STUDY ON THE SERUM AND GENE EXPRESSION PROFILE OF CYTOKINES AND THE ROLE OF CYTOKINE GENE POLYMORPHISM ON SUSCEPTIBILITY TO HEPATITIS C INFECTION IN MALAY MALE DRUG ABUSERS

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by

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LIST OF SYMBOLS AND UNITS

%	Percentage
α	Alpha
β	Beta
δ	Delta
Δ	Delta
°C	Celsius
µ	Micro
µL	Microlitre
Bp	Base pair
CT	Cycle threshold
dL	Decilitre
g	Gram
h	Hour
mg	Microgram
min	Minute
mL	Millilitre
mM	Millimolar
ng	Nanogram
pg	Picogram
rpm	Rotation per minute
s	Seconds

LIST OF ABBREVIATIONS

ATS	Amphetamine type stimulant
MMT	Methadone Maintenance Therapy
DNA	Deoxyribonucleic acid
PCR	Polymerase Chain Reaction
RT PCR	Reverse transcription polymerase chain reaction
TBE	Tris Borate EDTA
SD	Standard deviation
IQR	Interquartile range
BMI	Body mass index
SBP	Systolic blood pressure
DBP	Diastolic blood pressure
CI	Confidence interval
WHO	World Health Organization
PBMC	Peripheral blood mononuclear cells
RNA	Ribonucleic acid
cDNA	complementary DNA
MOH	Ministry of Health
MDMA	Methylenedioxy-metamphetamine
DSM	Diagnostic and statistical manual of mental disorder
APA	American Psychiatric Association
HIV	Human immunodeficiency virus
SNPs	Single Nucleotide Polymorphisms
TBE	Tris Borate EDTA

NCBI	National Centre and Biotechnology Information
$MgCl_2$	Magnesium Chloride
KCl	Potassium Chloride

KAJIAN MENGENAI PROFIL EKSPRESI SITOKIN SERUM DAN GEN DAN PERANAN POLIMORFISME GEN SITOKIN TERHADAP KERENTANAN JANGKITAN HEPATITIS C DALAM KALANGAN PENAGIH DADAH LELAKI MELAYU

ABSTRAK

Jangkitan virus hepatitis C kronik (HCV) adalah salah satu punca utama sirosis hati dan karsinoma. Patofisiologi jangkitan HCV kronik mungkin disebabkan oleh variasi dalam aktiviti sitokin sebagai imunomodulator dan bukannya tindakan langsung virus itu sendiri. Objektif kajian ini adalah untuk menyiasat tahap serum dan ekspresi gen sitokin dan kemungkinan perkaitan polimorfisme gen sitokin terhadap kerentanan kepada jangkitan hepatitis C dalam kalangan penagih dadah lelaki Melayu. Pemilihan subjek memberikan pengetahuan baru dalam memahami kejadian HCV kronik yang tinggi dalam kalangan penyalahguna dadah, kumpulan pesakit yang rentan dan sering diabaikan. Interleukin-10 (IL-10) rs1800896 dan rs1800871, interleukin 6 (IL-6) rs1800795, tumour necrosis factor (TNF)-α rs1800629 dan tumour growth factor (TGF) -β1 rs1800471 telah dipilih untuk penyiasatan ini mewakili jenis anti dan pro-radang sitokin. Sejumlah 162 subjek telah didaftarkan dari pelbagai klinik kesihatan di Kelantan, Malaysia, dan dibahagikan kepada dua kumpulan: 76 pesakit dengan jangkitan HCV kronik (HP) dan 86 kumpulan kawalan (HS). Polimorfisme gen dikesan melalui tindak balas rantai polimerase multipleks (PCR), ekspresi gen oleh "real time" tindak balas rantai polimerase (RT-PCR), dan tahap sitokin serum diukur dengan immunoassay. Terdapat perbezaan yang ketara dalam frekuensi genotip untuk IL-10 rs1800896 ($p = 0.0422$), IL-10 rs1800871 ($p = 0.0498$), dan pada tahap alel

untuk IL-10 rs1800896 A berbanding alel G (p = 02), TGF-β1 rs1800471 (p = 0.0051) dan pada tahap alel untuk TGF-β1 rs1800471 G berbanding alel C (p = 0.0082) dalam kumpulan HP dan kumpulan kawalan. Kajian itu menemui perbezaan yang signifikan dalam ekspresi gen untuk TNF-α (p = 0.0328) dan dalam tahap serum purata IL-6, dan TGF-β1 dalam kumpulan HP berbanding kumpulan HS (masing-masing p = 0.0180 dan p = 0.0005). Penemuan ini mencadangkan perkaitan yang ketara antara polimorfisme gen untuk IL-10 rs1800896, IL-10 rs1800871, TGF-β1 rs1800471 dan kerentanan kepada jangkitan HCV di kalangan penagih dadah lelaki Melayu dengan jangkitan HCV kronik. Ekspresi gen TNF-α dan ekspresi serum IL-10, IL-6 dan TGF-β1 menunjukkan hubungan yang signifikan dengan jangkitan HCV kronik. Kajian ini menyumbang kepada ilmu baru tentang peranan sitokin dan variasi genetik terhadap kerentanan kepada jangkitan HCV kronik di kalangan penagih dadah lelaki Melayu, menemui potensi biomarker genetik dan serum untuk penyakit ini, dan akhirnya ke arah ‘precision medicine’ untuk jangkitan HCV kronik, terutamanya di kalangan penagih dadah.

STUDY ON THE SERUM AND GENE EXPRESSION PROFILE OF CYTOKINES AND THE ROLE OF CYTOKINE GENE POLYMORPHISM ON SUSCEPTIBILITY TO HEPATITIS C INFECTION IN MALAY MALE DRUG ABUSERS

ABSTRACT

Chronic hepatitis C virus (HCV) infection is one of the major causes of liver cirrhosis and carcinoma. The underlying pathophysiology may be due to variations in immunomodulatory cytokine activities rather than the direct action of the virus itself. The objectives of this study were to investigate the serum levels and gene expression of cytokines and the possible association of cytokine gene polymorphism on susceptibility to hepatitis C infection in Malay Male drug abusers. The selection of the subjects provides novel knowledge in understanding the high occurrence of chronic HCV among drug abusers, a vulnerable and often neglected group of patients. Interleukin-10 (IL-10) rs1800896 and rs1800871, interleukin 6 (IL-6) rs1800795, tumour necrosis factor (TNF)-α rs1800629 and tumour growth factor (TGF)-β1 rs1800471 have been selected for the investigations representing anti and pro-inflammatory cytokines. A total of 162 subjects were enrolled from various health clinics in Kelantan, Malaysia, and divided into two groups: 76 patients with chronic HCV infection (HP) and 86 control group (HS). The gene polymorphisms were detected through multiplex polymerase chain reaction (PCR), gene expressions by real-time reverse transcriptase polymerase chain reaction (RT-PCR), and the serum cytokine levels were measured by immunoassay. There were significant differences in the frequencies of genotype for IL-10 rs1800896 ($p = 0.0422$), IL-10 rs1800871 ($p =$

0.0498), and at the allelic level for IL-10 rs1800896 A versus G allele (p = 0.0142), TGF-β1 rs1800471 (p = 0.0051) and at the allelic level for TGF-β1 rs1800471 G versus C allele (p = 0.0082) in the HP group and the control group. The study discovered a significant difference in gene expression for TNF-α (p = 0.0328) and in the mean serum levels of IL-6, and TGF-β1 in the HP group compared to the HS group (p = 0.0180 and p = 0.0005, respectively). These findings suggest significant associations between gene polymorphisms for IL-10 rs1800896, IL-10 rs1800871, TGF-β1 rs1800471 and susceptibility to HCV infection among Malay male patients with chronic HCV infection. Gene expression of TNF-α and serum expression of IL-10, IL-6 and TGF-β1 shows a significant association with chronic HCV infection. This study provides a novel insight into the role of cytokines and genetic variation on susceptibility to chronic HCV infection among Malay male drug abusers, discovering potential genetic and serum biomarkers for the disease, and eventually towards precision medicine for chronic HCV infection, particularly for drug abusers.

CHAPTER 1

INTRODUCTION

1.1 Background

Numerous cells, including immune cells like lymphocytes (T or B cells), mast cells, macrophages, endothelial, fibroblast, and stromal cells, produce glycoproteins or humoral immunomodulatory proteins known as cytokines (Oldman & Dillman, 2009). Cytokines govern and regulate the actions of target cells particularly in the haematopoietic system. By binding to receptor ligands, they start the second messenger pathway and the signal transduction cascade within the target cells. This will cause gene activation, which will be followed by mitotic division, growth and differentiation, migration, and death. Two broad groups of cytokines are lymphokines and monokines, which are generated by the monocyte lineage (produced by the lymphocyte lineage). They can also be divided into two categories based on their functional characteristics: type 1 cytokines, such as tumour necrosis factor (TNF), interferon (IFN), interleukin (IL)-2, and IL-12, are pro-inflammatory; type 2 cytokines, such as tumour growth factor (TGF), IL-4, IL-5, IL-6, IL-10, IL-13, etc., are anti-inflammatory (Zhang & An, 2007). While Type 2 favours antibody immunity, Type 1 will strengthen cellular immunity.

The pathophysiology and clinical prognosis of several viral, autoimmune, and neoplastic diseases may be impacted by the overall balance between the actions and effects of pro-inflammatory and anti-inflammatory cytokines (Ray, 2016). The body's normal feedback system will be disrupted by the imbalance in how these two types of cytokines interact, compromising the integrity of healthy tissue. For an instance, type 1 cytokine overexpression may have important negative impacts and wide inflammatory responses.

Many studies have been conducted to understand the relationship between individual variation in clinical features like susceptibility to infection or the course of illnesses and genetic variants within a particular gene that may impact the degree of cytokine production (Chapman & Hill, 2012). Both conservative and non-conservative mutations can result in gene polymorphism. Non-conservative mutations in the coding sequence of the structural protein may cause deletion, abrogation, or changed function in the produced proteins (Zhang & Lupski, 2015). Conversely, conservative mutations can affect the level, stability, and splicing of mRNA.

In-vitro or in-vivo research can be used to study genetic variation and how it relates to the course of illness. In-vitro gene expression studies look at the connection between certain polymorphic alleles of cytokine genes and the production of transcripts or cytokines in vitro in an effort to identify the genetic basis for inter-individual variance in immune response. To do this, cells in culture can be in vitro stimulated with a mitogen, specific gene promoter alleles can be isolated, and levels of cytokines, cytokine receptor mRNA, or cytokine or receptor proteins can be measured.

The in-vivo study compares the genotype of each cytokine directly to clinical parameters including susceptibility, severity, and duration of the disease in order to establish a relationship between particular cytokine gene polymorphism and clinical outcomes. Typically, the in-vivo research conducted as a case-control association investigation, which compares the frequencies of marker alleles in a group of patients and healthy controls statistically analysed the difference. In this work, we will investigate the relationship between cytokine gene variation and chronic hepatitis C virus (HCV) infection susceptibility in vivo. Other than that, we also examine the association between mRNA gene expression and serum levels of cytokines with

chronic HCV infection. One of the primary causes of chronic liver disease, liver cirrhosis, and one of the key indications for liver transplantation are common liver infections like HCV. The rationale of this study includes to better understand the pathophysiology of chronic HCV infection, discover potential markers of susceptibility, severity, and clinical outcome, target for therapy, and help in formulating strategies for preventing the progression of disease.

In our research, Malay male drug abusers have been chosen as study subjects. First, the reason why this study group has been selected is that drug abuses is identified as one of the main risk factors for HCV infections. Furthermore, the Malay subjects represent the largest ethnic group for Malaysia. This study is novel to Malaysia populations as to date, there is no other research has been done to study the association of genetic polymorphism, or gene and serum expression of cytokines with chronic HCV infection particularly in the context of Malay drug abusers. This study which was conducted in this vulnerable group hopefully could provide a better understanding of high occurrence of the disease among Malay male drug abuser and eventually contribute a novel knowledge towards improving the management of the disease in the near future.

Four types of cytokines have been selected to be examined in this research. The studied cytokines include IL-10, IL-6, TNF-α and TGF-β1. These cytokines were selected to represent both pro-inflammatory and anti-inflammatory cytokines. This selection allows us to examine which cytokine groups are associated with the chronicity of HCV infection. Il-10 & TGF-β1 are both anti-inflammatory cytokines, and TNF-α is the pro-inflammatory cytokines. Whereas IL-6 has been demonstrated to have both activities depending on the type of tissues in which it is being expressed. For the single nucleotide polymorphisms (SNPs) study, IL-10 rs1800896, rs1800871,

IL-6 rs1800795, TNF-α rs1800629 and TGF-β1 rs1800471 have been selected. Various studies on the association of these SNPs with certain inflammatory diseases have been carried out. However, some findings from previous studies demonstrated a contradictory result, particularly in chronic HCV infection, and they were mostly conducted in a Western population. Therefore, this study may provide novel information on the role of gene polymorphisms in the genes of interest with susceptibility to chronic HCV infection in the Malaysian population, specifically among Malay male drug abusers.

1.2 Problem statements

The mechanism of hepatitis C virus infection, as well as its development to chronicity and severe liver impairment, remains unknown. The virus does not appear to be directly damaging the liver cells. According to a number of studies, cytokines may contribute to the chronicity and persistence of HCV infection by modulating the immune system. This study will determine whether there is potential relationship between cytokine network and HCV infection as well as the association between gene polymorphism and susceptibility to chronic HCV infection among the male patients of Malay descent.

1.3 Research questions

1. Do Malay male drug abusers with chronic HCV infection exhibit any significant variations in cytokines gene polymorphism (IL-10, IL-6, TNF-α, and TGF-β1) from the control group?

2. Are there any significant variations in the gene expression of cytokines (IL-10, IL-6, TNF-α, and TGF-β1) between the Malay male drug users with chronic HCV infection and the control group?

3. Are there any significant variations in the serum expression of cytokines (IL-10, IL-6, TNF-α, and TGF-β1) between the Malay male drug users with chronic HCV infection and the control group?

4. Do cytokines (IL-10, IL-6, TNF-α, and TGF-β1) gene polymorphism, cytokine mRNA gene expression and serum levels affect Malay male drug abuser susceptibility to chronic HCV infection?

1.4 Hypothesis

The gene polymorphism, mRNA gene expression, and serum levels of cytokines (IL-10, IL-6, TNF-α, and TGF-β1) in Malay male drug abusers with chronic HCV infection will differ significantly from the control group when compared to these variables, and these variations impact the susceptibility to chronic HCV infection.

1.5 Objectives

1.5.1 General objective

The general objective of this study is to investigate the differences in serum and gene expression profiles of cytokines involved in hepatitis C infection in Malay male patients and healthy volunteers and to explore the role of cytokine gene polymorphism on susceptibility to infection among Malay male patients with chronic HCV infection.

1.5.2 Specific Objectives

The following are the specific objectives for this study:

1. To develop and optimize allele-specific multiplex polymerase chain reaction (PCR) techniques and use these methods to detect the polymorphisms of IL-10, IL-6, TNF-α and TGF-β1 genes among 2 groups of subjects: Malay male patients with chronic HCV infection (HP) and control group (HS).
2. To optimize real time-PCR technique and use the method to detect and quantify studied cytokines (IL-10, IL-6, TNF-α, and TGF-β1) mRNA expression in human blood among these 2 groups of subjects.
3. To determine the serum levels of IL-10, IL-6, TNF-α, and TGF-β1 among the subjects and to evaluate the change and clinical significance of cytokines in hepatitis C infection by Luminex and ELISA immunoassays.
4. To examine the associations of the studied genotypes, cytokine mRNA expressions, and serum cytokine levels with susceptibility to infection among Malay male patients with chronic HCV infection.

CHAPTER 2

LITERATURE REVIEW

2.1 Hepatitis C Virus Infection

Hepatitis C Virus (HCV) infection is one of the primary causes of chronic liver disease and hepatocellular cancer. HCV is a bloodborne virus that is commonly spread among intravenous drug users through the use of infected syringes, accounting for 60-80% of HCV infections worldwide (Schulte et al., 2015). Those with a 6-year or longer history of injections are at a much higher risk of contracting the virus (Diaz et al., 2001). Unscreened blood products for transfusion, unprotected sexual practises, and vertical transmission from the mother to the foetus are further risk factors for the spread (Yeung et al., 2014).

A person infected with HCV is frequently asymptomatic. Often, the condition is found by chance as a result of an abnormal blood test after a blood donation or standard medical screening. Otherwise, the patient may experience constitutional symptoms such persistent exhaustion, fever, and weight loss. The patient may develop jaundice, dark urine, easy bruising, limb oedema, and hepatic encephalopathy signs as the liver damage progresses (WHO, 2021). Extrahepatic symptom of chronic HCV infection includes arthralgia, paraesthesia, myalgia, pruritis, mixed cryoglobulinemia, etc (Cacoub et al., 1999).

Antiphospholipid syndrome (APS) is substantially associated with chronic HCV infection. According to a research, APS is most frequently caused by HIV and HCV (Mendoza-Pinto et al., 2018). Intraabdominal thrombosis and myocardial infection are the predominant symptoms of APS, an autoimmune illness defined by the development of antiphospholipid antibodies (APLS), arterial and venous

thrombosis, thrombocytopenia, and numerous foetal losses (Ramos-Casals et al., 2004).

In individuals with chronic HCV, the incidence of spontaneous viral clearance is quite low. About 20–40% of HCV patients spontaneously recover from the illness without receiving any therapy, however the majority of patients acquire chronic persistent HCV (Modi & Liang, 2008). With a high risk of consequences including liver cancer, liver failure, and increased mortality, liver cirrhosis affects between 5 and 20% of chronic HCV patients (Hallager et al., 2017). This may indicate genetic variations in the host, which might affect the disease's course and response. HCV is not cytopathic by itself. The lesion of chronic hepatitis C appears to be brought on by a local immune response, and cytokines have a significant impact on how this response is modulated (Napoli et al., 1996).

The management of chronic HCV infection involved non-pharmacological approaches and pharmacological intervention. The non-pharmacological approaches include peer-based support, HCV education, and clinical monitoring services. Pharmacological intervention must be preceded by a pre-treatment evaluation. This involves utilising the Child-Turcotte-Pugh Score to identify any co-morbidities and the presence of cirrhosis (CPS). Several oral DAA (Direct-acting antivirals) regimens have taken the place of pegylated interferon (PEG-IFN), which had low cure rates and significant adverse effects (Ministry of Health Malaysia, 2019).

By using pharmaceuticals that work well and have short treatment durations and few adverse effects, it is intended to provide a high cure rate, or SVR (sustained virological response). Chronic HCV infected patients should be evaluated for direct-acting antivirals (DAA) therapy unless they have a short life expectancy or significant non-liver-related co-morbidities. Those with cirrhosis or considerable fibrosis,

particularly those with decompensated cirrhosis and clinically significant extra-hepatic symptoms caused by HCV infection, should begin therapy immediately. DAAs include NS5B polymerase inhibitors such as sofosbuvir, NS5A inhibitors such as daclatasvir, NS3/4 A (protease) inhibitors such as glecapervir, and others. In cirrhosis, the medications used, and the duration of treatment are determined by the stage of the illness, and the HCV genotype should be considered. Advanced liver cirrhosis or liver cancer in HCV patients may demand liver resection or transplantation, which is exceedingly invasive and has a bad prognosis (Mukherjee et al., 2015).

2.2 Epidemiology of HCV infection

Liver infection with HCV is one of the world's most common viral illnesses, often acquired through the risky practise of intravenous drug use or a medical procedure. A chronic infection affects more than 71 million individuals globally, with majority of them (about 80%) are from under develop and developing nations. However, only one out of every five persons is aware of their illness. The HCV infection in Malaysia shows and increasing in trend with more persons are recognised as having HCV antibodies through regular screening. In Malaysia, 453,700 persons (2.5% of the population aged 15 to 64) were estimated to have HCV infection in 2009; 59% of these individuals received the infection by injection. Hepatitis C cases were reported in 550 cases in 2000, with a population incidence rate of 2.5/100,000; 741 cases in 2004, with a population incidence rate of 2.9/100,000; and 6,771 cases in 2013, indicating an increasing disease burden (Raihan, 2016).

Those with HCV have a greater risk of comorbidity and multimorbidity than the general population (Cooper et al., 2019). Despite the fact that multimorbidity was not associated to treatment, those with substance use disorders were less likely to

undergo HCV therapy. In 2019, 290 000 persons died from hepatitis C, largely from cirrhosis and hepatocellular cancer, according to the WHO (WHO, 2021). Although the HCV case fatality rate is low, those with proven HCV infection had a higher death rate than the general population, highlighting health inequities (Ireland et al., 2019).

2.3 Virology of HCV

HCV has been classified with GB virus B (GBV-B) and canine hepacivirus (CHV) in the Flaviviridae family's Hepacivirus genus (Moradpour & Penin, 2013). Other members of this family include Flavivirus (e.g., yellow fever virus and dengue virus), Pestivirus (e.g., bovine viral diarrhoea virus), and Pegivirus (e.g., human pegivirus/GB virus C). HCV features an internal ribosome entry site (IRES), an open reading frame (ORF) that codes for structural and nonstructural proteins, and a 3' noncoding region in its 9.6 kb positive-strand RNA genome. The p7 viroporin, NS2 protease, NS3-4A complex including protease and NTPase/RNA helicase activity, NS4B and NS5A proteins, and NS5B RNA-dependent RNA polymerase are examples of nonstructural proteins (RdRp).

There are 6 genotypes of HCV have been discovered: genotype 1 (subtypes 1a, 1b and 1c) genotype 2 (subtypes 2a, 2b and 2c) genotype 3 (subtypes 3a, 3 b) genotype 4 (subtype 4a) genotype 5 (subtype 5a) and genotype 6 (subtypes 6a, 6b). The HCV genotypes 1, 2 and 3 are the most frequent types globally, however, their relative frequency varies from one geographic region to another (Zein, 2000). In the United States of America (USA) and Europe the most frequent HCV genotypes are 1a and 1b. In Japan, the most frequent HCV genotype is 1b, whereas 2a and 2b are more common in North America, and 2c distribution is more common in Italy. HCV genotype 3a is the most frequent subtype among injection drugs abusers in Europe and the United

States. HCV genotype 4 appears to be common in North Africa and the Middle East, while genotypes 5 and 6 tend to be restricted to South Africa and Hong Kong, respectively.

HCV heterogeneity and genotypes have obvious value as epidemiologic indicators, albeit their impact on continuing clinical treatment for long-term HCV infection has yet to be demonstrated. HCV heterogeneity may potentially impair the sensitivity and specificity of virologic and serological tests for detection of HCV. Besides, HCV genotypes may impact the outcome of acute infection, as individuals with HCV genotype 1b infection had a 92% risk of evolution to chronicity following acute exposure, compared to those with other genotypes, who had a 33 to 50% rate (le Ngoc et al., 2019).

2.4 Pathophysiology of chronic HCV infection

Viral entrance plays a key role in hepatocyte tropism in HCV. Viral entrance into hepatocytes involves three important steps: viral attachment, receptor-mediated endocytosis of viral particles and endosomal fusion (Zeisel et al., 2013). HCV particles enter the circulation and pass through the liver's fenestrated endothelium of the sinusoids prior to interaction with liver's cells during the initial stage of infection (Dubuisson & Cosset, 2014). Direct contact between the virion and the basolateral surface of the liver parenchyma occurs in the Disse space. This enables the virus to communicate with the receptors and attachment factors on the cell's surface. The heparan sulphate proteoglycan syndecan-1 or syndecan- or the scavenger receptor B1 (SRB1), which is reliant on virion density, mediates the first attachment of HCV particles onto hepatocytes. Lipoproteins were implicated in virion binding to heparan sulphate proteoglycans or SRB1 (mostly ApoE). The HCV virion forms a complex

particle known as a lipoviroparticle when it is tightly coupled to lipoproteins. To begin its life cycle, it attaches to SRB1 and glycosaminoglycans (GAGs). The virus then undergoes a protracted, a multistep process comprising a range of distinct cellular entry components, such as the signalling proteins SRB1 and CD81, tight-junction proteins CLDN1 and OCLN, EGFR, and transferrin receptor (TfR). Figure 2.1 schematic diagram summarize the the life-cylce of HCV.

The HCV particle is ingested by clathrin-mediated endocytosis after binding to several components of the host cell and undergoes fusion in early endosomes (Zeisel et al., 2013). This resulted in the nucleocapsid release into the cytoplasm. The cell fusion is facilitated by the E1 envelope glycoprotein. Positive-strand genomic RNA is released from the viral nucleocapsid and is transported into the cytosol where it functions as mRNA for the production of HCV polyprotein (Manns et al., 2017). The translation of the HCV open reading frame is regulated by an internal ribosome entry site identified in the HCV 5 untranslated region. The enormous precursor polyprotein generated is translated by the endoplasmic reticulum membrane, which produces three structural proteins and seven non-structural proteins. At least two host cellular peptidases (signalase and signal peptide peptidase) needed for HCV structural proteins whereas HCV non-structural proteins require the utilisation of two viral peptidases (NS2 and NS3/4A). After processing, the viral proteins remain attached to the inner membranes.

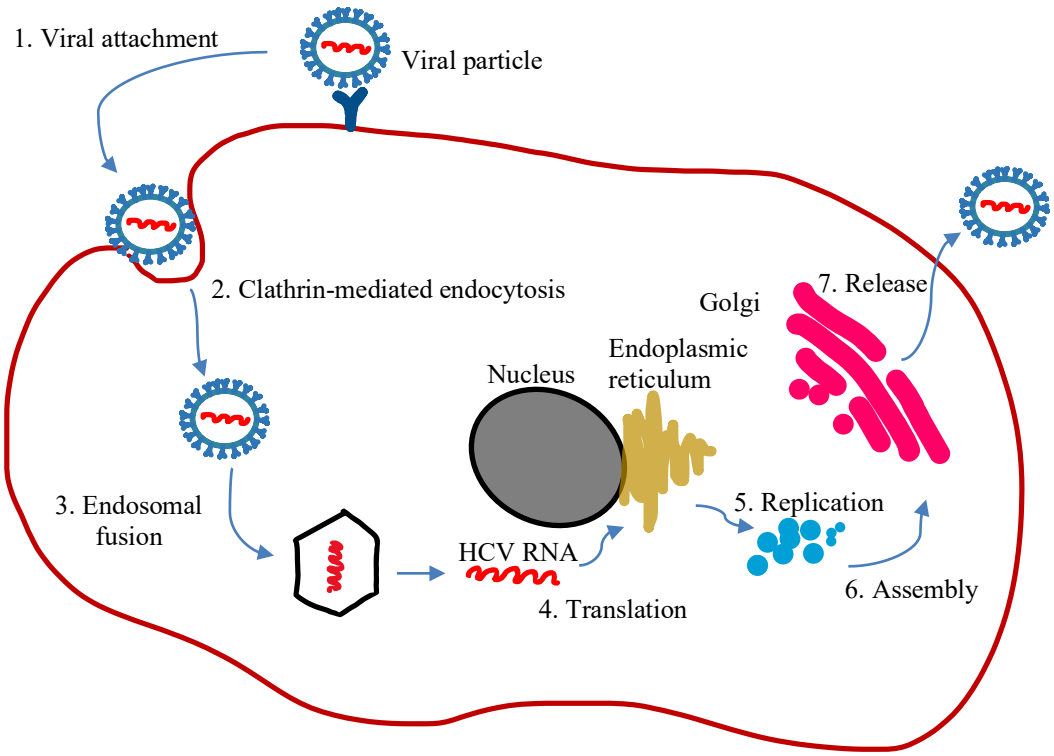

Figure 2.1 The schematic diagram represents the life cycle of HCV begins with the viral attachment, Clathrin-mediated endocytosis, endosomal fusion, RNA replication and finally viral assembly and release.

The NS5B protein is the catalyst for replication. NS5A served as a dimer with a basic channel that is involved in binding of RNA. Domains I and II of the NS5A protein are required for HCV replication in the replication complex (Lohmann, 2013). The HCV life cycle's balance between replication and subsequent stages is influenced by the NS5A phosphorylation state. The NS3 helicase is necessary for template and nascent RNA strand separation, RNA-binding protein dislodging, and secondary RNA structure unwinding (Scheel & Rice, 2013). The integral membrane protein NS4B promotes and compartmentalises HCV replication by facilitating HCV protein-induced membrane rearrangements that result in the creation of the complex of replication.

The formation of a negative-strand replication intermediate uses positive-strand genomic RNA as a template. Meanwhile, negative-strand RNAs serve as templates for the production of a huge number of positive polarity strands, which are subsequently used for polyprotein translation, the production of new replication intermediates, or the packaging of fresh virus particles (Lohmann, 2013). It has also been demonstrated that a number of host variables play crucial functional roles in the HCV life cycle. Peptidylprolyl isomerase A, also referred as Cyclophilin A, binds to both NS5A and NS5B and generates the conformational changes required for efficient HCV replication. The abundant liver-specific miRNA microRNA 122 (miR-122) interacts to two conserved sites in the internal ribosome entry site for efficient HCV replication and RNA stabilisation. Viral particles are manufactured as a result of the interaction of the core and NS5A proteins with the genomic RNA in cytoplasmic lipid droplets. In its latter stages of production and during release, HCV utilises the VLDL production pathway (Lindenbach, 2013).

The host immune system first activated the innate followed by adaptive immunity in response to HCV infection. The spontaneous viral elimination is linked with broad and multi-specific CD4+ and CD8+ T cell responses, while infection persistence is related to inadequate or premature T cell response loss. As with many other viral diseases, inherited immunity serves as a first line of defence against HCV infection. Infected cells release Type 1 IFN, which primes and urges cells to combat infection, inhibit viral replication, enhance adaptive immunity, and activate natural killer (NK) cells, dendritic cells (DCs), and Kupffer cells, among other cells (Irshad et al., 2013). PAMPs, or viral macromolecular motifs, are recognised as non-self by cellular pathogen recognition receptors once within the cell, triggering innate defence against HCV.

This stimulates the production of interferon regulatory factor-3 (IRF-3) and anti-viral/interferon-stimulated genes (ISG) (Gale & Liu, 2010). Following that, cytokines and IFN are produced, stimulating NK, DC, Kupffer cells, etc. Additionally, the development of T- and B-cell immunity depends on these cells (Saito & Gale, 2008). HCV has the ability to resist innate immunity, which lead to the persistence of viral infection. This is the result of HCV evolved to block the RIG-1 pathway and evade the immunological challenge that contributes to HCV chronicity (Schoggins & Rice, 2013). The non-structural protein complex of HCV NS3 and NS4A activates the NS protease domain to target IPS-1 cleavage. IPS-1 is unable to signal to activate IRF-3 and NF-B after cleavage, and infected cells stop generating IFN and expressing ISGs (Loo et al., 2006).

The responses of humoral antibodies and T-lymphocytes are commonly involved in adaptive immunity to combat viral infections (Manns et al., 2017). However, due to the heterogeneity of the virus and the presence of quasi-species

populations in a patient, most antibodies appear to have no meaningful effect against HCV. Neutralizing antibodies against certain epitopes, on the other hand, may be beneficial, and fast neutralising antibody production has been associated to infection control (Pestka et al., 2007). Following entrance and replication, viral molecules attach to the major histocompatibility complex (MHC) and are transported to the cell surface. This is then identified by T cells, resulting in the beginning of immunological responses. The bulk of CTL are CD8+, with only 10% being CD4+ (Irshad et al., 2013). During acute HCV infection, the onset, intensity, and length of the Th1 immune response are critical for effective HCV clearance (Aberle et al., 2006). Patients with a strong Th1 response exhibited efficient viral clearance and self-limited disease progression. Those that did not create IL-12 or IFN-γ, on the other hand, had the virus persist for a longer period of time (Fahey et al., 2014). In the majority of individuals who are unable to manage their illness, persistent infection with variable degrees of hepatitis and viremia develops (Valiante et al., 2000).

The fundamental mechanism causing the advancement of liver damage after chronic HCV infection is still unknown. However, the results of a number of studies suggest that HCV is not cytopathic, and that there is no correlation between the viral load of HCV and the severity of liver disease or its prognosis. Steatosis, which is characterised by an accumulation of lipids in hepatocytes, is the only lesion that may be attributed to a direct pathogenic action of HCV. Steatosis is the only lesion that has been linked to HCV. HCV genotype 3 is the only genotype known to produce virus-induced steatosis because the emergence of this condition in individuals with other HCV genotypes is primarily attributable to external metabolic factors (Poynard et al., 2003).

Chronic HCV infection damages the liver, resulting in degenerative lobular lesions, localised and bridging necrosis, and portal lymphoid infiltration (Pawlotsky, 2004). These lesions seem to be caused by oxidative stress and a local immune response directed at infected cells of the liver, which may be helped by an influx of certain T lymphocytes drawn by the production of adhesion molecules and chemokines (Neumann-Haefelin & Thimme, 2013; Pawlotsky, 2004). The bulk of lymphoid infiltration is composed of periportal CD4+ T cells, the majority of which have the Th1 phenotype such as production of IFN-γ, and periportal and lobular CD8+ T cells. According to several research, local Th1 cytokine expression correlates with the extent of the liver damages (Napoli et al., 1996; Piazzolla et al., 2000). Furthermore, cytotoxic T-cells responses appear to be important because they kill infected and uninfected cells through Fas-mediated apoptosis, production of TNF-α, and perforin-mediated pathways (Kondo et al., 1997).

The main factor contributing to chronic HCV infection's morbidity and mortality is fibrosis. As a result of a dynamic mechanism (fibrogenesis) of gene transcription and extracellular matrix component production, fibrosis develops when extracellular matrix components build up inside the liver parenchyma (Bedossa & Paradis, 2003). The advancement of liver fibrosis appears to be directly linked to continuing chronic inflammation, which is associated with cell death and regional cytokine and growth factor production. The hepatic stellate cell, which is essential for the development of liver fibrogenesis, lives in the perisinusoidal gaps (Pawlotsky, 2004). As a result of this complex activation process, myofibroblasts are formed, and they possess the capacity to generate considerable amounts of extracellular matrix components and smooth muscle α-actin (Friedman, 2000; Friedman, 2003). Furthermore, external variables have a significant influence in the development of

fibrosis to cirrhosis. This includes excessive alcohol intake, HIV or other viral hepatitis co-infection, obesity, and immunocompromised patients (Hezode et al., 2003; Peters & Terrault, 2002).

2.4 The role of cytokines in inflammation and diseases

Inflammation is a characteristic of many physiological and pathological processes. It is an adaptive response to unpleasant stimuli or situations, such as an infection or tissue damage (Medzhitov, 2008). During the acute phase of the inflammatory response, acute-phase proteins, and soluble mediators such as cytokines and chemokines, as well as other substances, aid the immune system's migration to the site of damage (Germolec et al., 2018). The acute phase may be sufficient to repair the damage and start the healing process, depending on the severity of the injury.

On the other hand, chronic inflammation is a protracted, abnormal, and maladaptive response that includes tissue damage, active inflammation, and attempts at tissue repair (Weiss, 2008). Chronic inflammation can result from either prolonged stimulation or an incorrect response to self-molecules. A variety of chronic human disorders and diseases are linked to such ongoing inflammation. Asthma, chronic obstructive pulmonary disease, metabolic syndrome, malignancy, diabetes mellitus, cardiovascular disease, inflammatory conditions (e.g., inflammatory bowel disease and rheumatoid arthritis) as well as other chronic health conditions are among those linked to inflammation, according to new research (Zhong & Shi, 2019). The mechanisms that are responsible for inflammatory control in chronic illnesses are still unknown, despite the fact that the significance of dysregulation of the immune system's inflammatory response has been established.

Cytokines are one of the essential factors known in controlling the inflammatory process. Cytokines are proteins that are soluble and have a low molecular weight (6-70 kDa) that affect immune cell formation, expansion, and response dynamically via sophisticated networks and serve as biological markers for a range of illnesses (Liu et al., 2021). Cytokines are secreted by a numerous type of cells, including lymphocytes, macrophages, stromal cells, mast cells and natural killer (NK) cells. Cytokines are categorised according to their cellular origin or function (Sprague & Khalil, 2009). CD4+ Th1 cells generate type 1 cytokines such as IL-12, IL-2, TNF-α and IFN, whereas CD4+ Th2 cells produce type 2 cytokines such as IL-6, IL-5, IL-4, IL-10, and IL-13. Depending on their activity, cytokines can also be classified as pro- or anti-inflammatory. Interferons, IL-1, IL-6, IL-8, IL-12, TNF-α, and other pro-inflammatory cytokines enhance inflammatory responses and excite immune-competent cells. Anti-inflammatory cytokines, on the other hand, such as TGF-β1, IL-1RA, IL-6, IL-10, IL-11, IL-13, and IL-4, reduce inflammation and restrict immune cells (Boshtam et al., 2017).

The immune system's failure to distinguish the body's regular components as "self" causes tissue damage and inflammation. Inflammation is the body's intricate biological reaction to damage and infection, which is controlled and governed by the equilibrium of inflammatory activity linked to cytokines (Liu et al., 2021). As a result, tissue homeostasis in response to environmental signals, as well as an imbalance in pro- and anti-inflammatory cytokine levels, can have substantial negative effects on health, especially in a susceptible host (Frey et al., 2019; Liu et al., 2021). Additionally, the potentially catastrophic cytokine release syndrome (CRS), a condition characterised by an out-of-control inflammatory response and an unbalanced immune system, can be influenced by the excessive or unregulated production of pro-

inflammatory cytokines (Tisoncik et al., 2012). CRS can result from a number of conditions, including infections, using drugs that contain natural and bispecific antibodies, and receiving adoptive T-cell therapy for cancer. The symptoms of CRS can range widely, from moderate flu-like symptoms to catastrophic, potentially fatal diseases of the overactive inflammatory response (Shimabukuro-Vornhagen et al., 2018).

2.5 Cytokines gene polymorphism and susceptibility to infectious diseases

The occurrence of many alleles for a certain gene as a consequence of variations in the nucleotide sequence occurring in $\geqslant$ 1% of the population is known as a gene polymorphism (Trent, 2012). Mutations lead to the occurrence of gene polymorphisms. A nucleotide shift from one sort to another, an insertion or deletion, or nucleotides rearrangements can all result in mutations. A polymorphism may be inherited once it has been created and can be transferred from parent to their children just like any other DNA sequence. Tandem repeat polymorphism, copy-number variations, and single-nucleotide polymorphisms, or SNPs, are the three forms of DNA polymorphisms (Ismail & Essawi, 2012).

Among the three, SNP is the most prevalent kind of polymorphism. The SNP identifies two alleles for which people in the population can have one of three genotypes: homozygous chromosomes, heterozygous chromosomes, or homozygous chromosomes with T-A on one chromosome and C-G on the homologous chromosome (Ismail & Essawi, 2012). There are approximately 3 million common SNPs in the human population; of these, approximately 1 million are routinely used in searches for SNPs that may be linked to complex disorders such as cardiovascular, endocrine, and neuropsychiatric diseases (Cargill et al., 1999).

It has been demonstrated that the genes for cytokines and their receptors are very polymorphic. The discovery that cytokine gene polymorphisms may affect the production of cytokines governing the immune response opened the door to understanding the intricate molecular basis of disease (Keen, 2002; Shastry, 2002). It is possible for polymorphisms to occur in either the coding or non-coding regions of the cytokine and cytokine receptor genes. Despite being quite common in non-coding regions like the promoter, intron, and untranslated regions, genetic polymorphism is less common in the coding region where amino acid substitution takes place. There is growing evidence that genetic variations in the cytokine gene's coding or non-coding regions affect the gene's capacity to produce cytokines. As a result, numerous studies have concentrated on establishing relationships between particular gene polymorphisms and the presence or absence of a disease, with the primary objective of classifying individuals into susceptibility groups. This is due to the fact that there is a possibility that a particular disease phenotype may be established as a result of the functional implications of polymorphisms (Dutra et al., 2009; Ollier, 2004).

Cytokine gene polymorphisms should be considered as variables that contribute to variance in the human immunological and inflammatory responses as well as potential susceptibility genes for relevant clinical conditions. The relationship between cytokine gene polymorphism and susceptibility to infections, illness development, viral persistence or clearance, as well as with responses to therapy, has been documented in a number of investigations. Examples are polymorphism of the IL-27 gene was found to be associated with susceptibility and disease progression in human immunodeficiency virus (HIV) infection (Pang et al., 2019), genes polymorphism of IL-17, TNF-α and TGF-β were associated with susceptibility and chronicity of hepatitis B virus infection and viral clearance (Suneetha et al., 2006;

Tayefinasrabadi et al., 2020) and IL-17 gene polymorphism was also found to be associated with Streptococcus pneumonia infection in Finnish children (Vuononvirta et al., 2015).

IL-4, IL-6, IL-10, IL-28B, and IFN-γ are among the cytokines gene polymorphisms that were discovered to be associated with either susceptibility, development of the disease to chronicity, as well as viral clearance and responsiveness to antiviral treatment in chronic HCV infection studies (Adnan et al., 2020; El-Bendary et al., 2017; Jing et al., 2020; Porto et al., 2015b; Ramos et al., 2012). The likelihood of developing liver cirrhosis is correlated with the polymorphism of the cytokine's gene, including IFN-γ and IL-10 (Mohamed et al., 2018). IL-1, IL-2, IL-10, IL-21, TNF-α, and IL-18 gene polymorphism has been linked to the development of hepatocellular carcinoma in a meta-analysis (Dondeti et al., 2016).

2.6 mRNA gene expression of cytokines in in inflammation and diseases

The proteins encoded by genes determine a cell's function. As a result, the tens of thousands of genes expressed in a single cell control the capability of the cell. Additionally, the cell possesses a possible control point for self-regulating its activities and this is accomplished by modifying the amount and kind of proteins generated at each step of the information transfer from DNA to RNA to protein (Phillips, 2008). The ratio of a protein's metabolic pathways for synthesis and degradation determines how much of it is present in a cell. The process of making proteins is completed by translation, which comes after transcription, which changes DNA into RNA and RNA into proteins. To ascertain which proteins and in what quantities are present in a cell, regulation of these activities is necessary. The manner in which a cell processes its

RNA transcripts and newly generated proteins is another factor that can influence the total amount of protein.

The quantity and kind of mRNA molecules in a cell indicate the functionality of the particular cell. mRNAs, which code for proteins, play an important role in eukaryotic gene expression. Precursor mRNA is transcribed from template DNA in the cell nucleus and undergoes 5' end-capping, 3' end-polyadenylation, and splicing (Romão, 2022). Splicing is the process by which introns are removed and exons are joined to form mature mRNAs. Mature mRNAs are transported to the cytoplasm for translation, storage, or destruction. mRNAs interact with RNA-binding proteins to form messenger ribonucleoprotein (mRNP) complexes, the protein composition of which fluctuates over time. This mRNP assembly is required for all mRNA metabolic activities as well as maintaining cellular homeostasis through correct gene expression. Noncoding RNAs (ncRNAs), which include snRNAs, miRNAs, lncRNAs, and circRNAs, interact with mRNPs to control mRNA metabolism and gene expression. Throughout the intricate life of mRNA, cells use surveillance systems to check its quality and quantity.

Transcriptional, post-transcriptional, and post-translational mechanisms govern gene expression and may be modulated at numerous levels to affect when and where proteins are created (Romão, 2022). Transcription is the first stage in gene expression control, where RNA production and protein expression may be altered. Post-transcriptional gene expression control allows a cell to adapt to its environment in a quicker and more reversible manner. Chemical changes that occur after transcription can have an impact on cellular RNAs. These modifications can affect mRNA structure, mRNA metabolism, and gene expression. In contrast, the mRNA translation process allows the cell to rapidly define its proteome in a spatiotemporal

manner. Because translational control is critical for cell homeostasis, growth, proliferation, and differentiation, any disruption or errors in its phases might lead to disease development.

The majority of common complicated diseases include malfunctions in several tissues and organs. Transcriptional changes in various tissues and organs are the primary cause of and reflect tissue and organ dysfunction. Notably, transcriptional variation modulates the causal relationships between genotype and complex characteristics (Basu et al., 2021). Cytokines regulate gene expression by binding to cell surface receptors. The biological roles of cytokines are dictated by the many gene expression patterns they generate (Shuai, 2006). Cytokines affect gene expression by using two main transcription factor families: signal transducer and activator of transcription proteins (STATs) and nuclear factor-B. (NF-B). STATs and NF-B become active inside the cytoplasm in response to stimulation by cytokines and then relocate to the nucleus, where they trigger transcription. These mechanisms for the cytokine-mediated activation of genes are subject to stringent regulation from both positive and negative regulators. The aberrant cytokine signalling contributes to the development of diseases such as malignancy and immune system problems.

However, the relative role of transcription in influencing protein levels remains controversial. A severe pathogen infection necessitates a complex response. Pathogens have evolved the ability to disrupt or influence almost every cellular process involved in gene expression (Barry et al., 2017). Host cells must be able to generate appropriate responses in the face of pathogen manipulation in order to develop an effective innate immune response to infection. The viral infection, on the other hand, can have a major influence on the host's gene expression programme. Viruses modify the host's gene