Kits	Manufacturer Company/ Origin	CAT.NO=
G-Spin Total DNA Extraction kit	Intron / Korea	14001
Human IL-17 A ELISA kit	Elabscience Biotechnology Inc.\USA	E-AB-70147
Viral Nucleic Acid Extraction kit	Intron / Korea	17151

3.3.3. Reagents and Buffers

Reagents and buffers used in this study are listed in table (3-3)

Table 3.3: Reagents and buffers used in this study with their Manufacturer Company and origin.

Reagents	Manufactured Company (Origin)
10X TBE DNA	Intron /S. Korea
Absolute Ethanol alcohol	Merck – Germany
Agarose E	Conda / Spain
DNA Ladder (1500-100bp)	Intron /S. Korea
Proteinase K	Intron /S. Korea
RNase A	Intron /S. Korea
De-ionized sterile distilled water	BioNeer / Korea
Master mix	Promega / USA
Nuclease free water	Promega / USA
DNA loading dye	Intron / Korea
RED Safe	Intron / Korea

3.3.4 Primes of Human Herpes Virus-6 A &B (HHV-6 A &B) and *IL-17 A* rs2275913 SNP.

Primers sets used in this study to detect the HHV-6 **A &B**, SNP of *IL-17 A* **rs2275913** polymorphism with their product size and source as well as origin are listed in table (3-4).

Table 3.4: Primers sets that used for detection of Human Herpes Virus-6 A&B (*HHV-6 A& B*), (*IL-17 A* rs2275913) gene polymorphism.

Gene	Sequence (5′-3′)	Product size (bp)	Source/ origin
HHV-6A (IF)	GGTTCCTGGCCCAAAACAGA	**529bp**	**IDT / USA**
(IR)	CGCGTGGATACGAAGAGACA		
HHV-6 B (IF)	CGGGATCAAAACCGCGAATC	**480 bp**	
(IR)	TTCATCGCCAGATGCCGTAG		
IL-17A **rs2275913 (IF)**	ACCTGGCCAAGGAATCTGTG	**526 bp**	**IDT / USA**
IL-17A **rs2275913 (IR)**	GCAAGAGCATCGCACGTTAG		
P14 rs3731249 **(IF)**	TATTTCCCATTTGCCGCCCT	**525 bp**	**IDT / USA**
P14 rs3731249 **(IR**	CCCTGGCTCTGACCATTCTG		

3.4 Methods

3.4.1 Detection of HHV-6 by Polymerase Chain Reaction (PCR)

3.4.1.1 Principles of Assay

Polymerase chain reaction (PCR) is based on two major processes: **Firstly, isolation** of viral genome (DNA\ RNA) from specimens, and **Secondly**, PCR amplification for each sample. In PCR the accumulating amplified product can be detected by gel electrophoresis.

3.4.1.2 Extraction of Viral Nucleic Acid from Clinical Specimens

By using specific viral DNA/RNA extraction kit (Intron/Korea); the viral genome was extracted, purified and migrated using agarose gel from the blood specimens as a first step to amplify the target Human Herpes virus-6 A&B DNA.

3.4.1.2.1 Principle of Viral Genome Extraction

Patho Gene-spinTM DNA/RNA extraction kit is specifically designed to isolate high-quality nucleic acids from a variety of pathogen and specimen using low elution volumes that allow sensitive downstream analysis. The purified RNA/DNA is free of proteins and nucleases, and is suitable for use in downstream applications that allow pathogen detection.

Patho G-spin™ DNA/RNA extraction kit uses the chaotropic salt in lysis buffer inactivates immediately DNase/RNase to ensure isolation of intact DNA/RNA. Patho Gene- spin™ DNA/RNA Extraction Kit uses advanced silica-gel membrane technology for rapid and effective purification of DNA or RNA without organic extraction or ethanol precipitation. Furthermore, the buffering conditions are finely adjusted to provide optimum binding of the DNA/RNA to the column.

The protocol is based on the lysis buffer that effectively dissolves and denatures virus envelope and capsid protein without additional enzyme treatment to easily elute viral gene. The binding buffer added after the elution helps to attach genes only to silica-gel membrane, and two different washing buffers efficiently remove proteins and other contaminants to get high purity viral gene. Swab and blood specimens were used same protocol.

3.4.1.2.2 Kit Contents

1) Lysis Buffer (35 ml).
2) Binding Buffer (35 ml).
3) Washing Buffer A (30 ml).
4) Washing Buffer B (10 ml). Was added 40 ml of EtOH before use.
5) Elution Buffer (20 ml).
6) Spin Columns inserted into a collection tubes. (2 ml tubes) (50 columns).
7) Instruction Manual (1 sheet).

3.4.1.2.3 Specimens Preparation

3.4.1.2.4 Assay Procedure

The procedure was carried out in accordance with the manufacturer's instruction with some modification as following:

1. Three hundred (300) microliters from blood plasma was transferred into 1.5ml micro centrifuge tube.
2. Six hundred (600) microliters Lysis buffer was added, then the lysate mixed by vortex for (25sec). Mixture was incubated at room temperature for (15 min).
3. Six hundred (600) microliters from binding buffer was added, and completely mix well by gently vortex. This step is conducive to efficient passage of cell lysates through a column and to increase binding onto column resins and it is important for effective deproteinization.
4. The lysates of cell were placed in a spin column that provided (2ml) collection tube.
5. Loaded lysates on the column and centrifuged for 2 min at 13,000 rpm.
6. Discarded solution in collection tube and place the spin column back in the same (2ml) collection tube.

7. Five hundred (500) microliters of washing buffer A was added to spin column and centrifuged for 2 min at 13,000 rpm.
8. The solution was discarded in collection tube and places the spin column back in the same (2ml) collection tube and centrifuged for 2 min at 13,000 g and then discarded solution.
9. Five hundred (500) microliters of washing buffer B was added to the spin column and centrifuged for 1min at 13,000 rpm.
10. The solution was discarded in the collection tube and places the spin column back in the same (2ml) collection tube. Centrifuged for 1min at 13,000 rpm. It is important to dry the membrane since residual ethanol may interfere with downstream reactions.
11. Placing spin column in an RNase-free (1.5ml) micro- centrifuge tube; then 50 µl of Elution buffer was added directly onto the membrane and was incubated at RT for 2min, then centrifuged for 2min at 13,000 rpm.
12. At this stage the supernatant was containing viral genome (DNA), then stored at -20°C.

3.4.1.3 Estimation of The Extracted DNA Concentration and Purity

After extraction of viral DNA from specimens; the concentration of RNA and DNA yield and purity were measured by using Nano drop (Korea), by applying 5 microliters of the extracted DNA in the instrument curette.

Extracted with purity in between (1.8-2) at absorption wave length (260/280) was included in this study, otherwise; DNA extraction of the sample was repeated.

3.4.2.Detection of *IL-17 A* rs2275913 *and P14* rs3731249 genes polymorphism by sequencing.

Total DNA for SNPs of *IL-17 A* **rs2275913** *and* ***P14*** **rs3731249** gene polymorphism were extracted from peripheral blood from patients using sequencing technique.

3.4.2.1 Principle of Assay

Is based on two major processes: extraction of total DNA from specimens and Polymerase chain reaction (PCR) is allows the amplification of a target region from a DNA template by using specific oligonucleotides. Finally, the PCR products of *IL-17 A* **rs2275913** *and* ***P14*** **rs3731249** SNPs sent to macrogene \ Korea to detection the variation of IL-17 A **rs2275913** *and* ***P14*** **rs3731249** polymorphism.

3.4.2.2 Extraction of Total DNA from Clinical Specimens

The G-spin™ Total DNA extraction kit is suitable for use fresh or frozen whole blood which has been treated with citrate, heparin, or EDTA. Pre-separation of leukocytes is not necessary. Purification does not require phenol/chloroform extraction or EtOH precipitation, and provides the simplest protocols. DNA is eluted in Buffer CE, TE (10:1), 10mM Tris (pH 7.5 ~ 8) or water, is prepared for direct addition to PCR or other enzymatic reactions. Alternatively, it can be safely stored at (–20°C) for later use. The purified DNA is protein-free, nucleases-free and does not include other contaminants or inhibitors. G-spin™ Total DNA extraction kit is optimized for extraction of (20-30kb) DNA fragments and able to extract up to 50 kb fragments.

All reagents required for the total DNA extraction were provided with DNA extraction kit (G-Spin total DNA extraction kit, Cat.No. 14001 Intron / Korea).

3.4.2.3 Kit Contents

The contents of G-Spin total DNA extraction kit are listed in table (3-5).

Table 3.5: List of reagents and buffers of G-Spin Total DNA extraction kit used in this study.

Label	Contents 50 Columns	Contents 200 Columns
Buffer CL	25 ml	90 ml
Buffer BL	25 ml	90 ml
Buffer WA	40 ml	160 ml
Buffer WB	14 ml	56 ml
Buffer CE	20 ml	40 ml
Spin Column / Collection Tube	50	200
RNase A (Lyophilized powder)	3 mg x 1 vial	3 mg x 4 vials
Proteinase K (Lyophilized powder)	22 mg x 1 vial	22 mg x 4 vials

3.4.2.4 Assay Procedure

The procedure was carried out in accordance with the manufacturer's instruction as following:

3.4.2.4.1 Extraction of Total DNA from Blood Specimens

1. Two hundred (200) microliters of whole blood was pipetted into a (1.5 ml) micro- centrifuge tube.
2. Adding 40 µl proteinase K Solution and 5 µl of RNase A solution into specimen tube and gently mixed.

3. Two hundred (200) microliters of Buffer BL was added into upper specimen tube and mixed thoroughly. This step is important in order to ensure effective decomposition, and it is important that the BL sample and buffer blood are thoroughly mixed to give a dissolution solution.
4. Placing the mixture at room temperature for (2minutes).
5. The lysate was incubated at 56°C for 10 min. For complete lysis, mix 3 or4 times during incubation by inverting tube. If it breaks down perfectly, the red color of lysate became dark green.
6. The 1.5 ml tube was centrifuged briefly to remove drops from inside the lid.
7. Adding (200 µl) of absolute ethanol into the lysate, and then mixed well by pulse vortex. After mixing, briefly centrifuged the (1.5 ml) tube to remove drops from inside of the lid. This step is an equilibration step for binding genomic DNA to column membrane. Also, this step conduces to pass efficiently cell lysate through a column.
8. The mixture from step 7 was applied carefully to the spin column (in a 2 ml collection tube) without wetting the rim, close the cap, and centrifuged at 13,000 rpm for 1 min. Discarded the filtrate and placed the spin column in a new (2 ml) collection tube (additionally supplied).
9. Seven hundred (700) microliters of Buffer WA was added to the spin column without wetting the rim, and centrifuged for 1 min at 13,000 rpm. Discarded the flow-through and reused the collection tube.
10. Seven hundred (700) microliters of Buffer WB was added to the spin column without wetting the rim, and centrifuged for 1 min at 13,000 rpm. Discarded the flow-through and placed the column into a new (2ml) collection tube (additionally supplied), then it was centrifuged again for additional 1 min to dry the membrane and discarded the flow-through and collection tube altogether.

11. Placing the spin column into a new (1.5 ml) tube (not supplied), and adding (50 µl) of Buffer CE directly onto the membrane, then incubated for (1 min) at room temperature and then centrifuged for 1 min at 13,000 rpm to elute, then stored at -20°C.

3.4.2.5 Measurement of Concentration and Purity of Extracted DNA

The DNA quantity and purity was determined using a spectrophotometer (Nano drop) at the absorbance at 260nm and 280nm respectively. The concentration of DNA was estimated at ng/ml and the purity calculated as 260/280 ratio, when the DNA solution is pure the ratio ranged (1.8 - 2).

3.5 Detection of HHV-6A&B ;*IL-17 A* rs2275913 *and P14* rs3731249 gene polymorphism by polymerase chain reaction (PCR)

3.5.1 Primers pairs dilution

The primers' source was from IDT / USA. Primers are commonly transported in a lyophilized state. The units of a lyophilized primer are given as a mass, in Pico moles. To create a stock of primers, one would reconstitute the primer in sterile, nuclease-free H_2O. The company supplies the amount of sterile, nuclease-free H_2O to be added to each primer to obtain master stock (100 mol/µl) that will be used again to obtain working solution. As following: The tube was spin down before opening the cap, then the desired amount of water was added according to the oligoes manufacturer to obtain a (100 pmol/µl master stock).

Vortex properly for re-suspend the primers evenly. Then 10µl of the master stock was transferred to a (2ml Eppendorf tube) that contains 90µl of sterile, nuclease-free H_2O (Working Solution 10 pmol/µl). The master stock is stored at -20 C^o and the working solution is stored at -20 C^o. Finally, the working solution was thawed on ice and vortex before using in PCR and then stored at -20 C^o.

3.5.2 PCR Experiments

PCR amplification was done using conventional thermal cycler (Biometra - Germany) as follows: Sample DNA (about 4 µl) was added into PCR master mix tubes. Forward and reverse primers were added (2 µl) into PCR master mix tubes (for each one). Distilled nuclease free water was added (5 µl) into PCR master mix tubes to a total volume of (25 µl) as the table (3-6).

Table 3.6: Recommended volumes and concentration for applying PCR into AccuPower® PCR tubes.

No.	Content of PCR Reaction Mixture	Volume/ µl
1	Master mix	12 µl
2	Forward primer	2 µl
3	Reverse primer	2 µl
4	Sample DNA	4 µl
5	Nuclease free water	5 µl
Total		**25 µl**

3.5.3 Thermal cycles conditions

Reactions were placed in a thermal cycler (Biometra-Germany) that had been preheated to 94°C and beforehand set up to the desired cyclic conditions. The target regions of **HHV-6A&B ;** ***IL-17 A*** **rs2275913 and** ***P14*** **rs3731249** polymorphism were amplified using specific primers according to the mentioned conditions in table (3-7).

Table 3.7: Amplification conditions of HHV-6 A&B and *(IL-17A* rs2275913) gene in patients with AML.

Gene	Initial denaturation	Denaturation	Annealing	Extension	Final extension	No. of cycles
HHV-6 A	95C^0 /5 min	95C^0 /1 min	54 C^0 /45 sec	72 C^0 /1 min	72 C^0 /5 min	40
HHV-6 B	95C^0 /5 min	95C^0 /1 min	54 C^0 /1 min	72 C^0 /2 min	72 C^0 /5 min	40
IL-17A rs2275913	95C^0 /5 min	95C^0 /1 min	53 C^0 /1 min	72 C^0 /2 min	72 C^0 /5 min	40
P14 rs3731249	95C^0 /5 min	95C^0 /1 min	58.6 C^0 /1 min	72 C^0 /2 min	72 C^0 /5 min	40

PCR products of target regions HHV-6 A& B; ; *IL-17 A* **rs2275913** and ***P14*** **rs3731249** polymorphism were electrophoresed on 1.5 % agarose at 85 V for 1h and visualized by safe stain. Photos were taken using gel documentation system.

3.6 Agarose Gel Electrophoresis Technique

The agarose gel electrophoresis was perfomed according to the method of Robinson and Lafleche (2000). This technique was used to detect viral genome; total DNA extracts, and PCR products.

3. 6.1 Preparation of Solutions and Buffers

3.6.1.1 Loading Buffer

The buffer was prepared from 0.25 % Bromophenol blue and 40% sucrose and stored at 4° C (Rushell and Sambrook, 2001).

3.6.1.2 TBE buffer (1X)

To prepare 500 ml of 1X TBE buffer, 50 ml of TBE (10X) stock solution was mixed with 450 ml of dH2O. Then the volume was completed to 500ml with dH2O.

3.6.2 Gel Electrophoresis Protocol

1. Device setup: The casting gates were sited on the ends of the gel tray and locked in place firmly against casting tray. This was done by engaging the "claws" of the gate in the recess of the side wall of the tray. The comb was sited into the slots of the gel tray, (1.0 mm above the base of gel casting tray) so that the sample wells are near the cathode.
2. Gel dissolving: 1.5g of agarose was dissolved in 100ml of (1X) TBE solution by melting to 100°C to prepare 1.5% agarose gel for migrated genomic DNA extracts, also 1% or/and 2% agarose gel was prepared in 1X TBE buffer for migrated PCR products or digested DNA by restriction enzymes respectively.
3. Gel casting: After agarose gel dissolving completely, it is let to cooling to approximately 60°C and (5µl) of the safe stain stock solution was added, then slowly pour the agarose into the gel- casting tray, and any air bubbles were removed. The comb was positioned at approximately (1.5cm) from one edge of the gel. The agarose was allowed to solidify at room temperature at least 30 min. After that, the claws were disengaged from the gel tray and the comb was separated gently. Then the gel was placed in the gel tank in such a way that the wells should be on end with the cathode.

(1X) TBE buffer (depending the purpose) was added to the buffer tank until it was about (5 mm) above the top of the gel.

4. Loading the samples: Each (4µl) of the genomic DNA sample was mixed with (1µl) loading dye briefly and loaded into the wells. Whereas, the PCR products were loaded without loading dye because of the PCR master mix contained loading dye.
5. Gel electrophoresis conditions: After sample loading the electric field was turned on at 5 V/cm (85V) for (60-120) min until bromophenol blue dye reached at the end edge of the gel.
6. The gel was photographed using gel documentation system (Cleaver Scientific - UK).

3.7. Sequencing

3.7.1 Nucleic Acids Sequencing of PCR Amplicons

The resolved PCR amplicons were commercially sequenced from both directions, forward and reverse directions, following the instruction manual of the sequencing company (Macrogen Inc. Geumchen, Seoul, South Korea). Only clear chromatographs obtained from ABI (Applied Bio system) sequence files were further analyzed, ensuring that the annotation and variations are not because of PCR or sequencing artifacts. By comparing the observed nucleic acid sequences of local samples with the retrieved nucleic acid sequences, the virtual positions, and other details of the retrieved PCR fragments were identified.

3.7.2 Interpretation of Sequencing Data

The sequencing results of the PCR products of the targeted samples were edited, aligned, and analyzed as long as with the respective sequences in the reference database using Bio Edit Sequence Alignment Editor Software Version 7.1 (DNASTAR, Madison, WI, USA). The observed variations in each sequenced

sample were numbered in PCR amplicons as well as in their corresponding position within the referring genome. The observed nucleic acids were numbered in PCR amplicons as well as in their corresponding positions within the referring genome. Each detected variant within the genome sequences was annotated by Snap Gene Viewer ver. 4.0.4 (https://www.snapgene.com).

3.8 Evaluation of *IL-17 A* Concentration in Blood Serum of Patients and AHC

The concentration of *IL-17 A* in the serum of patients with AML and AHC was evaluated by enzyme linked immunosorbent assay (ELISA).

3.8.1 Assay Principle

This kit is an Enzyme-Linked Immunosorbent Assay (ELISA). The plate has been pre-coated with Human *IL-17 A* antibody. *IL-17 A* present in the sample is added and binds to antibodies coated on the wells. And then biotinylated Human *IL-17 A* Antibody is added and binds to *IL-17 A* in the sample. Then Streptavidin-HRP is added and binds to the Biotinylated *IL-17 A* antibody. After incubation unbound Streptavidin-HRP is washed away during a washing step. Substrate solution is then added and color develops in proportion to the amount of Human *IL-17 A*.

The reaction is terminated by the addition of acidic stop solution and absorbance is measured at 450 nm.

3.8.2 Reagent Provided

Reagent provided of ELISA kits to evaluate IL- *IL-17 A* are listed in table (3-8).

Table 3.8: Reagent provided of ELISA kits to evaluate IL-17A.

Components	Quantity 96T	Quantity 48T

Components	Quantity 96T	Quantity 48T
Pre-coated ELISA Plate	12 * 8 well strips x1	12 * 4 well strips x1
Standard Solution (1600pg/ml)	0.5ml x1	0.5ml x1
Standard Diluent	3ml x1	3ml x1
Streptavidin-HRP	6ml x1	3ml x1
Stop Solution	6ml x1	3ml x1
Substrate Solution A	6ml x1	3ml x1
Substrate Solution B	6ml x1	3ml x1
Wash Buffer Concentrate (25x)	20ml x1	20ml x1
Biotinylated Human *IL-17 A*	1ml x1	1ml x1
User Instruction	1	1
Plate Sealer	2 pics	2 pics
Zipper bag	1 pic	1 pic

3.8.3 Assay Procedure

1. Preparing all reagents, standard solutions and samples as instructed and Bringing all reagents to room temperature before use. The assay is performed at room temperature.
2. Determining the number of strips required for the assay and Inserting the strips in the frames for use. The unused strips should be stored at 2-8°C.
3. Adding 50µl standard to standard well. Note: Don't add antibody to standard well because the standard solution contains biotinylated antibody.
4. Adding 40µl sample to sample wells and then add 10µl anti-IL-17 Aantibody to sample wells, then add 50µl streptavidin-HRP to sample wells and standard

wells (Not blank control well). Mix well. Cover the plate with a sealer. Incubate 60 minutes at 37°C.

5. Removing the sealer and wash the plate 5 times with wash buffer. Soak wells with at least 0.35 ml wash buffer for (30 seconds to 1 minute) for each wash. For automated washing, aspirate or decant each well and wash 5 times with wash buffer. Blotting the plate onto paper towels or other absorbent material.
6. Adding 50µl substrate solution A to each well and then add 50µl substrate solution B to each well. Incubate plate covered with a new sealer for 10 minutes at 37°C in the dark.
7. Adding 50µl Stop Solution to each well, the blue color will change into yellow immediately.
8. Determining the optical density (OD value) of each well immediately using a microplate reader set to 450 nm within 10 minutes after adding the stop solution.

3.8.4 Calculation of Results

Construct a standard curve by plotting the average OD for each standard on the vertical (Y) axis against the concentration on the horizontal (X) axis and draw a best fit curve through the points on the graph. These calculations can be best performed with computer-based curve-fitting software and the best fit line can be determined by regression analysis.

3.8.5 Typical data

This standard curve is only for demonstration purposes.

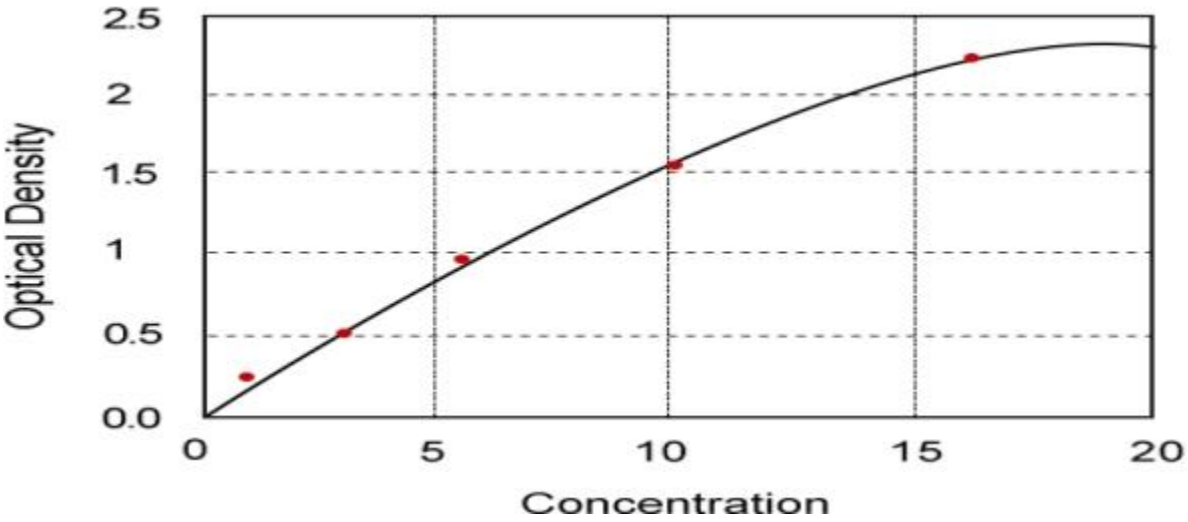

Figure 3.2: A standard curve should be generated with each assay.

3.9. Statistical Analysis

Statistical analyses were performed using Statistical Package of the Social Sciences (SPSS) version 23.0. Two-way ANOVA, One-way ANOVA, a hi-Square test (χ^2); Binomial test (Z- test); KolmogorovSmirnov (Z) test and Spearman's rho and Chi square (X2) were done to establish relationships of expression immunological variables levels according to the ELISA test results between patients with AML and without AML.

The correlation matrix between the selected variables and HHV-8 infection in current study was estimated by using Spearman's correlation coefficient analysis. Correlation coefficients were considered significant at P-values less than 0.05 by using SPSS version 23.0. Asterisk (*) indicates that the differences were statistically significant when compared with control group with patient groups. Chi square test (X2) was used to compare the selected groups ** $p<0.01$; *** $p<0.001$. Hierarchical cluster analysis based on similarity coefficient was used in this study to identify the relatively homogenous results using expression immunological variables levels according to the ELISA test results between patients with AML

and without AML using PRIMER-E7 software package (http://www.primer-e-.com/)(Clarke and Gorley, 2014).

Ethical Approval

The study was conducted in accordance with the ethical principles that have their origin in the Declaration of Helsinki. It was carried out with patients verbal and analytical approval before the sample was taken. The study protocol and the subject information and consent form were reviewed and approved by a local ethics committee according to the document number 7/17/1336 (including the number M220904 and the dte in 28/9/2022) to get this approval.

GHAPTER FOUR

Results

4. Results:

4.1. Clinicopathological Findings:

4.1.1. Distribution of study populotion, according to their age:

The blood specimens collected in this study were related to Acute myeloid leukemia patients whom ages were ranged from two years to eighty years. The mean age of the patients with Acute myeloid leukemia (**39.3 ±12.41** years). While, the mean age of apparently healthy control (AHC) was (**37.9± 11.17**) years. However, there was no significant difference between AML and AHC in age distribution Table (4.1).

Table 4.1: Distribution of Study Groups According to the Mean and Range of their Age (years).

Study groups	No.	Mean of age (years)	S. D	S. E	Range(years)	
					Minimum	Maximum
AML	100	39.3	12.41	1.979	2	80
AHC	100	37.9	11.17	2.403	8	70
Total	200	*p*-value = 0.3 No. sign. (*p*<0.05)				

4.1.2. Distribution of patients with acute myeloid leukemia, according to their age groups.

In Acute myeloid leukemia group, the most affected age group were from 21-40 years were constituting (48%), followed by the age group of 41 – 60 years (25%) and the lowest group of Acute myeloid leukemia was the age group of 2-20 and of 61 - 80 years were constituted 12%and 15%, respectively. The statistical analysis shows significant differences (P>0.05) among age groups as shown in the Table (4.2).

Table 4.2: Distribution of Age groups According to the Histopathological Diagnosis of Studied Groups.

Age groups /Year		STUDY GROUPS		
		A.H. Control	AML	Pearson Chi-Square (P-value)
≤ 20	N	20	12	
	%	20.0%	12.0%	
21 – 40	N	50	48	
	%	50.0%	48.0%	P=0.02
41 – 60	N	25	25	*Sign.
	%	25.0%	25.0%	(P>0.05)
61 – 80	N	5	15	
	%	5.0%	15.0%	
Total	N	100	100	

	%	100.0%	100.0%	

***** Significant differences for all age groups using Pearson Chi- square test at **P**>0.05 level.

4.1.3.: Distribution of The Patients with Acute myeloid leukemia According to Their Sex :

In this study, The highly percentage of sex of patients with AML results was males 57 % (57 out of 100 cases) hightly than females 43% (43 out of 100 cases)(Figure 4.1). The statistical analysis showed a significant difference (P>0.05) between Acute myeloid leukemi patients and control groups according to sex Table (4.3).

Table 4.3: Distribution of Study Groups According to Their sex.

Sex			STUDY GROUPS		
			A.H. Control	AML	Pearson Chi-Square (P-value)
	Male	No.	45	57	P=0.02 *Sign. (P>0.05)
		%	45.0%	57%	
	Female	No.	55	43	
		%	55.0%	43%	
Total		No.	100	100	

	%	100.0%	100.0%	

* Significant differences using Pearson Chi- square test at P>0.05 level.

4.2.Detection of Human Herpes Virus -6 (HHV-6) by Conventional Polymerase Chain Reaction (PCR) Technique

4.2.1. HHV-6 Associated with apparently healthy control :

In this study, all cases were negative; therefore they are excluded from the statistical analysis.

4.2.2. HHV-6A Genome Detection in AML Patients By Using PCR:

According to PCR, only 14% (14 out of 100) of the AML specimens results are positive for HHV-6A genome detection, while 28 out of 100 (28%) are negative for HHV-6A genome detection, as indicated in Tables (4-4) and Figures (4-1). The results of these groups of patients showed statistically significant differences (p = 0.008).

Table 4.4: The PCR results of HHV-6A DNA in AML specimens.

HHV-6A	AML (no.=100)	%	P-value
Negative	28	14%	Z test P=0.008
Positive	14	28%	Sign. (P<0.01)

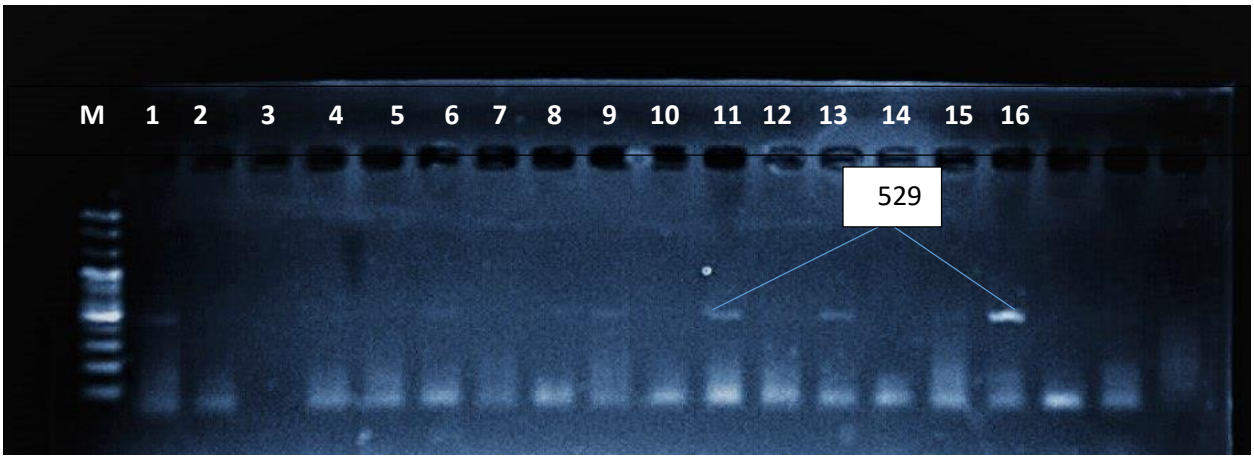

Figure 4.1: The electrophoresis pattern of HHV-6A DNA (529bp) detection in blood sample patients with AML. Lanes (9,11,13,16 and others) refers to HHV-6A DNA specimens; Electrophoresis conditions, 1.5% agarose,85 V, for 1h.

4.2.3. Detection Rates of HHV-6B in patients with AML by PCR:

According to PCR detection results, 16% (16 out of 100) of the specimens have HHV-6B genome, while 26%(26 out of 100) specimens showed negative results for HHV-6B genome detection, and as indicated in table (4-5) and figure (4-2). The statistical analysis of the differences between these 2 groups were significant ($p = 0.04$).

Table 4.5: The PCR results of HHV-6B DNA in AML specimens.

HHV-6B	AML (no.=100)	%	P-value
Negative	26	26%	Z test P=0.04
Positive	16	16%	sign. (P>0.05)

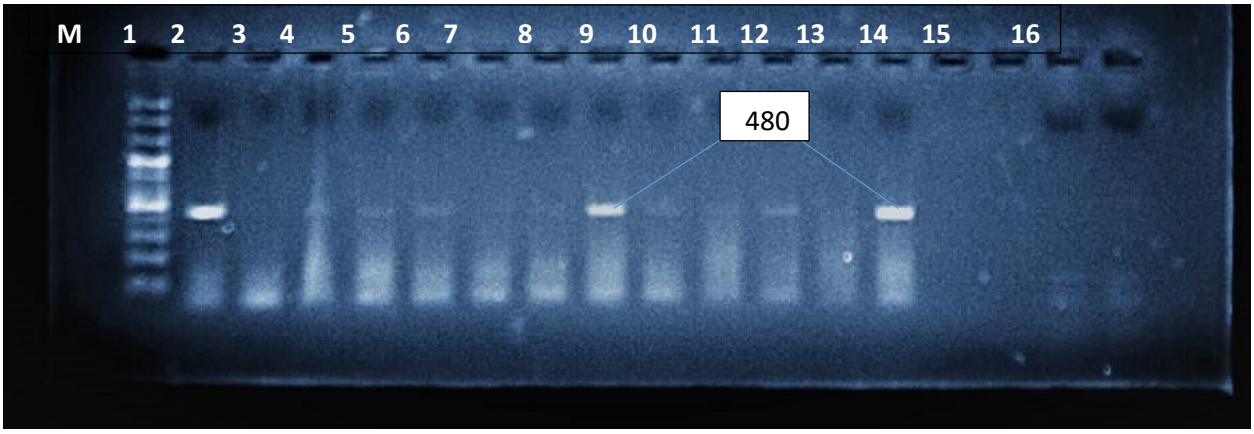

Figure 4.2: The electrophoresis pattern of HHV-6B DNA (480bp) detection in blood sample patients with AML. Lanes (1,8,13 and others) refers to HHV-6B DNA specimens; Electrophoresis conditions, 1.5% agarose,85 V, for 1h.

4.2.4. The Results of HHV-6 in the Study Group According to the Age of Patients:

Table (4-6) illustrated the positive results of HHV-6-DNA-PCR detection in patients with AML. The frequency percent of HHV-6A according to age groups (1-20 years), (21-40 years), (41-60 years) and (61-80 years) were 7.1%; 42.9 % ; 28.6% and 21.4 %, respectively. While, the frequency percent of HHV-6B according to age groups (1-20 years), (21-40 years), (41-60 years) and (61-80 years) were 12.5%; 43.8 % ; 31.3% and 12.5%, respectively. Significant differences (P<0.05) were found according to age groups(p-value > 0.05).

Table 4.6: HHV-6A & B-DNA according to the age of patients with AML.

	Groups		Total	P value
	HHV-6A	HHV-6B		

		Positive	Negative	Positive	Negative		
Age group	≤ 20	1	11	2	10	12	0.04
		7.1%	12.8%	12.5 %	11.9%	12%	
	21 – 40	6	42	7	41	48	
		42.9 %	48.8%	43.8 %	48.8%	48 %	
	41 – 60	4	21	5	20	25	
		28.6%	24.4%	31.3 %	23.8%	25 %	
	61 – 80	3	12	2	13	15	
		21.4 %	13.9%	12.5%	15.5%	15%	
Total		14	86	16	84	100	
		100%	100%	100%	100%	100.0%	

4.2.5. The Results of HHV-6(A&B)-DNA-PCR Detection in the Study Groups According to the Sex of Patients:

Table (4-7) illustrated the positive results of HHV-6-DNA-PCR detection ,The highly percentage of sex of patients with AML that have positive HHV-6 A-DNA PCR results was males 64.3% (9 out of 14 cases) followed by females 35.7% (5 out of 14 cases).

The highly percentage of sex of patients with AML that have positive HHV-6 B-DNA PCR results was males 62.5% (10 out of 16 cases) followed by females 37.5% (6 out of 16 cases). Statistical analysis revealed significant differences in sex whom are positive for HHV-6A&B-DNA PCR ($P<0.05$).

Table 4.7: HHV-6 A&B -DNA-PCR according to the sex of patients with AML.

		AML				
Sex		**HHV6A**		**HHV6B**		**P value**
		Positive	Negative	Positive	Negative	
Sex	**Male**	9	48	10	47	0.038
		64.3%	55.9%	62.5%	55.1%	
	Female	5	38	6	37	
		35.7%	44.1%	37.5%	44.1	
Total		14	86	16	84	
		14%	86%	16%	84%	

4.4. The Results of *IL-17 rs2275913* and P14 rs3731249 SNPs.

4.4.1. Extraction Total Genome DNA From Blood Specimens:

By using specific Total genome DNA extraction kit (G-Spin total DNA Extraction kit, Intron / Korea) the genomic DNA Figure (4-4) was extracted, purifying and migrated using agarose gel from the blood specimens of patients with AML as well as apparently healthy control(AHC) groups.

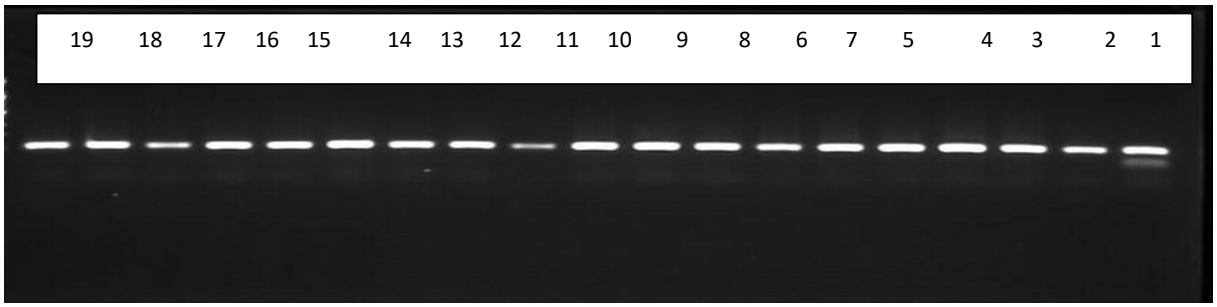

Figure 4.3: The electrophoresis pattern of gnomic DNA extracted from blood samples of patients with AML and healthy control groups. Lane 1 lane 19 refers to genomic DNA samples (1-10 patients with AML & 11-19 control) ; Electrophoresis conditions, 1.5% agarose, 85 V, 20 mA for 1h (5 µl in each well), stained with red safe solution.

4.4.2.1. Genotyping of *IL-17 rs2275913* Among Study Groups.

To summarize the results obtained from the sequenced 526 bp fragments, the detailed positions of the observed variations are described in the NCBI reference sequences as shown in Table (4-8).

The results showed that DNA polymorphism distribution were DNA polymorphism distributions according to A\A; A\G; A\C ;G\C and C\C were 23.3% ; 26.7% ;33.3% 16.7% and 0%, respectively in patients with AML and 35% ; 40%;15% ;10% and 0% , respectively in AHC group. The difference in frequency of genotype distribution of the polymorphism between patients and controls groups was statistically significant Table (4-8).

Table 4.8: Comparison between patient with and without AML based on percentages of*IL-17 rs2275913*expressed gene polymorphism.

Conformational Polymorphism of *IL-17rs2 275913* gene	**Type of Mutation**	**Study group**				**OR Patients**	***P value***
		AML NO\ (30)		**AHC NO\20**			
		NO	**%**	**NO**	**%**		
A\A	Transition	7	23.3	7	35	0.45 (0.49-1.55)	0.05
A\G	Transition	8	26.7	8	40	0.53 (0.55-1.18)	0.04
A\C	Transversion	10	33.3	3	15	1.26 (0.46-3.43)	0.02
G\C	Transition	5	16.7	2	10	1.26 (0.46-3.43)	0.04
C\C	Transition	-	-	-	-	-	-
TOTAL		30	100	20	100		
ALLE							

LE							
A		40		38		1.32(0.72-2.41)	
C		60		62			

4.4.2.2. The Alignment Results of *IL-17 rs2275913*gene, located on chromosome 6q region by bio edit program version 7.2.5:

The alignment results of the 526 bp samples revealed the presence of only one variation in all of the analyzed samples in comparison with the referring reference DNA sequences

The sequencing results observed that many SNPs between the one resolved haplotypes and between the ***IL-17 rs2275913***, exon4 for Primer3 plus reference sequence . The results appeared in the presence of fourteen SNPs Figure (4-7). Which revealed that which located at position 59 a substitution mutation (A→G) , position 99 substitution (C→ A) , position 336 substitution (C→ A) , position 387 substitution (A→ G) , position 421 substitution (A→ C) , position 423 substitution (G→A) , position 436 substitution (C→ G), and last one at position 456 substitution (A→ G) **(Appendix 1).**

```
          10        20        30        40        50        60        70        80        90       100
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Reference
ACCTGGCCAAGGAATCTGTGAGGAAAAGAAAGATCAAATGGAAAA
TCAAGGTACATGACACCAGAAGACCTACATGTTACTTCAAACTTTT
TCTTCCTCA
sample1   .....................................................................C.....A.G.....................
sample2   .....................................................................................
```

sample3 .....
sample4 .....
sample5 .....
sample6 .....C.....ACG.....
sample7 .....
sample8 .....C.....ACG.....
sample9 .....G.....ACG.....
sample10 .....

110 120 130 140 150 160 170 180 190 200
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|

Reference
TGAACCATTAAAATAGAGCATAACTCTTCTGGCAGCTGTACATATG TTCATAAATACATGATATTGACCCATAGCATAGCAGCTCTGCTCAG CTTCTAAC
sample1 .....
sample2 .....
sample3 .....
sample4 .....
sample5 .....
sample6 .....
sample7 .....
sample8 .....
sample9 .....
sample10 .....

210 220 230 240 250 260 270 280 290 300
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|

Reference
AAGTAAGAATGAAAAGAGGACATGGTCTTTAGGAACATGAATTTCT GCCCTTCCCATTTTCCTTCAGAAGGAGAGATTCTTCTATGACCTCA TTGGGGGC
sample1 .....A.....
sample2 .....
sample3 .....
sample4 .....
sample5 .....A.....
sample6 .....A.....

sample7 **.....**
sample8 **.....**
sample9 **.....**
sample10 **.....A.....**

310 320 330 340 350 360 370 380 390 400

....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|

Reference
GGAAATTTTAACCAAAATGGTGTCACCCCTGAACCCACTGCGACAC GCCACGTAAGTGACCACAGAAGGAGAAAAGCCCTATAAAAAGAGA GACGATAGC
sample1 **.....**
sample2 **.....**
sample3 **.....**
sample4 **.....**
sample5 **.....**
sample6 **.....**
sample7 **.....**
sample8 **.....**
sample9 **.....G.....**
sample10 **.....**

410 420 430 440 450 460 470 480 490 500

....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|

Reference
GCTACATTTTGTCCATCTCATAGCAGGCACAAACTCATCCATCCCC AGTTGATTGGAAGAAACAACGATGACTCCTGGGAAGACCTCATTGG TGGTGAGT
sample1 **.....**
sample2 **.....**
sample3 **.....**
sample4 **.....**
sample5 **.....**
sample6 **.....**
sample7 **.....**
sample8 **.....**
sample9 **.....**
sample10 **.....**

```
              510       520
         ....|....|....|....|....|.
Reference  CCTGCACTAACGTGCGATGCTCTTGC
sample1    .........................
sample2    .........................
sample3    .........................
sample4    .........................
sample5    .........................
sample6    .........................
sample7    .........................
sample8    .........................
sample9    .........................
sample10   .........................
```

4..4.2.3. New recording in gene bank NCBI and American bank ACCESSION NUMBERS was recorded

LC770961 ; LC770962

4.4.3. Genotyping of P14 rs3731249 SNP in AML and AHC

For **P14 rs3731249** genotyping, the genomic DNA was amplified using specific primers The results revealed that the presence a single band (525 bp) of the target sequence of **P14 rs3731249** gene in agarose gel Figure (4-5).

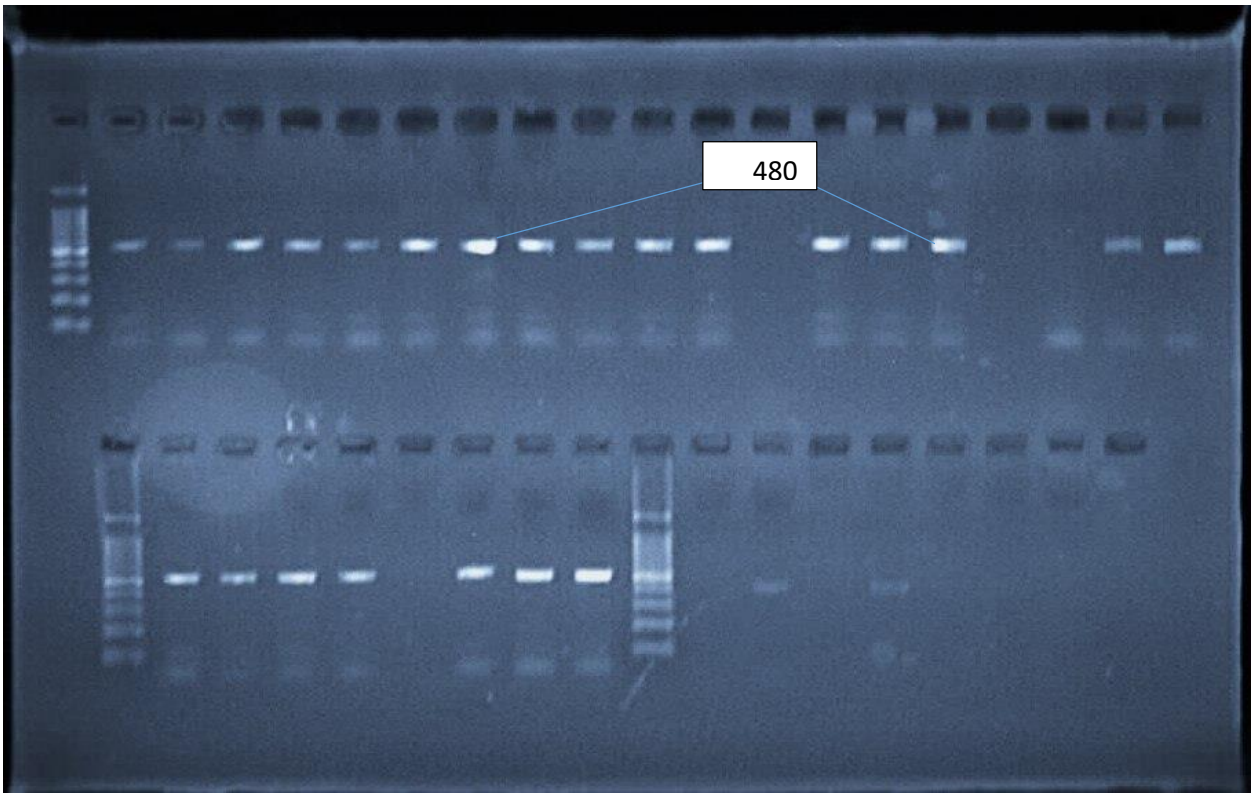

Figure 4.5: Agarose gel electrophoresis of an amplified product patterns of P14 rs3731249 exon4 region. Electrophoresis conditions, 1.5% agarose, 85 V, 20 mA for 1h (5 µl in each well), stained with red safe solution.

4.4.3.1. Genotyping of P14 rs3731249 Among Study Groups.

To summarize the results obtained from the sequenced 525 bp fragments, the detailed positions of the observed variations are described in the NCBI reference sequences as shown in Table (4-9).

The results showed that DNA polymorphism distribution were DNA polymorphism distributions according to A\A; C\G; A\C ;C\T; T\G ;T\A andC\C were 20% ; 30 % ;36.7% ; 6.7%,5% ;5% and 0% respectively in patients with AML and 45% ; 35%;10% 10% ;0%;0% and 0% , respectively in AHC group.

The difference in frequency of genotype distribution of the polymorphism between patients and controls groups was statistically significant Table (4-9).

Table 4. 9: Comparison between patient with and without AML based on percentages of P14 rs3731249 expressed gene polymorphism.

Conformational Polymorphism of P14 rs3731249 Gene	Type of Mutation	Study group				OR Patients	*P value*
		AML NO\ (30)		AHC NO\20			
		NO	%	NO	%		
A\A	Transition	6	20	9	45	0.48 (0.43-1.11)	0.07
C\G	Transition	9	30	7	35	0.59 (0.50-1.48)	0.06
A\C	Transversion	11	36.7	2	10	1.38 (0.80-3.78)	0.02
C\T	Transition	2	6.7	2	10	1.06 (0.34-	0.04

						3.74)	
T\G	Transversion	1	5	-	-	-	0.02
T\A	Transversion	1	5	-	-	-	0.02
C\C	Transition	-	-	-	-	-	-
TOTAL		30	100	20	100		
ALLELE							
G		45		34		1.90(0.65-2.22)	0.04
C		55		66			

4.4.3.2. The Alignment Results of P14 rs3731249 gene, located on chromosome 9q region by bio edit program version 7.2.5:

The alignment results of the 525 bp samples revealed the presence of only one variation in all of the analyzed samples in comparison with the referring reference DNA sequences**.**

The sequencing results observed that many SNPs between the one resolved haplotypes and between the **P14 rs3731249** , exon4 for Primer3 plus reference sequence . The results appeared in the presence of fourteen SNPs . Which revealed that which located at position 30 a substitution mutation (G →C) position 46 a substitution mutation (G→C) , position 63 substitution (T→ A) , position 72 substitution (C→ A) , position 94 substitution (G→ A) , position 212 substitution (C→ G) , position 214 substitution (G→C) , position 354 substitution (G→ T), position 366 substitution (T→ G), , position 378 substitution (C→ T), position 407

substitution (G→ A), position 457 substitution (G→ A), position 485 substitution (G→ A), position 503 substitution (G→ A) and last one at position 504 substitution (A→ G) **(Appendix 2).**

```
          10        20        30        40        50        60        70        80        90       100
     ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Reference
TATTTCCCATTTGCCGCCCTGGCGGGGCAGGGCGATAGGGAGACT
CAGGCCGTCCCACCGATTGGCGCGTGAGCTGAGGCAAGACCGGAG
ACTGGTCTCC
sample1   ..........................................................................................
sample2   ..........................................G.............A........C.....................
sample3   ..............................C~..........................................................
sample4   ..........................................G.............................................
sample5   ..........................................................................~.~.............
sample6   ..........................................~.............................................
sample7   ..............................C.............................................
sample8   ..........................................................................A......
sample9   ..........................................................................
sample10  ..........................................................................

          110       120       130       140       150       160       170       180       190
200
     ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Reference
CGGGCTGAACTTTCTGTGCTGGAAAATGAATGCTCTGAGCTTTGGA
AGCTCTCAGGGTACAAATTCTCAGATCATCAGTCCTCACCTGAGGG
ACCTTCCG
```

sample1 .....
sample2 .....
sample3 .....
sample4 .....
sample5 .....
sample6 .....
sample7 .....
sample8 .....
sample9 .....
sample10 .....

210 220 230 240 250 260 270 280 290 300
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|

Reference
CGGCATCTATGCGGGCATGGTTACTGCCTCTGGTGCCCCCCGCAG CCGCGCGCAGGTACCGTGCGACATCGCGATGGCCCAGCTCCTCAG CCAGGTCCAC
sample1 .....
sample2 .....
sample3 .....
sample4 .....
sample5 .....**G.C**.....
sample6 .....
sample7 .....
sample8 .....
sample9 .....
sample10 .....

310 320 330 340 350 360 370 380 390 400
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|

Reference
GGGCAGACGGCCCCAGGCATCGCGCACGTCCAGCCGCGCCCCGGC CCGGTGCAGCACCACCAGCGTGTCCAGGAAGCCCTCCCGGGCAGC GTCGTGCACG
sample1 .....
sample2 .....
sample3 .....
sample4 .....

sample5 .....G.....
sample6 .....
sample7 .....
sample8 .....T.....
sample9 .....
sample10 .....T.....

410 420 430 440 450 460 470 480 490 500
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|

Reference
GGTCGGGTGAGAGTGGCGGGGTCGGCGCAGTTGGGCTCCGCGCC GTGGAGCAGCAGCAGCTCCGCCACTCGGGCGCTGCCCATCATCAT GACCTGCCAGA
sample1 .....
sample2 .....
sample3 .....
sample4 .....
sample5 .....
sample6 .....A.....G.....
sample7 .....
sample8 .....
sample9 .....
sample10 .....T.....

510 520
....|....|....|....|....|

Reference GAGAACAGAATGGTCAGAGCCAGGG
sample1 ..AG.....
sample2 .....
sample3 .....
sample4 .....
sample5 .....
sample6 .....
sample7 .....
sample8 .....
sample9 .....
sample10 .....

4..4.3.3. New recording in gene bank NCBI and American bank ACCESSION NUMBERS was recorded
LC770963 ; LC770964

4.5. Evaluation of Serum IL-17 concentration By ELISA Among Study Population:

Table (4-10) shows the mean of serum IL-17 concentration for AHC and patients with AML groups were 8.00 ±0.31pg./ml and 14.00 ±0.59 pg./ml ,respectively.

Statistically, significant difference ($p<0.05$) was found on comparing the mean of serum IL-17concentration among these study groups.

Table 4.10: Results of serum IL-17 concentration by ELISA for AHC and AML patients

IL-17	AHC (pg/ml)	AML (pg/ml)
Mean± SE	8.00 ±0.31	14.00 ±0.59
LSD	**5.65**	
P value	***P*<0.05 (0.001) ***	

4.6.Correlation between HHV6A&B; *IL-17 Concentration* ; SNPs of *IL-17 rs2275913* Gene Polymorphism Among Study Population.

Human Herpesvirus-6A&B was statistically significantly correlated with the patients with AML group when compared with apparently healthy control group (p >0.001, OR=22.42).

The SNP ***IL-17 rs2275913*** polymorphism was significantly correlated with the AML group who had viral genome when compared with the healthy control group (p>0.001, OR= 37.8). The ***IL-17 Concentration*** was significantly correlated with AML group who had viral genome when compared with the healthy control group (p <0.001, OR 48.4) Table (4-11).

Table 4. 11: Correlation between between HHV-6A&B ; *IL-17 Concentration* and SNPs of *IL-17 rs2275913* gene polymorphism among study population.

	Patients with viral genome (n=42)	Control (n=25)	P-value	OR(95%CI)
HHV-6	28(56%)	0(0%)	P> 0.01	22.42 (0. 9 – 1)
IL-17 rs2275913	48(64%)	12(48%)	P> 0.01	37.8 (1.850-0.98)

IL-17 Concentration (rs2275913)	**38 (50.7%)**	**10(40%)**	**P> 0.01**	**48.3 (1.667-1.37)**

4.7. Spearman's Rho Statistical Testing of Age, gender, HHV-6A&B and SNPs of *IL-17 rs2275913* ; P14 rs3731249 To Evaluate The Studied Markers In Study Population.

A strong positive relationship (with highly significant correlation) was found between HHV-6A&B and SNP ***IL-17 rs2275913***in AML (r = 0.968, P = 0.007). Similarly, there is a strong positive relationship (with highly significant correlation) between HHV-6A&B and IL-17 in AML (r = 0.984 , P = 0.008).

In addition, A strong positive relationship (with highly significant correlation) was found between HHV-6A&B and SNPs of ***IL-17 rs2275913*** as well as **P14 rs3731249** according to ages patients who AML (r=0.855, P= 0.001) ; (r=0.788, P= 0.009) and (r=0.739, P= 0.004), respectively. However, there are no significant correlations among HHV-6A&B and SNPs of ***IL-17 rs2275913*** according to the sex of study population (and as illustrated in Table 4 -12).

Table 4. 12: Spearman's Rho Statistical Testing of Age, Gender, HHV-6A&B ;SNPs of *IL-17 rs2275913* and P14 rs3731249 To Evaluate The Studied Markers In AML.

Spearman's rho		**Age groups** (years)	***IL-17 rs2275913***	**P14 rs3731249**	**HHV-6A&B**
HHV-6A&B	R	0.855**	**0.986	**0.984	

	P	0.001	0.007	0.0008	
IL-17 rs2275913	R	0.788**			
	P	0.009			
P14 rs3731249	R	0.739**			
	P	0.004			
Sex	R	0.166	-0.149	0.123	0.145
	P	0.249	0.477	0.512	0.034

GHAPTER FIVE

discussion

5. Discussion

5.1 Risk factors of Leukemıa

The pathogenesis of AML is currently unclear. Although many studies have revealed that viruses may be the main factor. In addition, some cytotoxic substances (including alkylating agents and topoisomerase inhibitors, ionizing radiation, benzene, and other risk factors) can also cause chromosome breakage and susceptibility. This can make the position of the oncogene move and become activated and cell mutation and immune function to decline, which is conducive to the occurrence of leukemia (Ye *et al*.,2019; Young *et al*.,2019).

However, the medical community has not yet clarified whether the causes of hair dyeing, smoking, obesity, green tea, radiation, and other lifestyle factors have a certain impact on the occurrence of leukemia. With the continuous modernization of lifestyles, these possible risk factors for the onset of leukemia have become more and more closely related to people's lives (Atteya *et al*.,2020).

Besides, multiple studies reported that race is a vital factor affecting the mortality rate of AML (Yi *et al*.,2020; Guo *et al*.,2022).

China is a major hepatitis country, and one-tenth of population is a carrier of HBV. At the same time, whether family history of tumors is a risk factor for predicting the onset of AML is also a controversial issue (Logan *et al*.,2020).

For children, in addition to the above factors, they will also be affected by maternal factors related to pregnancy. For example, relevant studies show that maternal hair perming, drinking and smoking during pregnancy will increase the risk of leukemia in children (Timms *et al*.,2019). Therefore, it is necessary to explore the causes of AML.

5.2.Evaluation of Some Clinico-Pathologic Criteria of Patients acute myeloid leukemia

The current study updates the recent insight of acute myeloid leukemia (AML) is a cancer of the blood and bone marrow. It is the most common type of acute leukemia in adults. This type of cancer usually gets worse quickly if it is not treated. AML is also called acute myelogenous leukemia and acute nonlymphocytic leukemia.

In the first part of the study, some clinical and pathological information of acute myeloid leukemia were investigated to explore this challenging group of malignancies in relation to their existing information.

5.2.1. Distribution of the patients with AML according to their age :

Acute myeloid leukemia (AML), with a yearly incidence in Europe of 5–8 cases per 100,000 individuals, is cancer predominantly of the elderly, with a median age at diagnosis of 67 years. In the last decades, unlike for young adults, overall survival (OS) has not changed meaningfully for the elderly ones, with less than 10% of patients older than 65 years being alive 5 years after the diagnosis(Palmieri *et al*.,2020).

The current study , the mean age of the patients with Acute myeloid leukemia (39.3 ±12.41 years). While, the mean age of apparently healthy control (AHC) was (37.9± 11.17) years. the most affected age group were from 21-40 years were constituting (48%), followed by the age group of 41 – 60 years (25%) and the lowest group of Acute myeloid leukemia was the age group of 2-20 and of 61 - 80 years were constituted 12%and 15%, respectively. These results consistent with Hossain and Xie , (2015) who detected sex difference in the survival of AML patients diagnosed at ages 0-24 years.

While , current results inconsistent with Yoo *et al*.,(2021) who revealed an age of patients with AML increased, those in the favorable-risk group decreased rapidly, especially among those older than 50 years of age.

The median age, among unselected subjects, in most studies from Europe and North America are in the range of 58–63 years (the majority being above 55 years) (Preiss ,2003).

Reports from Japan and Australia show a mean age of 51 and 52 years, respectively (Nakase, 2016) .

Considering that the life expectancy of people aged 65 in the Western world is of approximately 15–20 additional years, the negative social impact of AML is evident (OMS ,2019). The reasons for poor outcomes of older patients can be attributed to both patient- and disease-related features. Advanced age is frequently associated with low performance status (PS), comorbidities, and organ impairment, most of them likely to evolve into organ failures, discouraging the use of intensive remission-oriented therapies (Mohammadi *et al*.,2020).

Several registry-based studies have reported the existence of a strong relationship between age and the clinical characteristics of AML and poor outcomes of older adults have been well described, regardless of intensive treatments or transplantation . Some potent factors including decreased performance status, comorbidities, and different compositions of cytogenetic risk are considered to correlate with worse outcomes of AML in conjunction with aging (Juliusson *et al.*,2009; Klepin *et al.*,2014).

The reasons for the difference between age at presentation in subjects from the various regions are not clear. Whether this represents a true geographic/ethnic difference or merelythe result of varying referral patterns is difficult to ascertain.

5.2.2. Distribution of the patients with acute myeloid leukemia according to their sex :

AML is a heterogeneous entity with many clinical and biologic factors influencing treatment response and survival. In our study, gender, age, and region of diagnosis were independently associated with overall survival in AML (Utkarsh *et al*.,2018).

The current results were showed highly percentage of sex of patients with AML results was males 57 % (57 out of 100 cases) followed by females 43% (43 out of 100 cases).These results compatible with Hossain, and Xie, (2015) who compared to females, male patients had substantially increased risk of mortality in the following subgroups of: ages 20–24 years at diagnosis (aHR1.30), Caucasian (1.14), acute promyelocytic leukemia (APL) (1.35), acute erythroid leukemia (AEL) (1.39), AML with inv(16)(p13.1q22) (2.57), AML with minimum differentiation (1.47); and had substantially decreased aHR in AML *t*(9;11)(p22;q23) (0.57) and AML with maturation (0.82).

Sex differences in tumor biology may be an important factor in the observed sex differences in survival after a childhood cancer diagnosis (Lindsay *et al*.,2019).

This study suggests that female gender may serve as a favorable risk factor in AML, which is consistent with a publication reporting on younger patients with AML . While the exact mechanism accounting for this disparity is unclear, it certainly alludes to the potential role of hormonal variations in altering disease biology and social influences

Utkarsh *et al*.,(2018) who explained patients were included in our analyses were increasing age (HR 1.2,p<.0001),male gender (HR 1.05,p¼.01), and geographic region of Midwest (HR 1.07,p¼.002) were associated with inferior 3-year overall surviva in univariate analysis, and these parameters remained independent prognostic factors in multivariate analyses.

5.3 Detection of HHV-6 in Patients with AML by PCR

5.3.1 Detection of HHV-6 Genome in Patients with AML by PCR .

Chromosomal integration of *human herpesvirus 6* (HHV-6) is a novel situation found in a small percentage of individuals. While active HHV-6 infection is treatable using antivirals, the abnormally high level of HHV-6 DNA found in chromosomal integration of HHV-6 (CI-HHV-6) is not affected by such drugs (Petr *et al*.,2009).

As this is up to what we know the first study in Mid-Euphrates of Iraq for detection of HHV-6 DNA in AML, it's reasonable to compare the present result with relatively similar world-wide studies done for similar purposes of detection HHV-6-DNA in the blood specimens by PCR.

The current results were revealed only 14% (14 out of 100) and **16**% (16 out of 100) of the AML specimens results are positive for HHV-6A and HHV-6 B genome detection ,respectively. The present result of HHV6 is compatible with Petr *et al*.,(2009) who found the percent of HHV6 in in patients with AML was 26.8% (107 samples from 91 patients).

The identification of only HHV-6 B species in this study is in accordance with the results obtained by seror *et al*., (2008), but differs from those obtained by Hermouet *et al*. (2003) who identified 89% of HHV-6A species, and 11% of HHV-6B . This may be linked to the high number of children included in our

study, the frequent occurrence of HHV-6 infections during childhood and to inter-laboratory technical discrepancies. The clinical symptoms related to HHV-6 were studied to establish a link with HHV-6 activation but no correlation was observed in these patients.

Voigt *et al*.,(2021) analyses of the cerebrospinal fluid (CSF) and blood serum were positive for HHV-6.

According to study of Handous *et al*.,(2020) who findings, the pre-chemotherapy serologic status for HHVs (EBV, VZV, CMV, HSV, HHV-6) were very high, ranging from 81 to 97.9%. Thus, most of the patients were seropositive before chemotherapy, and most probably viral DNAemia during chemotherapy was due to reactivation or reinfection under immunosuppressive conditions rather than primo-infection.

In contrast , current results inconsistent with Handous *et al*.,(2020) who found the percent of HHV6 in in patients with AML was 9.7% .

Faten *et al*., (2015) was detected the HHV-6 in 16%, 9%, 29%, and 25% at diagnosis, aplasia, remission, and relapse, respectively. The prevalence of HHV-6 was significantly higher at remission than at diagnosis (P ¼ 0.04) or during aplasia (P ¼ 0.002) and higher at relapse than at diagnosis (P ¼ 0.07) or during aplasia (P ¼ 0.01). No significant differences were observed for the HHV-6 loads at different disease stages in the blood samples.

In the latters, the prevalence of HHV-6 was higher at remission and relapse than at diagnosis and aplasia, confirming the reactivation of HHV-6 after chemotherapy (Faten *et al*., 2012).

These data showed a correlation between HHV-6 load in blood and lymphocyte counts and a high correlation with the count of polymorphonuclear leukocytes in

favor of a predominant contribution of the polymorphonuclear leukocytes to HHV-6 load in whole blood, as reported in a previous study (Palleau *et al*., 2006).

The concept of the relationship between HHV-6 and AML is based on the identification of HHV-6 genome sequences in blood, and antigen has also been found in villous tissue (Faten *et al*., 2020).

Human herpesvirus 6 (HHV-6) can integrate into human chromosomes, resulting in genetic transmission from parent to child. Individuals of either sex with inherited chromosomally integrated human herpesvirus 6 (iciHHV-6) harbor the virus in every cell. Viral reactivation from the integrated HHV-6 genome can occur in pregnancy (Miura *et al.*,2021). The authors suggest two possible mechanisms by which reactivated HHV-6 from an integrated viral genome could cause AML : (1) direct cellular damage or (2) bystander effects (such as upregulation of inflammatory cytokines) induced by the viral reactivation.

The majority of congenital HHV-6 infections are thought to be secondary to chromosomal integration of the virus into different human chromosomes within the whole genome, which is a proven phenomenon (Hall *et al*.,2008). Thus, there are 5 possibilities of congenital HHV-6 infection in an infant like our patient:

1.She had ciHHV-6 but no active infection; however, these patients would be asymptomatic with evidence of HHV-6 DNA but no IgM antibodies.
2.She had HHV-6 with active infection from a HHV-6+ mother who reactivated the virus during pregnancy and subsequently transmitted the active virus, but it is impossible to differentiate active infection in a HHV-6 patient using PCR methods alone.

3.She did not have HHV-6 but had evidence of active infection from a HHV-6 mother who reactivated and transmitted the replicating HHV-6 virus transplacentally.

4. She acquired it transplacentally from a mother re-infected with HHV-6 or whose latent HHV-6 reactivated with no evidence of maternal HHV-6.

5. She acquired it postnatally from another person in the NICU about 2 weeks prior to the onset of symptoms.

The opinion of authors of current study , the differences in percentages of HHV-6 detection among the present as well as these studies could be attributed to the site of infection ,genetic as well as environmental factors, the quality & sensitivity of the techniques used in these studies. However, most of the studies done in this respect have included a small numbers of AML cases and for better revealing of the importance of HHV6 in AML patients,this may need enrollment of large case-control studies.

5.4 The Results of Gene Polymorphism of *IL-17rs2275913* SNP.

Th17 cells with their secretory cytokines are considered essential controllers in inflammatory and autoimmune disorders .They represent a curious research point. Many studies proposed the potential effect of Th17 cells on solid tumors but their role in haematological malignancies is still under investigations (Abousamra *et al.*,2013).

To our knowledge, the published data on the role of the IL-17A polymorphisms in AML are limited *;* thus, our report is considered a novel finding not previously studied in Iraqi population. The authors did not contribute Il-17 polymorphism to the disease incidence in Egyptian cases with AML. Respectively, these results should be re-assessed in a more extended study, including multicentric patients.

The results showed that DNA polymorphism distribution were DNA polymorphism distributions according to C\A; A\G; A\C ; G\A; and C\G heterozygote were 2% ; 9% ; 1% ; 1% ; and 1%, respectively in patients with AML and 20% ; 0% ; 0% ; 20% and 40% , respectively in AHC group. These results agreement with Rania *et al.*,(2020) who found IL-17A homozygote mutation was more frequent in AML patients compared to control subjects ($P = 0.034$) and conferred 2.8 fold increased risk of AML (OR = 2.755 [95% CI: 1.078–7.042]). While IL-17F mutation showed no correlation with acute myeloid leukemia susceptibility. Also neither IL-17 A nor IL 17-F mutation showed significant correlation to therapy outcome. In conclusion, IL-17 A homozygote mutation may be associated with AML susceptibility.

Regarding IL-17 ; our results were in consistent to Wrobel *et al.*,(2015) and Zhu *et al.*,(2014) who stated that **IL-17** [rs763780; **A7488G]** polymorphism was proved to be correlated with a predisposition to AML in Poland and China respectively. IL-17 G homozygous genotype was significantly more frequently observed among patients than healthy individuals. While, the other showed statistically significant increase in IL-17F GG homomutant genotype among AML patients compared to control group (Mahfouz *et al*., 2018).

In contrast these results incompatible with Elsissy *et al.*,(2019) who found IL-17F, IL-17A mutant genotypes and alleles showed no significant relation with acute myeloid leukaemia incidence. Also, ELISA results proved that serum IL-17 did not vary between acute myeloid leukaemia patients and healthy subjects.

IL-17A; A mutant allele was proved to be a critical factor involved in IL-17 regulation in comparison to G wild allele; as the IL-17 A allele exhibited a higher affinity to transcriptional factors crucial for the regulation of T cell-mediated IL-17

gene transcription and thus affecting the production of IL-17 from T cells (Espinoza *et al*., 2011).

However a slight tendency toward a higher frequency of the IL-17A heterozygosity was observed; the IL-17A GG, GA and AA genotypes were detected in 23 (37.1%), 25 (40.3%) and 14 (22.6%) patients, and in 38 (30.4%), 67 (53.6%) and 20 (16%) controls, respectively (Wróbel *et al*., 2014). Two recent studies were conducted on Egyptian AML patients, their results are not matching to ours. The discrepancies in results between different studies might be due to differences in sample size, age groups, AML subtypes included, clinical characteristics or ethnic origin of the studied patients. It should be noted that most the cases in our study were AML/M4 (40%) which was not the case in the other studies. Significant association of IL-17A and IL-17F genes mutations with AML risk should be evaluated further on a larger scale with respect to age, different AML subtypes, environmental factors, ethnicity and race.

Concerning the different genotypes of IL-17A and IL-17F in the Egyptian population in general, multiple studies explored this, regarding different haematological and immunological diseases, and most of them stated that normal control subjects included in their studies showed similar percentages like ours with little differences as most of them showed for IL-17A; GG > AG > AA genotypes; and for IL-17F; AA > GA > GG genotypes. These findings confirms that the Egyptian population is homogeneous for IL-17A and IL-17F genotypes (Mohammed *et al*., 2017; Botros *et al*., 2018; Elfasakhanya *et al*., 2018).

The diseases under investigation in those studies included chronic idiopathic thrombocytopenic purpura, primary immune thrombocytopenia, juvenile lupus and lupus related nephritis, vitiligo and rheumatoid arthritis. Unlikely; most of those

studies showed no significant associations between IL-17A and IL-17F SNPs and the studied group of patients in correlation with each disease concerned in the study except for Yousry *et al*., (2016) who found that IL-17F SNP has a significant association with chronic idiopathic thrombocytopenic purpura in Egyptian patients and that it may play a role in the genetic cause of corticosteroid resistance in idiopathic thrombocytopenic purpura .

These non-significant correlations between IL-17A and IL-17F SNPs and different immunological and haematological diseases in the Egyptian population might be explained by the fact that they are multifactorial diseases influenced by factors other than genetics; where all factors work together synergistically to produce the disease. IL-17 SNP might be associated with diseases in which the genetic component takes the upper hand. This strengthens the fact that the pathogenesis of AML is complex, and it relays on both genetic and micro-environmental factors playing significant roles in the development of the disease.

5.5. Evaluation of Serum IL-17 concentration By ELISA Among Study Population:

IL-17-axis has been targeted by many studies representing a pivotal role in the pathogenesis of AML; Th17 cells, cytokines regulating their differentiation and functions (TGF-β and IL-6), their main effector cytokines (IL-17A and IL-17F), and IL-17A and IL-17F SNPs have been the concern of a lot of studies to explore their association with AML development, progression, prognosis and response to treatment (Elsissy *et al*., 2018).

The current results; the mean of serum IL-17 concentration for AHC and patients with AML groups were 8.00 ±0.31pg./ml and 14.00 ±0.59 pg./ml ,respectively. These results are agreement with Abousamra *et al*.,(2013) who

proved that Th17 cells were increased in AML patients and were significantly higher than in healthy controls.

Also ,Wu *et al*.,(2009) first stated that Th17 cell frequencies were increased significantly in untreated AML patients, compared to healthy volunteer subjects, and that increase in Th17 cell frequencies was accompanied by increase in IL-17 concentrations.

TGF-β and IL-6 concentrations were increased in the untreated AML patients as well, and IL-6 concentrations showed a positive correlation with Th17 cell frequencies (Wu *et al*., 2009). Furthermore, pretreatment Th17 cells were reduced significantly in patients who achieved complete remission after induction therapy. Serum levels of IL-17 showed positive correlation with circulating Th17 cells and were significantly elevated in AML patients. Positive correlation between circulating Th17 cells and survival in AML patients was reported (Abousamra *et al*., 2013).

Han *et al*., (2014) who demonstrated that the frequency of Th17 cells was significantly increased in AML patients compared to healthy donors. Plasma levels of IL-17, IL-6, and TGF-β1 were also significantly increased in AML patients compared to healthy donors. And that patients with high Th17 cell frequency had poor prognosis. These data verifies that Th17 cells play a crucial role in the pathogenesis of AML and may be an important therapeutic target and prognostic predictor.

IL17-A had no impact on AML incidence; as there was no significant relation between cases and controls, regarding polymorphic genotype and mutant allele (p-value 0.898 and 0.124) respectively. This finding was in agreement with Ersvaer *et al*., (2010).

Espinoza *et al*., (2010) who proved that T lymphocytes from normal individuals revealed an elevated level of IL-17 protein after provoking A allele. However, we were not able to prove the significant differences concerning IL-17A polymorphism and IL-17 level (data not shown). Also, we did not find a significant relationship between the IL-17 levels in AML patients and healthy controls, as observed in previous studies Ersvaer *et al*., (2010)., Wrobel *et al*., (2003).

Wu *et al*., (2009) found that an increase in IL-17 concentration is associated with an elevation in Th17 cell number in the patient group. It is inhibited in cases with complete response undergoing therapy . Abusamra *et al*.,(2013) proposed that the number of circulating Th17 cells significantly increased in ALL and AML patients compared with controls. These patients showed a decrease number of ofTh17 cell number when reaching complete remission after therapy .On the other hand, Tian *et al*.,(2013) indicated that compared with normal controls, IL-17 level decreased obviously in acute lymphoblastic leukaemia patients.

Variation in results may be due to the difference of patient ethnic groups, the correlation between IL-17F polymorphic genotypes. Also, plasma IL-17 values might be correlated to racial variations.

5.7.The Results of P14 rs3731249 Gene Polymorphism.

Expression analysis by quantitative RT-PCR revealed that mdm2, p14ARF, p16INK4A and c-myc are expressed at high levels in AML blasts. Three of these genes are closely connected within one pathway: c-myc leads to p14ARF induction, which in turn regulates mdm2–p53 interaction and activity. Both c-myc as well as mdm2 are oncogenic, and overexpression of mdm2 is a common mechanism of p53 inactivation (Michael and Oren,2002).

Mutations of the p53 tumor suppressor itself are relatively rare in de novo AML(Stirewalt *et al*., 2001).

Ohsaka, and Nishino,(2011) who found nucleotide alterations in the promoters, including substitutions at positions −2610 and −1536 and deletions at positions −4489, −4488 to −4483, −2241 to −2240, and −2221 to −2218 in *p14 ARF* and substitutions at positions −1643 and −1270 in *p15 INK4b*, and they found that each individual harbored polymorphisms in the locus promoters and/or introns. Some polymorphic nucleotides were included in the same set of associatively altered nucleotides. Reporter gene analysis by using luciferase revealed that altered nucleotides, including those containing the set, changed luciferase gene activity in some cell lines.

Thus, p53 activity could be abrogated in AML by mdm2 overexpression. Expression of the p14ARF tumor suppressor gene was increased in many AML samples. The high expression of p14ARF in AML might at least partially be a result of the c-myc overexpression(Eischen *et al*., 1999).

The p16INK4A gene was also expressed at higher levels in leukemic samples compared to normal controls. A weak correlation was found between p14ARF and p16INK4A expression. This coregulation of p14ARF and p16INK4A is likely to depend on their genomic proximity and their sharing of exons 2 and 3 on the mRNA level (Quelle *et al*., 1999).

So far, the prognostic relevance of the c-myc–ARF pathway was unknown for AML patients. Deletions of the ARF/INK4A locus have recently been reported in a small percentage of AML patients (5%), and deletions indicated a poor prognosis(Faderl *et al*., 2000).

However, neither the strong prognostic role of p14ARF for all patients nor which one of the tumor suppressors at the ARF/ ink4A locus might be relevant has been described.

Conclusions

and

Recommendations

Conclusions

The Following Conclusions are obtained from the Present Study

- Age factor has a significant association with viral infection, rather than with the differences of mean ages of the HHV-6.
- Highest age – specific frequency was noticed in patients with AML in the age stratum of ≤40 years.
- The ratio of males patients whom suffering from AML was higher than their Females counterparts.
- HHV-6 might be one of the most recently identified viruses in Iraqi patients suffering from AML in the Iraqi population. The positive results of HHV-6 DNA PCR in blood sample from patients group, these findings lead to the proposal that HHV-6 acts as cofactor in patients suffering from acute myeloid leukemia.
- Our study indicated that ***IL-17rs2275913*** **as well as** ***P14*** **rs3731249** genes polymorphism may be associated with risk in the Iraqi patient. But, the exact role and effects of ***rs2275913*** and **rs3731249** polymorphism in AML is not fully identified. Thus , ***IL-17rs2275913*** **as well as** ***P14*** **rs3731249** genes polymorphisms may be considers as risky factors for AML Patients.
- Serum IL-17A levels can be considered a useful diagnostic and prognostic factor in AML patients, like IL-17A ***and*** ***P14*** **rs3731249** gene polymorphisms.
- The significant correlation between the gene polymorphism of *IL-17A* with HHV-6 infection could indicate highly important role of these molecular factors in patients suffering from AML.

Recommendations

The recommendations of these study dependent on current results are:

1. HHV-6 could be added as another potential cause of AML.
2. Routine work uses of viral detection such as (HHV-6, HHV-8,Rubella, HCMV, HSV1&2 …etc) from blood for in centrals health especially in young age to prevent the pathogenesis; inflammation or carcinogenesis.
3. Further prospective studies are required with a large number of cases are needed to validate the results of the current study which may lead to a better understanding of the role of HHV-6 in patients with AML .
4. However, the exact role and effects of *IL-17A* and ***P14* rs3731249** polymorphism in AML is not fully identified. Therefore, for better understanding of the association of this polymorphism with AML, further studies on different races and geographic areas with larger sample sizes are recommended to identify the effects of ***rs2275913*** and **rs3731249** polymorphism on AML.
5. Studying the role of other Genetic biomarkers in deregulation of cell cycle pathway that play essential role in AML.
6. The addition of highly sensitive RT-PCR test beside ELISA and PCR techniques are important to confirm the real HHV-6 infection especially in health centers in order to early detection of viral infection.
7. To study the sequence and the strains prevalent in Iraq and their differences from the global prevalence and importance, preparing traits against prevalence of HHV-6 in our country via proposing a vaccine which fits the Iraqi isolates is important.
8. To study the complete hole genome sequence and the detection the new genotypes and strains prevalent in Iraq and their differences from the global prevalence and importance in patients with AML .

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Appendices

Appendices

Appendices 1

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REFERENCE 1 (bases 1 to 526)
AUTHORS Isam,Z.
TITLE Direct Submission
JOURNAL Submitted (12-JUN-2023) to the DDBJ/EMBL/GenBank databases.
Contact:Zahraa Isam
Babylon University, Biotechnology; 40 Street, Babel, Hilla 51001,
Iraq
REFERENCE 2
AUTHORS Najah,W. and Al-Alwany,S.H.
TITLE Molecular detection of IL17 polymorphism and HHV.6 A in patients
with Acute Myeloid Leukemia
JOURNAL Unpublished (2023)
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AUTHORS Isam,Z.
TITLE Direct Submission
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Contact:Zahraa Isam
Babylon University, Biotechnology; 40 Street, Babel, Hilla 51001,
Iraq
REFERENCE 2
AUTHORS Najah,W. and Al-Alwany,S.H.
TITLE Molecular detection of IL17 polymorphism and HHV.6 A in patients
with Acute Myeloid Leukemia
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Appendices 2


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Catarrhini; Hominidae; Homo.
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Babylon University, Biotechnology; 40 Street, Babel, Hilla 51001,
Iraq
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Contact:Zahraa Isam
Babylon University, Biotechnology; 40 Street, Babel, Hilla 51001,
Iraq
REFERENCE 2
AUTHORS Najah,W. and Al-Alwany,S.H.
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الخلاصه

الخلاصة

• تم عزل فيروس الحلا البشري نمط 6 لأول مرة من كريات الدم البيضاء للدم المحيطي للمرضى الذين يعانون من اضطرابات التكاثر اللمفاوي بما في ذلك سرطان الغدد الليمفاوية وسرطان الدم. تتهم الخلايا التائية المساعدة 17 بالتسبب في الإصابة بسرطان الدم النخاعي الحاد. الخلايا التائية المساعدة 17 هي نوع فرعي من الخلايا الليمفاوية المساعدة4 (الايجابية سي دي 4 +) يقومون ب انتاج الحركي الخلوي 17 ا و ف . قد تؤدي التغايرات الوراثية المتعددة المتعددة الجينية في محور الحركي الخلوي 17 إلى تغيير القدرة على استنباط استجابات مناعية فعالة.

•هدفت هذه الدراسة إلى تحديد النسبة المئوية لفيروس الحلأ البشري 6 نمط ا و ب وما إذا كانت تعدد الاشكال في الحركي الخلوي -17 اضافتا الى الجين الكابت للورم -14 تعدل خطر لحدوث سرطان الدم النخاعي الحاد في مجموعة من عينات المجتمع العراقي.

•تضـــمنت الدراســـة الحالات - الســـيطرة والمختارة بصـــوره عشـــوائية **200** عينة من الدم (**100** حالة للاشخاص المصابين بسرطان الدم النخاعي الحاد و **100** حالة كمجموعة سيطرة ظاهريًا اصحاء) (**57** ذكرًا و **43** أنثى مصابون بسرطان الدم النخاعي الحاد: 45 ذكر و55 انثى مجموعة الاصحاء ظاهريا) تم جمعها من مســـتشـــفيات تعليمية مختلفة في مناطق الفرات الأوســـط ومحافظة بغداد في العراق. وتتفاوت أعمار المجموعات المدروسة من **2** إلى **75** سنة. وقد أجريت الدراسة في قسم علم الأحياء المجهرية، كلية العلوم، جامعة بابل خلال الفترة بين تشرين الأول/أكتوبر 2022 وحزيران/يونيو 2023.

•تم جمع خمســـة مل من عينة الدم (2 مل في مانع تخثر الدم و3 مل في انبوب الجل لعزل المصـــل)، ثم تم الحصول على الجينوم الفيروسي والحمض النووي الدنا الإجمالي وتخزينها في درجة حرارة -20س°/ -80 س° حتى الاستخدام.

•تم الكشف عن فايروس الحلا البشري نمط 6 ا و ب باستخدام تقنية تفاعل البلمرة المتسلسلة (PCR) . تم إجراء تحاليل روتينية أخرى لتعدد الاشكال لجين مستقبل الحركي الخلوي 17 (***rs2275913)*** والجين الكابت للورم 14(rs3731249 (. ايضا تم اجراء الاختبارات المصلية لقياس مستوى تركيز الحركي الخلوي-17 امصال الاشخاص الاصحاء والمرضى الذين يعانون من سرطان الدم النخاعي الحاد بوساطة تقنية المقايسة الامتصاصية المناعية للانزيم المرتبط .

الخلاصه

• تم تلخيص النتائج المتحصل عليها على النحو التالي:

• كان متوسط عمر المرضى الذين يعانون من سرطان الدم النخاعي الحاد (39.3±12.41 سنة) . في حين ان متوسط عمر للمجموعة السليمة ظاهريًا (37.9±11.17 سنة).

• في مجموعة سرطان الدم النخاعي الحاد ، كانت الفئة العمرية الأكثر تأثرًا هي من 21-40 سنة وتمثل (48%)، تليها الفئة العمرية من 41-60 سنة (25%)، وكانت أقل منها مجموعة هي الفئة العمرية ≥ 20 ومن 61-80 سنة حيث تشكلت (12%) و (15%) على التوالي.

• كانت 57٪ من حالات سرطان الدم النخاعي الحاد ذكورا، بينما كانت النسبة المتبقية 43٪ اناثا. فيما يتعلق بمجموعة الاصحاء ظاهريا فقد كانت نسبة الإناث أعلى (55٪: 55) من نسبة الذكور (45٪: 45).

• تم اكتشاف 42 عينة من حالات سرطان الدم النخاعي الحاد ايجابيه للجينوم الفيروسي في هذه الدراسة، مما يشكل42 ٪ من العدد الإجمالي للمرضى المصابين ب سرطان الدم النخاعي الحاد ، في حين تم اكتشاف 5 عينة من مجموعة الاصحاء ظاهريا ايجابيه للجينوم الفيروسي في هذه الدراسة، مما يشكل 5٪ من العدد الإجمالي لمجموعة السيطرة.

• وفقًا لتقنية تفاعل البلمرة المتسلسلة (PCR)، كانت نتائج 33.3٪ (14 من أصل 42) من عينات سرطان الدم النخاعي الحاد إيجابية لكشف جين الفايروس الحلا البشري نمط 6 ا، بينما كانت 28 من أصل 42 (66.7٪) سلبية لكشف جين الفايروس الحلا البشري نمط 6 ا. أما بالنسبة الفايروس الحلا البشري نمط 6 ب، فقد كانت 38.1٪ (16 من أصل 42) من العينات تحمل جين الفايروس الحلا البشري نمط 6 ب، بينما أظهرت 61.9٪ (26 من أصل 42) من العينات نتائج سلبية لكشف جين الفايروس الحلا البشري نمط 6ب.

• وفقًا لتقنية تفاعل البلمرة المتسلسلة لكشف جين الفايروس الحلا البشري نمط 6 ا وب كانت اعلى في جنس الذكور 64.3٪ (9 من أصل 14) و 62.5٪ (10 من أصل 16) من عينات سرطان الدم النخاعي الحاد على التوالي .بينما كانت نسبة إيجابية جين الفايروس الحلا البشري نمط 6 ا وب في جنس الاناث 35.7% (5 من اصل 14 حالة) و 37.5% (6 من اصل 16) على التوالي.

• أظهرت نتائج IL-17 rs2275913 تعدد الأشكال أن توزيع تعدد أشكال الحمض النووي كان توزيعات تعدد أشكال الحمض النووي وفقًا لـ C \ A ؛ اي جي؛ أ \ ج ؛ G \ A ؛ و C \ G متغايرة الزيجوت كانت 2 ٪ ؛ 9٪ ؛ 1٪ ؛ 1٪ ؛ و 1٪ على التوالي في المرضى المصابين بابيضاض الدم النقوي الحاد و 20٪. 0٪ ؛ 0٪ ؛

الخلاصه

20٪ و 40٪ على التوالي في مجموعة AHC. كان الاختلاف في تواتر توزيع النمط الجيني لتعدد الأشكال بين المرضى والضوابط ذات دلالة إحصائية.

أظهرت النتائج rs3731249 14-pتعدد الاشكال ان توزيع تعدد اشكال الحمض النووي كان توزيعات تعدد اشكال الحامض النووي وفقا لـA/A:"A/G;A/C;Aو C/Tمتغايرة الزيجوت كانت ;36.7%;30%;26.7%و6.7% على التوالي في المرضى المصابين بابيضاض الدم النقوي الحاد و45% ; ;10 ;35 و 10% على التوالي في مجموعه AHC. كان الاختلاف في تواتر توزيع النمط الجيني لتعدد الاشكال بين المرضى والضوابط ذات دلاله احصائيه

● كان متوسط تركيز المصل من للحركي الخلوي -17 بالنسبة للمصل لمجموعة الاصحاء والمرضى الذين لديهم سرطان الدم النخاعي الحاد هو 8±0.31, 14 ± 0,59 على التوالي .

• تشير التحليلات الإحصائية إلى أن هناك ارتباطًا معنوياً قوياً بين انتشار ؤ-6 و سرطان الدم النخاعي الحاد (p = 0.0070,968:)،والتغاير الوراثي للحركي الخلوي 17 ***rs2275913*** .بشكل مشابه هنالك ارتباط معنوي بين فيروس *الحلا البشري نمط 6* والحركي الخلوي 17في مرضى سرطان الدم النخاعي الحاد .(p = 0.9840,008)

• وجدت علاقة إيجابية قوية (مع ارتباط كبير جدا) بين فيروس الحلا البشري –6 والتغاير الوراثي للحركي الخلوي 17- ***rs2275913*** حسب اعمارمرضى سرطان الدم النخاعي الحاد ((r0.001 = 0.8550). .ولكن لاتوجد علاقة (لا ارتباط معنوي) بين فيروس الحلا البشري – 6والتغاير الوراثي للحركي الخلوي-17 ***rs2275913*** حسب اجناس مجتمع المرضى المصابين مرضى سرطان الدم النخاعي الحاد.

• نحن نستنتج من هذه النتائج : بالرغم من الأعداد الصغيرة نسبياً التي تضمنتها دراستنا، تشير النتائج الحالية إلى احتمال أن يؤدي الاصابة بفيروس الحلا البشري نمط--6 والتغاير الوراثي للحركي الخلوي-17 دوراً في بيولوجيا سرطان الدم النخاعي الحاد وقد يسهم في تطورها.

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة بابل
كلية العلوم
قسم علوم الحياة

علاقه فايروس الحلا البشري السادس (ا , ب) مع تعدد الاشكال الجيني للانترلوكين 17 و P14 في مرضى سرطان الدم النخاعي الحاد

رسالة مقدمة الى مجلس كلية العلوم في جامعة بابل، وهي جزء من متطلبات نيل درجة الماجستير في علوم الحياة

من قبل
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