Manipulation of Interleukin-6 (IL-6) and Transforming Growth Factor Beta-1(TGFβ-1) towards viral induced liver cancer pathogenesis

By:

Maha Fatima (00000239472)

Iqra Majoka (00000240094)

Laiba Aslam (00000240238)

Huda Munawar (00000239399)

Atta-ur-Rahman School of Applied Biosciences (ASAB)

National University of Sciences and Technology (NUST), Islamabad

2021

Manipulation of Interleukin-6 (IL-6) and Transforming Growth Factor Beta-1(TGFβ-1) towards viral induced liver cancer pathogenesis

By:

Maha Fatima (00000239472)

Iqra Majoka (00000240094)

Laiba Aslam (00000240238)

Huda Munawar (00000239399)

Supervised By: Ms. Yasmin Badshah

Atta-ur-Rahman School of Applied Biosciences (ASAB)

National University of Sciences and Technology (NUST), Islamabad

2021

Manipulation of Interleukin-6 (IL-6) and Transforming Growth Factor Beta-1(TGFβ-1) towards viral induced liver cancer pathogenesis

By:

Maha Fatima (00000239472)

Iqra Majoka (00000240094)

Laiba Aslam (00000240238)

Huda Munawar (00000239399)

A thesis submitted to the National University of Science and Technology, Islamabad, in partial fulfillment of the requirements of the degree of

Bachelor of Science in

Applied Biosciences

Thesis Supervisor: Ms. Yasmin Badshah

Atta-ur-Rahman School of Applied Biosciences (ASAB)

National University of Sciences and Technology (NUST), Islamabad

2021

THESIS ACCEPTANCE CERTIFICATE

Certified that final copy of BS FYP Thesis written by **Maha Fatima, Iqra Majoka, Laiba Aslam, Huda Munawar** (Registration No 00000239472, 00000240094, 00000240238, 000239399) of 'Atta-Ur-Rahman School of Applied Biosciences' has been vetted by undersigned, found complete in all respects as per NUST Regulations, is free of plagiarism, errors, and mistakes and is accepted as partial fulfilment for award of BS degree. It is further certified that necessary amendments as pointed out during final presentation of the scholar, have also been incorporated in the said thesis.

Signature: _____

Name of Supervisor: Yasir Bashir

Date: 25/6/2021

Signature: _____

Name of Co-Supervisor: Dr. Maria Shabbir

Date: 25/6/21

Signature (HOD): _____

Dr. Touqeer Ahmed
Head of Department (HoD)
Deptt of Healthcare Biotechnology
Atta-ur-Rahman School of Applied Biosciences (ASAB), NUST Islamabad

Date: 28/6/2021

Signature (Dean/Principal): _____

Dr. Husnain A. Janjua
Principal
Atta-ur-Rahman School of
Applied Biosciences (ASAB)
NUST, Islamabad

Date: _____

AUTHOR'S DECLARATION

I Maha Fatima, Iqra Majoka, Laiba Aslam, Huda Munawar hereby state that this BS FYP thesis titled "Manipulation of Interleukin-6 (IL-6) and Transforming Growth Factor Beta-1(TGFβ-1) towards viral induced liver cancer pathogenesis" is my own work and has not been submitted previously by me for taking any degree from this university 'National University of Sciences & Technology' or else anywhere in the country/world.

At any time if my statement is found to be incorrect even after my graduation, the university has the right to withdraw my BS degree.

Name of student:

Maha Fatima: Mahafatima

Iqra Majoka: Iqra!

Laiba Aslam: Laiba

Huda Munawar:

Date: 25-06-21

Certificate for Plagiarism

It is certified that BS FYP Thesis Titled "Manipulation of Interleukin-6 (IL-6) and Transforming Growth Factor Beta-1(TGFβ-1) towards viral induced liver cancer pathogenesis" by Maha Fatima, Iqra Majoka, Laiba Aslam, Huda Munawar has been examined by me. I undertake the following: -

a. Thesis has significant new work / knowledge as compared to already published or are under consideration to be published elsewhere. No sentence, equation, diagram, table, paragraph or section has been copied verbatim from previous work unless it is placed under quotation marks and duly referenced.
b. The work presented is original and own work of the author (i.e., there is no plagiarism). No ideas, processes, results or words of others have been presented as Author's own work.
c. There is no fabrication of data or results which have been compiled / analysed.
d. There is no falsification by manipulating research materials, equipment or processes, or changing or omitting data or results such that the research is not accurately represented in the research record.
e. The thesis has been checked using TURNITIN (copy of originality report is attached) and found within limits as per HEC / NUST plagiarism Policy and instructions issued from time to time.

Date: 25-06-21

Student Signature

Maha Fatima: Mahafatima

Iqra Majoka: Iqra!

Laiba Aslam: Laiba

Huda Munawar: HM

Dr. Yasmin Bilal
Assistant Professor
Atta-ur-Rahman School of Applied Biosciences (ASAB)
NUST

Date: 25-06-21

Signature & Stamp of Supervisor

This thesis is dedicated to our beloved parents, friends, and family whose encouragement and guidance has been unmatched.

ACKNOWLEDGEMENTS

First and foremost, we thank our research supervisors, Ms.Yasmeen Badshah and Dr. Maria Shabbir for their assistance and dedicated involvement throughout our final year project. We would like to thank them for their constant support and understanding over these past four years.

We wish to express our sincere appreciation to Dr. Hussnain A. Janjua and the entire ASAB department for providing us with all the necessary facilities to conduct our final year project.

We would also like to show special gratitude to the ASAB diagnostic lab staff, particularly Mr. Izzat Ullah Khan and Mr. Nasir. They helped and guided us during our lab working hours and we fondly think of our time spent in the lab. We are grateful to Ms. KhushBakht Khan who helped us with the in-silico work and guided us throughout our thesis write-up and compilation process.

Most importantly, none of this could have been possible without our parents, family, friends, and classmates who offered their encouragement all these years. We thank them for their constant support and for always believing in our abilities.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

%	Percentage
A	Alpha
B	Beta
Γ	Gamma
°C	Degree Celsius
µL	Microliter
AFP	Alpha FetoProtein
ARMS	Amplification Refractory Mutation System
ALT	Alanine Transaminase
BCLC	Barcelona Clinic Liver Cancer
BP	Base Pair
CI	Confidence Interval
CRP	C-reactive Protein
CT	Computerized Tomography
DNA	Deoxyribonucleic Acid
DR	Dopamine Receptor
EDTA	Ethylenediaminetetraacetic Acid
ER	Endoplasmic Reticulum
ERK	Extra-cellular Signal Regulated Kinase
HBV	Hepatitis B Virus
HCC	Hepatocellular Carcinoma
HCV	Hepatitis C Virus
HIV	Human Immunodeficiency Virus
HKLC	Hong Kong Liver Cancer
IL-6	Interleukin 6
LAP	Latency Associated Peptide
LT	Liver Transplantation
JNK	JUN N-Terminal Kinase

MAPK	Mitogen-Activated Protein Kinase
MRI	Magnetic Resonance Imaging
NFKB	Nuclear Factor Kappa-B
NF-1	Nuclear Factor 1
OR	Odds Ratio
PPAR- α	Peroxisome proliferator-activated receptor alpha
PCR	Polymerase Chain Reaction
PDB	Protein Data Bank
PDGFR	Platelet derived growth factor receptor
RNA	Ribonucleic Acid
RFA	Radiofrequency Ablation
ROS	Reactive Oxygen Species
RR	Relative Risk
RXR- α	Retinoid X receptor-alpha
SNP	Single Nucleotide Polymorphism
STAT	Signal Transducer and Activator of Transcription
TACE	Trans-arterial Chemoembolization
TE	Tris-EDTA
TBE	Tris Borate EDTA
TGFβ-1	Transforming Growth Factor Beta-1
TNF	Tumor Necrosis Factor- Alpha
US	Ultra-Sound
VEGFR	Vascular Endothelial Growth Factor Receptor
ZEB	Zinc finger E-box-binding homeobox
ZO1	Zonula-occludens-1

ABSTRACT

Background: Hepatocellular carcinoma (HCC) is the most common liver malignancy. Early diagnosis of HCC has always been challenging. Hepatitis-C Virus (HCV)-induced HCC makes up for 25% of HCC cases annually. Many studies have reported the association of TGFβ-1 and IL-6 gene polymorphisms with the predisposition of HCC. This study aims to assess the pathogenicity and the prevalence of IL-6 -174G/C (rs1800795) and TGFβ-1 +29C/T (rs1800470) polymorphisms in HCV-infected HCC patients.

Methodology: This study uses multiple bioinformatics tools to analyse the pathogenicity of the TGFβ-1 +29 C/T and IL-6 -174 G/C polymorphisms. AliBaba2 was used to predict the transcription factor binding sites in the mutant and wild type IL-6 genes. The structural changes in the mutant TGFβ-1 structure were determined through project HOPE. To assess the polymorphic prevalence of IL-6 -174 G/C and TGFβ-1 +29 C/T genotypes in HCC and control subjects, ARMS-PCR was performed on 213 diseased and 216 control samples. GraphPad Prism was used for the statistical analysis of the results. CB Dock was used for the molecular docking analysis of tetrahydroxyflavanone with IL-6 and TGFβ-1 proteins to predict its inhibitory potential against the two cytokines.

Results: In-silico analysis revealed the regulatory nature of both IL-6 -174 G/C and TGFβ-1 +29 C/T polymorphisms but with limited functional significance. The structural variation in mutant TGFβ-1 such as Pro10Leu substitution might contribute to the structural instability. An additional transcription factor binding site of nuclear-factor-1 was identified in the mutant IL-6 structure. ARMS-PCR results revealed that the individuals carrying TT genotype for TGFβ-1 gene have an increased risk of developing HCC ($p<0.0001$, OR=5.403, RR=2.062) as compared to individuals with CT and CC genotype. Similarly, GC genotype carriers for IL-6 gene exhibit an increased risk of HCC susceptibility ($p<0.0001$, OR=2.276, RR=1.512) as compared to the people carrying the GG genotype. The molecular docking results revealed considerable docking efficiency of tetrahydroxyflavanone with IL-6 (-7.3 kJ/mol) and TGFβ-1 (-7.7 kJ/mol) proteins. Compared to the conventional chemotherapy, tetrahydroxyflavanonone shows a promising potential for treating HCC.

Conclusion: The TT genotype of TGFβ-1 gene and the GC genotype of IL-6 gene are found to be associated with the development of HCC in patients. Tetrahyrdoxyflavanone is proposed as a promising treatment option for HCC.

CHAPTER 1

INTRODUCTION

1.1. Overview of Hepatocellular Carcinoma (HCC)

Hepatocellular carcinoma (HCC) is a type of primary liver cancer. It is the sixth most common cancer and is the leading cause of death worldwide. The distribution of HCC is different according to different geographic regions. The prevalence of the disease is high in Eastern/South-Eastern Asia, Africa and intermediate in Southern Europe (Bosetti, Turati, & La Vecchia, 2014). Similarly, Italy, Greece and Spain have an intermediate rate while North and South America have a relatively low prevalence rate of HCC (Mittal & El-Serag, 2013). Hepatitis B and C viruses (HBV and HCV, respectively) are recognized to be the major culprits for causing HCC in South-East Asia, specifically in Pakistan. The risk of HCC is high in people with chronic liver diseases such as cirrhosis caused by HBV, HCV, and other factors (Balogh et al., 2016). Alpha fetoprotein (AFP) is a serum glycoprotein that is frequently used as a biomarker for HCC detection. However, this biomarker is not very specific as its elevated levels are also observed in other medical conditions (Bergmann et al., 2017).

1.2. Overview of Genes

1.2.1. Interleukin-6

Interleukin-6 (IL-6) is a cytokine that mediates proliferation and differentiation of hematopoietic stem cells (Manfredini et al., 2003). In liver, IL-6 is found to be involved in liver regeneration and in inducing acute phase responses against infections (Schmidt-Arras & Rose-John, 2016). IL-6 plays a vital role in the mitogenesis and homeostasis of hepatocytes. An increased activation of IL-6 signalling pathway can lead to liver tumorigenesis. Several single nucleotide polymorphisms (SNPs) in IL-6 gene have been linked to cancers. Studies have predicted the association of three IL-6 promoter polymorphisms (rs1800795, rs1800796, rs1800797) with increased risk of cervical cancer, colorectal cancer, breast cancer, prostate cancer, lung cancer, glioma, non-Hodgkin's lymphoma, and Hodgkin's lymphoma (Peng et al., 2018; Slattery, Wolff, Herrick, Caan, & Potter, 2007; Yu, Wang, Zhai, Dang, & Sun, 2012) .

1.2.2. Transforming growth factor beta-1 (TGFβ-1)

Transforming growth factor beta-1 (TGFβ-1) belongs to a family of cytokines involved in cell differentiation and cell growth. The TGFβ-1 protein is mostly found in skeleton tissues, where it controls the development and growth of bone and cartilage. The signalling pathway of TGFβ-1 is found to play a vital role in the progression of liver diseases (Tu et al., 2019). Studies have shown that the over-activation of TGFβ-1 pathway causes liver inflammation and fibrosis leading to HCC. Various studies have predicted the association of TGFβ-1 polymorphisms with the predisposition of different types of cancers. Several studies have reported SNPs in TGFβ-1 (exon 1: 327 C>T,

rs1982073; exon 5: 73 C>T, rs1800472) to be associated with breast, colorectal, lung and nasopharyngeal carcinomas and non-Hodgkin lymphomas (Castillejo et al., 2009).

This study employs an *in-silico* approach using bioinformatics tools to determine pathogenic variants of TGFβ-1 and IL-6 genes (rs1800470 and rs1800795, respectively) followed by their validation through allele-specific ARMS-PCR. This approach will aid the earlier diagnosis of HCC by screening the patients against pathogenic SNPs. Moreover, this research aims to predict therapeutic potential of tetrahydroxyflavanone for the inhibition IL-6 and TGFβ-1 in HCC patients. Tetrahydroxyflavanone belongs to flavonoid family of plant secondary metabolites comprising of polyphenolic compounds. Flavonoids have shown various anti-cancerous, antiviral, and antimicrobial properties (García, Gutierrez, Melo, Novaes, & Gonçalves, 2018; Liao et al., 2015). This study uses CB-Dock for the molecular docking analysis to propose tetrahydroxyflavanone as an alternative therapeutic option for HCC.

1.3. Problem Statement

800,000 cases of HCC are reported annually out of which 700,000 people die from HCC every year across the world (García et al., 2018). 25% of the world's HCC cases are caused by HCV infections (Bruix, Gores, & Mazzaferro, 2014) and 71 million people in the world are infected with HCV (Dash, Aydin, Widmer, & Nayak, 2020). HCC is ranked third amongst all other cancers for cancer-related mortalities (Hoshida, Fuchs, Bardeesy, Baumert, & Chung, 2014). Current diagnostic techniques are inefficient to diagnose HCC at early stages. Moreover, the treatment options prove ineffective to restore the health of the patient. Therefore, early diagnostic markers are needed to diagnose HCC at the early stage. Furthermore, new compounds need to be tested against the overexpressed cytokines in HCC to facilitate regression of the tumor and to lower down the disease burden. Improved diagnostic markers would help to increase the lifespan of HCC patients by interfering at an early disease stage. Better treatment options would aid to lower down the annual death ratio.

1.4. Purpose Statement

The purpose of this study is to compare the prevalence of IL-6 -174 G/C and TGFβ-1 +29 C/T polymorphisms in the diseased and control samples of HCV-infected HCC patients followed by analysing the docking capacity of tetrahydroxyflavanone with IL-6 and TGFβ-1 to lower their overexpression.

1.5. Hypothesis

We hypothesize that the IL-6 -174 G/C and TGFβ-1 +29 C/T polymorphisms increase the risk of HCC development in patients infected with HCV. We also hypothesize that tetrahydroxyflavanone can interact with IL-6 and TGFβ-1 protein structures to downregulate their over-expressed signalling pathways.

1.6. Objectives

1. To assess the pathogenicity of the IL-6 -174 G/C and TGFβ-1 +29 C/T polymorphisms through *insilico* analysis.
2. To assess the association of IL-6 -174 G/C polymorphism with the risk of HCV-induced HCC in Pakistan.
3. To assess the association of TGFβ-1 +29 C/T polymorphism with the risk of HCV-induced HCC in Pakistan.
4. To identify the role of IL-6 -174 G/C and TGFβ-1 +29 C/T polymorphisms as early diagnostic markers of HCC in HCV-infected patients.
5. Assessing the inhibitory potential of tetrahydroxyflavanone against the overexpression of TGFβ-1 and IL-6 proteins.

CHAPTER 2

LITERATURE REVIEW

2.1. Hepatocellular Carcinoma

HCC is a hepatocyte derived primary liver cancer. It is the sixth most common cancer with an average diagnosis rate of 800,000 people per year. The significant mortality rate of 700,000 people per year is quite alarming (García et al., 2018). The history of HCC is dated back to the early 20th century (But, Lai, & Yuen, 2008). Eggel in 1910 and Yangiwa in 1911 proposed the earliest classification of HCC (Okuda, 2002). HCC was initially classified as nodular, diffused, and widespread (But et al., 2008). The particular interest in HCC emerged when the increasing number of HCC cases were reported in Mozambican males in 1951. Following this, worldwide studies on HCC increased leading to the development of the understanding that the pathological and epidemiological features of HCC vary from one region to another (But et al., 2008; Okuda, 2002).

When observing the South African population, the tumours are large with poor differentiation and limited or chronic disease progression in surrounding tissues (But et al., 2008; Okuda, 2002). Cancer afflicted livers of African populations weigh more than 4kg when compared to the Japanese population weighing only 2 kg (Okuda, 2002). Outside of Africa, the tumour is reported to be significantly shrunk in size with higher differentiation patterns (But et al., 2008). Such considerable differences can be attributed to diverse causative factors in different populations. In African settings, aflatoxin B1 (a mycotoxin secreted by *Aspergillus* species) is the carcinogenic molecule (But et al., 2008; Okuda, 2002). Whereas hepatitis caused cirrhosis is reported to be prevalent outside of Africa. One interesting type of non-viral HCC with fibrolamellar characteristics is unique to the young Caucasian population and is non-existent in the far East (But et al., 2008; Okuda, 2002).

2.2. Key Mediators of HCC

2.2.1. Viral Infections

Viral infections (HBV and HCV) are associated with 80% of HCC cases worldwide (But et al., 2008; McGlynn, Petrick, & London, 2015). HBV, HCV, and aflatoxin are reported to be the most established risk factors of HCC (McGlynn & London, 2011). Around 52% of the HCC cases are caused by HBV followed by considerable percentages of HCV and alcohol. HBV mediated HCC occurs in cirrhotic patients in Europe. However, the trend is quite opposite in Asia where HBV-related HCC occurs in non-cirrhotic patients (Bergmann et al., 2017). Aflatoxin is responsible for a mutation in the tumour suppressor gene of p53 and is reported to play a synergistic role with HBV in HCC development (Bergmann et al., 2017; Guerrero & Roberts, 2005; Tarocchi, Polvani, Marroncini, & Galli, 2014).

2.2.2. Other Factors

Other probable causes include already present liver cirrhosis, alcoholic liver disease, autoimmune diseases, Wilson's disease, and hemochromatosis (But et al., 2008; McGlynn & London, 2011). HCC can also be due to alcohol abuse and co-morbidities (Arbuthnot & Kew, 2001). Obesity and type 2 diabetes are also reported to be HCC associated factors (Bergmann et al., 2017). Alcohol plays a synergistic role with other causative factors such as HBV, HCV, and metabolic risk factors to develop HCC. Hereditary hemochromatosis and biliary cirrhosis are aggravating factors for HCC (Bergmann et al., 2017).

However, the most prevalent risk factors differ in the high and low incidence region. Aflatoxin and HCV are the major cancer-causing factors in Asia and Africa (high-incidence areas). Whereas, non-viral risk factors are prevalent in low-incidence regions (Western countries) (McGlynn & London, 2011; McGlynn et al., 2015).

2.3. Incidence of HCC

2.3.1. Region Susceptibility

The highest number of HCC cases are reported in Asia and Africa. Out of the 70% cases occurring in Asia, 50% originate in China (McGlynn & London, 2005; McGlynn et al., 2015). A paper published in 2008 reported the highest incidence of HCC in China i.e., 52.1 per 100,000. It decreases significantly with 5.1 cases per 100,000 when studying populations in Northern Europe (But et al., 2008). Along with China, other regions with a high prevalence of HCC included sub-Saharan Africa, Eastern & South-eastern Asia, and Melanesia (But et al., 2008). African and Asian populations show a distinct trend of HCC from the western world. They usually acquire the viral infection during early childhood. The virus stays dormant followed by a prolonged period of immune tolerance (But et al., 2008). The areas of low incidence include Northern Europe, Oceania, the Middle East, North America, and South America. The ratio of cases in Central Europe is intermediate (McGlynn & London, 2005, 2011; McGlynn et al., 2015).

When compared with the statistics in the past, the areas that used to have higher incidence report a decrease in cases that can be attributed to successful HBV vaccination programs (But et al., 2008; McGlynn et al., 2015). Other probable causes include a lower infection rate of HCV in China & Singapore and the declining percentage of people who got infected with HCV in the earlier 20th century in Japan (McGlynn & London, 2011). Some of the areas from the western side of the world showed an upsurge in HCC cases in recent studies that might be linked to an increased viral load of HCV in these areas or HBV carried by immigrants from endemic areas (But et al., 2008). The increasing incidence of obesity, diabetes and the improved survival rates of patients with cirrhosis also contribute towards this trend in previously low-incidence countries (McGlynn & London, 2011).

HBV is acquired during prenatal years whereas HCV is contracted during adulthood (McGlynn & London, 2005; Tarocchi et al., 2014). It takes around 2 to 8 decades to

develop HCC in the virus-infected patient. Several changes in the liver can be observed including fibrosis, cirrhosis, chronic inflammation, and an increased rate of hepatocyte death and regeneration (McGlynn & London, 2005).

2.3.2. Gender Susceptibility

The HCC incidence in males is 2-3 times higher than in females and this gender disparity can be observed in the epidemiology data of nearly all the countries (Bergmann et al., 2017; McGlynn & London, 2005, 2011; McGlynn et al., 2015). The risk of HCC in an infected man is estimated to be 10-25% whereas it is lower in women (But et al., 2008).

Some of the possible explanations for this disparity might include the higher susceptibility of males to HBV and HCV infection, higher rate of cigarette smoking & alcohol consumption, and the availability of comparatively large numbers of aflatoxin markers in the male population (McGlynn & London, 2005, 2011). Higher oestrogen levels in females cause downregulation of IL-6 which is produced naturally by Kupffer cells. This inhibitory effect results in diminished liver injury and normal proliferation of hepatocytes (Bergmann et al., 2017).

2.4. Hepatitis C mediated Carcinogenesis

As per a report published by WHO, 3% of the world population is HCV positive (Goossens & Hoshida, 2015; Hoshida et al., 2014; Selimovic et al., 2012). HCV is attributed to 25% of the world's HCC cases (Bruix et al., 2014). 71 million people are infected with HCV. Only 20-30% of them develop cirrhosis followed by 1-7% people developing HCC per year (Dash et al., 2020; Hoshida et al., 2014). HCC risk increases by 17% in HCV positive patients whose intensity can be defined by the degree of liver fibrosis, and when the patient acquired the infection (But et al., 2008).

HCV is acquired in adulthood as opposed to HBV which causes infection in the perinatal period or early childhood (McGlynn & London, 2005). Long term infection of HCV leads to the development of HCC. Older age and alcohol consumption aid the development of HCC in HCV infected patients (McGlynn & London, 2005). The development of HCV related HCC is a multi-step process comprising chronic inflammation, fibrosis, genomic alterations, and formation of malignant microenvironment (Goossens & Hoshida, 2015). The inability to clear the HCV infection leads to chronic Hepatitis C followed by the production of lesions, fat accumulation, and progressive fibrosis leading to cirrhosis in the upcoming 30-40 years of patient's life (Dash et al., 2020; Goossens & Hoshida, 2015).

HCV is more lethal than HBV in the development of HCC in the western countries as compared to the Asian and African region where HBV is the primary HCC causing factor (Dash et al., 2020). When compared with the Asian countries, the diagnostic markers of HCC are found in a higher proportion in Spain (60-75%), France (27-58%), and Italy (44-66%). Japan also has 80-90% incidence of HCV-induced HCC cases (But et al., 2008; McGlynn & London, 2005). Duration of the disease, viral genotype, and

ethnicity play a role in the intensity of HCC (Axley, Ahmed, Ravi, & Singal, 2018). The degree of fibrosis in the patient's liver determines the degree of the disease. Many co-morbidities and other HCC risk factors such as obesity, diabetes, non-alcoholic steatohepatitis, and HBV & HIV infection enhance the HCV associated HCC progression (Axley et al., 2018; Dash et al., 2020; Goossens & Hoshida, 2015).

2.4.1. Structure of Hepatitis C Virus

HCV is a 9.6 kb enveloped single-stranded RNA virus belonging to the family Flaviviridae (Chen & Morgan, 2006; Hoshida et al., 2014; Selimovic et al., 2012). The genome is a positive sense RNA that encodes 10 structural and non-structural proteins. The structural proteins of HCV include core, E1, E2 whereas the non-structural proteins are p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B. The natural hosts of HCV are humans and chimpanzees (Chen & Morgan, 2006; Hoshida et al., 2014). Various routes of transmission have been reported by different studies including intravenous drug abuse, unsafe sexual intercourse, unscreened blood transfusions, and vertical transmission (Chen & Morgan, 2006). The receptors of HCV are found on the surfaces of hepatocytes, monocytes, lymphocytes, and secretory cells (Goossens & Hoshida, 2015).

2.4.2. Mechanism of Pathogenesis

HCV cannot integrate itself into the host genome unlike HBV, which integrates via reverse transcriptase enzyme. HCV, therefore, causes HCC by indirect mechanisms such as chronic inflammation, proliferation, cirrhosis, and necrosis (But et al., 2008; Goossens & Hoshida, 2015; Hoshida et al., 2014). Cirrhotic HCV patients have a greater incidence (5.8%) of developing HCC when compared to non-cirrhotic patients (But et al., 2008). Owing to a wide range of studies, it is considered that HCV-related HCCs are mostly found in patients with cirrhosis. HCV causing HCC without cirrhosis is less frequently reported (But et al., 2008). As per a study, 15% of the patients develop HCC without cirrhosis. HCC can also cause non-cirrhotic infection in a patient with non-alcoholic fatty liver disease and coinfected with HBV (Dash et al., 2020).

2.4.2.1 Disruption of Host Proteins

HCV mediated HCC is a long-term process spanning over 20-40 years (Axley et al., 2018; Hoshida et al., 2014). The pathogenicity of HCV is mediated by both structural and non-structural proteins of HCV (Hoshida et al., 2014). Both viral-induced mechanisms and host immune response play a role in the development of carcinogenesis (Axley et al., 2018). HCV causes HCC by interfering with cell proliferation and cell survival pathways (Goossens & Hoshida, 2015). HCV proteins are reported to damage normal lipid metabolism and oxidative stress response (W. Li, Wu, & Wan, 2007).

The core and non-structural proteins mediate the signalling of TGF-β1 by activating reactive oxygen species production, p38, MAPK, JNK, ERK, and NFKB pathways (Hoshida et al., 2014; Lin et al., 2010; Schulze-Krebs et al., 2005). HCV downregulates the anti-tumour activity of TGF-β1 by interacting with SMAD 3 (Hoshida et al., 2014).

Various tumor suppressor genes TP53, TP73, & RB1 and down regulator of cell cycle i.e., CDKN1A are suppressed by HCV proteins to promote tumor progression (Alisi et al., 2003; Hoshida et al., 2014; Kao, Chen, Chen, & Lee, 2004; Machida et al., 2009). HCV core proteins suppress retinoblastoma protein and p53 tumour suppressor gene. The suppressor effect synergistically leads to the development of carcinogenesis (Lemon & McGivern, 2012). HCV also interacts with RXR-alpha (Retinoid X receptor-alpha), and PPAR-alpha (peroxisome proliferator-activated receptor alpha) to disturb the normal cell proliferation, cell differentiation, and fatty acid transport (N. Tanaka et al., 2008). Due to disturbed mitochondrial function by HCV, PPXR-alpha causes steatosis, promotes oxidative stress, and upregulates cell growth signals (Koike, 2009). Several somatic mutations in telomerase reverse transcriptase promoter have also been reported in HCV mediated HCC (Axley et al., 2018; Nault et al., 2013).

2.4.2.2. Cytokines & Inflammatory factors

Anti-tumour immunity is upregulated by HCV core proteins. They inhibit the NFKB mediated immune responses (Nault et al., 2013). Proinflammatory signals such as reactive oxygen species (ROS) create an inflammatory microenvironment by upregulating the JNK pathway in liver cells (Hui, Zatloukal, Scheuch, Stepniak, & Wagner, 2008). HCV leads to the development of steatosis by inhibiting the release of triglyceride-rich proteins (Perlemuter et al., 2002).

2.5. Interleukin-6

Interleukin-6 (IL-6) is defined as a pleiotropic cytokine that mediates proliferation and differentiation of hematopoietic stem cells. It has also been reported in the pathogenesis of various diseases (Manfredini et al., 2003). IL-6 expression is mostly found in hepatocytes, monocytes/macrophages, neutrophils, and lymphocytes (Schett, 2018).

The role of IL-6 has been reported in inflammation, regeneration, and tumour progression. IL-6 is actively involved in acute phase response, inflammation reactions, hematopoiesis, hepatic regeneration, metabolism, bone formation, cancer development, cardiovascular function, and neural development (Schett, 2018). IL-6 also plays a central role in activating the innate and adaptive immune system. IL-6 is attributed to the differentiation of monocytes into the macrophages and immunosuppressive function in dendritic cells. IL-6 also promote T cell survival by upregulating anti-apoptotic factors (Schett, 2018). IL-6 promotes hepatocyte proliferation by upregulating the production of acute-phase proteins i.e., C reactive protein (CRP), serum amyloid A, fibrinogen, and α1-antichymotrypsin (Schett, 2018).

2.5.1. Structure of IL-6

The gene for IL-6 is located on the short arm of chromosome 7 i.e., 7p21. The IL-6 transcript contains four introns and five exons. IL-6 exists in the form of single-chain glycoprotein (Simpson, Hammacher, Smith, Matthews, & Ward, 1997). IL-6 cytokine family includes IL-6, IL-11, IL-27, IL-20, and IL-31. IL-6 consists of four alpha-helical chains. Helical cytokine structure exists in the form of up-up-down-down topology. A

and B helix are structured diagonally opposite to the C and D helix (Simpson et al., 1997). Linking between helices is maintained by a long loop between helix A and B, a short loop between B and C, followed by again a long loop between helix C and D (Somers, Stahl, & Seehra, 1997).

IL-6 belongs to a superfamily that has remarkable structural similarities between cytokine and its receptor (Somers et al., 1997). The helices are marked as Helix A (Thr20-Lys46), AB loop (25 amino acid long), Helix B (Glu80-Asn103), Helix C (Glu109-Lys129), and Helix D (Gln156-Met184). An additional small helix is formed by Pro141-Gln152 as the Helix E (Simpson et al., 1997; Somers et al., 1997). Helix E is positioned within the CD loop. The remaining residues of the CD loop following helix E are disordered, possess low electron density and are not visible in the crystal structure (Gelinas et al., 2014).

Several serine residues in the IL-6 structure are phosphorylated subjected to the site of its presence. IL-6 structure contains four cysteine residues that are highly conserved in humans and other mammal species. These cysteine residues form two disulfide bonds i.e., Cys44-Cys50 and Cys73-Cys83. Disulfide bonds maintain the structural integrity of receptor binding sites (Simpson et al., 1997). Out of the 5 methionine residues (Met49, Met117, Met184, Met161, Met67), Met161 play a role in receptor binding. Trp157 lies closer to the receptor-binding site and is believed to perform a role in receptor binding (Gelinas et al., 2014; Simpson et al., 1997). A hydrophobic signal of 24-28 residues in cleaved form is present at the N-terminal of unfolded protein. There have been reported some differential cleavage which states that the mature protein can differ by 2 amino acid residues on N-terminal (Simpson et al., 1997).

2.5.2. IL-6 Attachment with Receptor

IL-6 binds to the IL-6 receptor consisting of two chains. The alpha chain is IL-6R which is an 80 kDa protein and binds with low affinity to IL-6. However, it is necessary for forming an IL-6-IL-6R complex to bind with the second transmembrane beta chain i.e., gp130 which is a 130 kDa protein (Manfredini et al., 2003; Simpson et al., 1997; Somers et al., 1997). IL-6R structure consists of cytokine binding type I domain, Ig-like domain, and short cytosolic domain. The domains in the gp130 structure include Ig-like domain, cytokine type I domain, a contacting signalling like region, a transmembrane domain, and a cytosolic domain necessary for signalling with a motif such as box1 and box2 (Somers et al., 1997).

IL-6 structure constitutes three contact points to bind with its receptor. The site-I is a conserved region that forms a bond with a non-signalling receptor i.e., IL-6R (Manfredini et al., 2003; Simpson et al., 1997; Veverka et al., 2012). It includes the C terminal portion of helix D, C terminal region of AB loop/N terminal part of Helix B (Manfredini et al., 2003; Simpson et al., 1997). Site II has a composite interface that recruits gp130 and the interactions are mediated by residues on helix A and C

(Manfredini et al., 2003; Paonessa et al., 1995). Site III consists of the N terminal residues of the helix D/CD loop and some residues of the initial part of the AB-loop that make additional contact with gp130 (Manfredini et al., 2003; Paonessa et al., 1995; Simpson et al., 1997; Somers et al., 1997).

2.5.2.1. Site 1

Eight residues in Site I (Met 161, Leu 158, 165, 167, 174, 181) play an active role in binding to the IL-6R contributing maximum of the binding energy. Leu 165 is present on the interface between loop AB and helix D. Leu 174, and 181 are buried within this region. These residues are likely to be involved in structural stability rather than in binding with receptor (Simpson et al., 1997). Ser176 and Arg179 are identified to be actively involved in receptor binding (Simpson et al., 1997; Somers et al., 1997). A region comprising the AB loop (Gly77-Glu95) play a crucial in binding to sIL-6R (Simpson et al., 1997). Gln175, Arg182, Phe74, Ser 177, Ala180, Leu 178, and Leu 181 have been studied through mutagenesis studies and are identified to be vital in binding interactions with the receptors (Somers et al., 1997).

2.5.2.2. Site 2

Four amino acid residues within helix A (Arg24, Lys27, Gln28, and Tyr31) along with 2 residues of helix C (Val 121, Phe125) serve as the contact points of site II interaction with gp130 (Simpson et al., 1997). Gly35 along with Tyr 31 is separately reported by a study to be involved in gp130 binding (Paonessa et al., 1995; Somers et al., 1997). Ser118 is located close to the binding site (Somers et al., 1997).

2.5.2.3. Site 3

Lys41, Ala56 located within helix A and AB loop are involved in IL-6R mediated binding of IL-6 to gp130. A single binding determinant is formed by these 2 amino acids with two more amino acids located in CD loop-Helix D i.e., (Glu159, Thr162) (Simpson et al., 1997; Somers et al., 1997). Glu 51-59 in the AB loop serve to stabilize the interaction between IL-6 and gp130 and are not directly involved in binding (Simpson et al., 1997). Trp157 and Asp160 are also reported by a study to be the site III binding residues (Paonessa et al., 1995; Somers et al., 1997).

2.5.3. Role of IL-6 in Liver

Liver regeneration is an immediate response to a liver injury. An increased level of tumor necrosis factor-α (TNF-α) and IL-6 has been observed in the patients undergoing hepatectomy. Studies performed in IL-6 knockout mice resulted in a low proliferation of hepatic progenitor cells followed by poor liver regeneration. Studies conducted in transgenic mice revealed the serum binding protein role of IL-6R to improve the half-life of IL-6. IL-6 involvement with IL-6R plays a dramatic role in a high rate of liver regeneration. As already discussed, gp130 is more widely expressed by the cells than IL-6R, therefore, IL-6 association with sIL-6R causes more widespread hepatic

regeneration by recruiting widely expressed gp130 molecule (Schmidt-Arras & Rose-John, 2016).

2.5.4. Signaling Mechanism of IL-6

IL-6 signalling takes place through two pathways. One is a direct pathway whereas the other is the trans-signalling pathway (Bergmann et al., 2017; Schett, 2018; Schmidt-Arras & Rose-John, 2016). Along with IL-6R, IL-6 associated signalling is dependent upon the participation of gp130 to form a complex (Bergmann et al., 2017; Jones, Scheller, & Rose-John, 2011; Wormald et al., 2006). gp130 forms part of α chains of receptor complex to initiate a signalling cascade. gp130 is also reported to form a complex with several cytokines such as IL-6, IL-11, IL-27, Leukemia inhibitory factor, cardiotrophin 1, and others. It is found on the surface of both hematopoietic and non-hematopoietic stem cells. gp130 expression on a cell is related to the extent of cell proliferation activity (Wormald et al., 2006).

2.5.4.1. Direct Pathway

Direct pathway requires the presence of IL-6 receptor i.e., IL-6R on the cells (Bergmann et al., 2017; Jones et al., 2011; Schett, 2018). Along with IL-6R, gp130 is also required to form a complex. gp130 is ubiquitously present on the cells whereas IL-6R is exclusively present on the surface of hepatocytes, leukocytes, and epithelial cells etc (Schmidt-Arras & Rose-John, 2016). IL-6 binds to CD126 or IL-6R which serves as a non-signalling α-receptor (Bergmann et al., 2017; Giannitrapani, Soresi, Balasus, Licata, & Montalto, 2013; Jones et al., 2011; Schett, 2018).

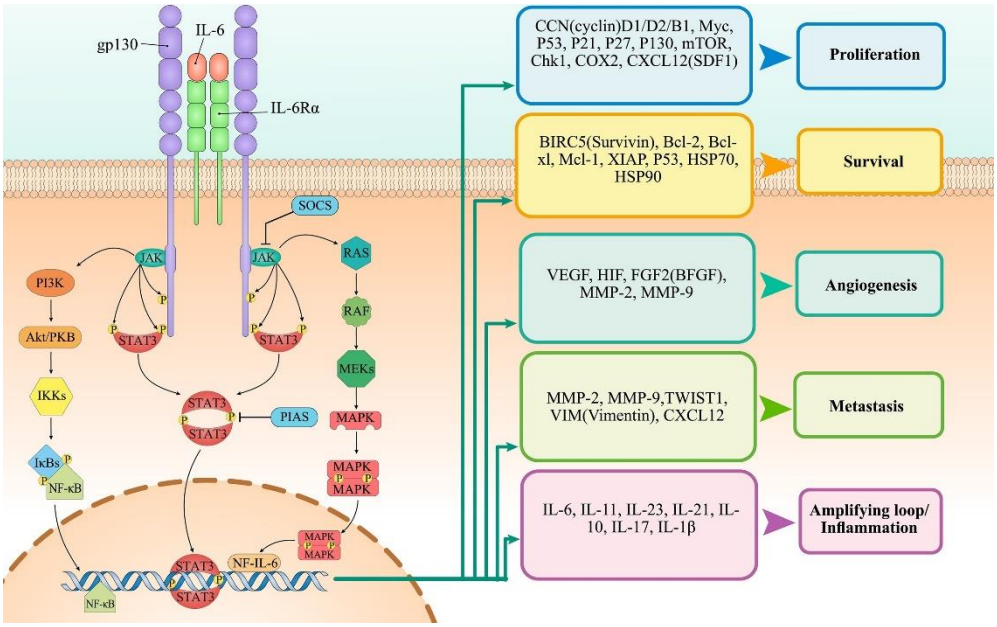

Figure 1. Signalling Mechanism of IL-6(Masjedi et al., 2018)

IL-6-IL-6Rα complex dimerizes with signal-transducing β-subunit gp130 following binding to form a heterohexameric complex (Bergmann et al., 2017; Giannitrapani et

al., 2013; Schmidt-Arras & Rose-John, 2016). This hexameric complex contains 2 molecules each of IL-6, IL-6Rα, and gp130 (Bergmann et al., 2017). The tyrosine residues located in the intracellular pocket of gp130 get phosphorylated. The complex causes activation of receptor-related kinases (JAK1, JAK2, and Tyk2) (Schmidt-Arras & Rose-John, 2016). JAK/STAT is the most important signalling pathway activated by gp130 mediated signalling. Normally, JAK1, JAK2, and Tyk2 remain associated with gp130 in inactive form but the complex formation leads to their phosphorylation and activation (Bergmann et al., 2017; Schmidt-Arras & Rose-John, 2016). Phosphorylation of the aforementioned molecules leads to phosphorylation of STAT molecules (STAT 1, STAT 3, and STAT5). STAT is a transcription factor that translocate into the nucleus to upregulate gene transcription (Bergmann et al., 2017; Schmidt-Arras & Rose-John, 2016; Wormald et al., 2006). STAT 3 also upregulates the expression of suppressor of cytokine signalling 1 (SOCS1) and suppressor of cytokine signalling 3 (SOCS3) to terminate the IL-6 signalling. SOCS1 and SOCS3 bind to phosphorylated JAK and gp130 respectively to downregulate their activity (Schett, 2018; Schmidt-Arras & Rose-John, 2016).

2.5.4.2. Trans Signalling Pathway

In the trans-signalling pathway, IL-6R is not present on the surface of cells. It is rather present in a soluble form as soluble IL-6R (sIL-6R). IL-6R is present as both membranes bound form on hepatocytes and inflammatory cells as well as in soluble form near the site of inflammation (Bergmann et al., 2017). sIL-6R release is mediated by ectodomain shedding via α-disintegrin and metalloprotease 17 from hepatocytes or inflammatory cells (Bergmann et al., 2017). sIL-6Rα is produced via alternative splicing or proteolytic cleavage (Schett, 2018). IL-6 forms a complex with sIL-6R by engaging gp130 molecules (Bergmann et al., 2017). IL-6 has an affinity for IL-6R but not for gp130. Binding to gp130 is only possible through the IL-6-IL-6R complex (Schmidt-Arras & Rose-John, 2016). IL-6-IL-6R-gp130 complex primarily causes cell proliferation in the tissues where transmembrane IL-6R is not inherently expressed such as smooth muscle and endothelial cells (Schett, 2018). As gp130 is widely expressed by the cells, therefore, the trans-signalling pathway results in a widespread activation of the signalling cascade (Bergmann et al., 2017). Various signalling pathways such as Ras-MAPK, p38, JNK MAPK pathways, PI3 K-Akt, and MEK-ERK pathway are upregulated by IL-6. Both pathways i.e., classical and trans-signalling project a synergistic effect when activated together (Bergmann et al., 2017).

2.5.5. Role of IL-6 in Carcinogenesis

It has been demonstrated from various studies that the single nucleotide polymorphisms affect the susceptibility and progression of HCC (Adnan et al., 2020). Several polymorphisms in IL-6 have been linked to cancer (Giannitrapani et al., 2013). IL-6 polymorphisms play role in viral persistence in the case of HCV (Adnan et al., 2020; Giannitrapani et al., 2013). Moreover, HCV employs IL-6/STAT3 pathway to cause chronic liver damage (Adnan et al., 2020). A similar role of IL-6 has also been

suggested in HBV infection which increases the chances for HCC development (Giannitrapani et al., 2013). IL-6 is actively involved in regulating differentiation, proliferation, acute phase response, and maintaining a balance between pro-inflammatory and anti-inflammatory cytokines (Giannitrapani et al., 2013). IL-6 causes chronic inflammation due to its dual role of pro and anti-inflammatory cytokine role that helps it to upregulate cell growth and induce anti-apoptotic effects (Adnan et al., 2020).

The presence of G/C polymorphism in the promoter region of the IL-6 gene is related to the rate of IL-6 transcription and its plasma levels (Giannitrapani et al., 2013) (Adnan et al., 2020; Zheng et al., 2015). -174 G/G and -174 G/C are responsible for high IL-6 levels whereas -174 C/C is linked to low IL-6 levels in the Caucasian population. G/G and G/C polymorphisms play role in viral persistence whereas C/C is reported to be responsible for spontaneous viral clearance (Giannitrapani et al., 2013). Dysregulated expressions of IL-6 play a role in the pathogenesis of chronic inflammation, hematopoiesis and dysfunctional immune response (Adnan et al., 2020). Several studies have been done to draw a correlation between the levels of IL-6 and the aforementioned polymorphisms. Still, more studies need to be done to validate the relationship (Adnan et al., 2020; Giannitrapani et al., 2013; Zheng et al., 2015). This relationship between SNPs and HCC development can also be used as a prognostic biomarker in future treatments (Adnan et al., 2020).

An elevated level of IL-6 and IL-6R has been observed in Hepatic cancer patients e.g., in chronic hepatitis, liver cirrhosis, and HCC (Giannitrapani et al., 2013; Schmidt-Arras & Rose-John, 2016; Zheng et al., 2015). To further validate such observations, studies were performed on sgp130Fc transgenic mice which were fed with a high dose of carcinogen diethylnitrosamine (DEN) (Bergmann et al., 2017; Schmidt-Arras & Rose-John, 2016). DEN gets activated by the cytochrome P450 (Bergmann et al., 2017). A high dose of DEN results in DNA damage (DNA adducts) and liver cells death. In response to the injury, Kupffer cells are activated via TLR and EGFR to release IL-6 and TNF-α (Bergmann et al., 2017; Schmidt-Arras & Rose-John, 2016). IL-6 tries to compensate for the DNA damage by inducing hepatic proliferation.

IL-6 trans-signalling is a major driver of hepatocellular carcinoma. It mutilates the apoptotic ability of DNA damaged cells by downregulating the expression of p53. p53 has been correlated linearly with the levels of STAT1 which in turn is responsible for stable expression of p53. Thus, positive upregulation of p53 through STAT1 causes apoptotic death of malignant cells. Trans signalling also supports the proliferation of abnormal hepatocytes resulting in the accumulation of damaged DNA, high β-catenin production which thus lead to carcinogenesis. Angiogenesis is also promoted by IL-6 trans-signalling which serves as the core factor of tumour progression (Bergmann et al., 2017; Schmidt-Arras & Rose-John, 2016).

2.6. Transforming Growth Factor β-1

The transforming growth factor β-1 (TGFβ-1) is a negative cell growth regulator. It consists of a disulfide-linkage and exists in three isoforms (Y. Wang, Deng, Tang, Liu, & Shen, 2013). TGFβ-1 and TGFβ-2 have 70% structural homology (Ito et al., 1990). All three isoforms have quite similar functions (Y. Wang et al., 2013) but only TGFβ-1 has a significant role in tumour progression (S. Liu, Chen, & Zeng, 2018). TGFβ-1 is produced by Kupffer cells, storage cells, and endothelial cells (Malaguarnera et al., 2010). The expression of TGFβ-1 in normal liver function is very low and is not usually detectable (Ito et al., 1990). TGFβ-1 plays quite an important role in-vivo function. It binds to its two sets of receptors and triggers a downstream signalling pathway. The transcriptional factor involved with TGFβ-1 in the SMAD complex. Ras and MEKs propagate TGFβ-1 mediated signalling through non-SMAD pathways (S. Liu et al., 2018).

The normal functions of TGF-β1 include keeping a check on adipogenesis, hematopoiesis, epithelial cell growth, and myogenesis (Ito et al., 1990). It prevents auto-immunity by keeping the immune response moderated (Silver & Hunter, 2010; Y. Wang et al., 2013). Therefore, its role can also be attributed to transplant immunity (Y. Wang et al., 2013). It inhibits aforementioned functions by arresting the cell growth in the G1 phase, thus blocking the proliferation and causing apoptosis (Malaguarnera et al., 2010). It blocks the cell cycle progression by downregulating the expression of cyclin D1 (Malaguarnera et al., 2010). TGFβ-1 is considered a tumour suppressor in premalignant cells but promotes metastasis in cancer cells. 14-3-3ζ is reported to destabilize p53 (a SMAD partner) which then turns off the tumour suppression ability of TGFβ-1. The same protein promotes TGFβ-1 mediated metastasis by stabilizing the expression of Gli2 and its complex formation with SMADs in cancer cells (S. Liu et al., 2018).

2.6.1. Signaling Mechanism of TGFβ-1

TGFβ-1 signalling takes place through SMAD and non-SMAD pathways. Three isoforms of TGFβ i.e., TGFβ-1, TGFβ-2, and TGFβ-3 are the three ligands that respond to a common receptor (S. Liu et al., 2018; Xie et al., 2004).

2.6.1.1. SMAD Pathway

TGFβ-1 protein is produced in an inactive state and is cleaved by proteases for activation (Goumans, Liu, & Ten Dijke, 2009; S. Liu et al., 2018). It is activated by the plasminogen which activates TGFβ-1 by dissolving its pro-segments along with additional confirmation changes induced by matrix metalloproteinase MMP9 and MMP2. TGFβ-1 signalling is mediated through two types of TGFβ receptors i.e., TGF-βR1 and TGF-βR2 which belong to the family of serine/threonine kinase transmembrane receptors (Goumans et al., 2009; S. Liu et al., 2018; Xie et al., 2004). Upon activation by metalloproteinases, TGFβ-1 can bind efficiently to TGF-βR2 This leads to the formation of a heterotetrameric active receptor complex (S. Liu et al., 2018). TGF-βR2 then phosphorylates TGF-βR1 on serine and threonine residues located in the intracellular juxtamembrane pockets (Goumans et al., 2009; S. Liu et al.,

2018; Xie et al., 2004). TGF-βR1 is responsible for the recruitment and phosphorylation of receptor-regulated SMAD1/3 (RSMAD) in two serine residues of the carboxy terminal (Goumans et al., 2009; S. Liu et al., 2018). RSMAD forms complex with co-SMAD4. This R-SMAD-CoSMAD heteromeric complex translocate to the nucleus to upregulate the expression of DNA transcription factors (Goumans et al., 2009; S. Liu et al., 2018; Xie et al., 2004). SMAD dependent activation is involved in upregulating tumour suppression in early-stage tumorigenesis through TGFβ-1 mediated antiproliferative activity (Xie et al., 2004).

2.6.1.2. Non-SMAD Pathway

TGF-β1 can also be activated through a non-SMAD pathway or otherwise known as non-canonical signalling. Signalling cascade running through MAPK/Erk or PI3K/Akt pathways activate TGFβ-1 through non-SMAD pathways (S. Liu et al., 2018; Malaguarnera et al., 2010). Non-SMAD pathways either work independently or in conjugation with SMAD complexes to activate the signalling cascade of TGFβ-1. TGFβ-1 has been reported to upregulate cell proliferation through PI3K/Akt signalling. Cancer cell invasion and metastasis via epithelial-to-mesenchymal transformation (EMT) are promoted through TGFβ-1 activated MMPs, p38MAPK, Zinc finger E- Zinc finger E-box-binding homeobox 1 (ZEB1), ZEB2, Snail and Slug (S. Liu et al., 2018). Non-canonical signalling play role in TGFβ-1 induced EMT to promote metastasis and cell invasion (Malaguarnera et al., 2010).

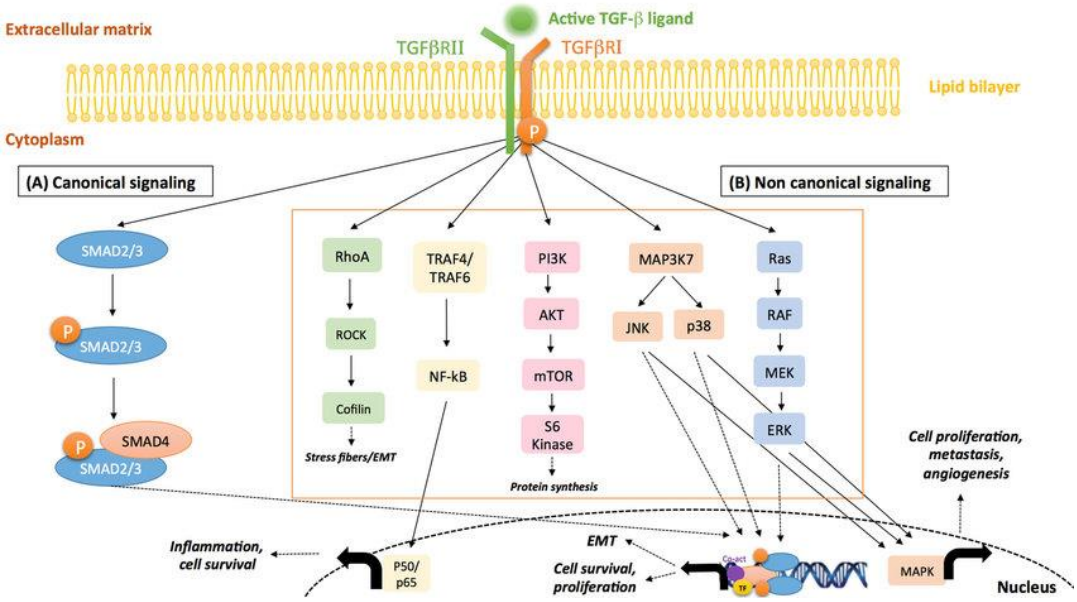

Figure 2. Signalling Mechanism of TGFβ-1(Costanza, Umelo, Bellier, Castronovo, & Turtoi, 2017)

2.6.2. Role of TGF-β1 in Carcinogenesis

The tumour microenvironment of hepatic cancer has reported quite significant levels of TGFβ-1. TGFβ-1 plays a role in tissue differentiation and development. The level of tumour differentiation depends upon the level of TGFβ-1 (Ito et al., 1990; Malaguarnera

et al., 2010). TGFβ-1 acts as a strong basis of collagenesis and promotes tissue fibrosis (Ito et al., 1990). Angiogenesis and inhibition of immune-mediated cell death is also inhibited by TGFβ-1. It creates a microenvironment for tumour growth by inhibition of immune cells and the activity of major histocompatibility class I and II (S. Liu et al., 2018). TGFβ-1 promotes the expression of tumour-associated macrophages and the ROS with genotoxic potential. Tumor-associated macrophage (TAMs) with an M2 phenotype are responsible for tumour invasiveness as they enhance the adhesive property of cancer cells to their microenvironment (S. Liu et al., 2018). ROS in tumour cells is responsible for a higher rate of mutation, genetic instability, and metastasis (S. Liu et al., 2018). The expression of TGFβ-1 has also been linked with the presence of other tumour biomarkers i.e., AFP, ALT (Malaguarnera et al., 2010) .

High levels of TGFβ-1 play role in cell malignancy and tumour metastasis. It promotes metastasis, and tumour invasion by upregulating the EMT (S. Liu et al., 2018; Y. Wang et al., 2013). Normally, the epithelial phenotype is characterized by the structural and functional polarization of the cell surface that promotes cell adhesion and tight junctions. Tight junctions are regulated by E-cadherins that promote contacts between neighbouring cells. Tight junctions are connected to microfilaments through zonula-occludens-1 protein (ZO1) (Malaguarnera et al., 2010). EMT is the state of absence of epithelial boundary and normal cell-to-cell contact (Y. Wang et al., 2013). The loss of epithelial phenotype, downregulation of E-cadherin and ZO1 proteins, and reorganization of the actin cytoskeleton are associated with the development of EMT (Malaguarnera et al., 2010). EMT is also characterized by a state with inhibited epithelial marker and enhanced mesenchymal marker. Upregulated expressions of transcription factors such as ZEB1, ZEB2, Snail, and Slug) via SMAD pathway, and MMPs, as well as p38MAPK through the non-SMAD pathway, contribute to the development of EMT (S. Liu et al., 2018). TGF-β1 further helps tumour progression by positively regulating angiogenesis (S. Liu et al., 2018; Y. Wang et al., 2013). TGFβ inhibits immune surveillance in the microenvironment. Therefore, it helps malignant cells escape from the immune system (Y. Wang et al., 2013).

TGFβ-1 serves the role of a growth inhibitor in the early stages of cancer. However, as cancer progresses, TGFβ-1 promotes angiogenesis, invasion, and metastasis. Mutations in the TGFβ-1 receptor aggravates the condition by upregulating the downstream signalling pathway. Cancer cells thus easily escape from TGFβ-1 induced immunosuppression (Y. Wang et al., 2013). Moreover, TGFβ-1 released by the cancer cells cause CD4 to transform into the Treg cells (S. Liu et al., 2018).

IFN-γ is responsible for local immune surveillance in tumour cells where it collaborates with natural killer cells to boost the process of antigen presentation. When cancer cells release TGFβ-1, the expression of IFN-γ in CD8 is reduced. TGFβ-1 also suppresses dendritic cells to further weaken the immune surveillance. Therefore, TGFβ-1 is a crucial target in restoring immunity to fight off cancer (Y. Wang et al., 2013).

Polymorphisms in the TGFβ-1 gene have been studied for their association with the aetiology of multiple diseases. Studies have identified the possible role of TGFβ-1+29

C/T polymorphism in the pathogenesis of osteoarthritis (Čengić et al. 2015), breast cancer (Gautam et al. 2015), urinary bladder cancer (Gaussin et al. 2012), development of cervical lesions (Trugilo et al. 2019), sarcopenia (Fuku et al. 2012), and chronic periodontitis (Heidari et al. 2014). development of HCC. However, the association of the TGFβ-1+29 C/T polymorphism with the development of HCC has not been studied before.

2.7. Diagnosis

Ultrasonography is recommended these days for detection of HCC due to its high sensitivity (65-80%) and specificity (90%). HCC is detected as echogenic, hypoechoic, or isoechoic surrounded by a capsule. Unfortunately, these are not specific to HCC therefore a nodule must be detected for further evaluation. Some demerits of US are the technical limitation (operator dependent), adverse effects due to radiation, and high cost (H. Tanaka, 2020).

A serologic marker-alpha fetoprotein (AFP) is quite frequently used for HCC detection. However, it is not specific for HCC as its elevated levels are even observed in HBV or HCV infection without any trace of HCC. It is also reported to increase in conditions such as cholangiocarcinoma and in gastric cancer (Kew, 2015).

Des-gamma carboxyprothrombin or accessing the ratio of glycosylated AFP to total AFP concentration are some useful markers for diagnosis at advanced stage. For surveillance stage, scientists still rely on US. An interval period of 6 months is preferred for follow-up as it is the average time during which tumour doubles regardless of the causative factors (Volk, Hernandez, Su, Lok, & Marrero, 2007). Nodules <1 cm in size are not considered a risk factor for HCC. Nodules greater than 1cm require application of further diagnostic strategies. Diagnosis of such small nodules proves challenging due to high risk of sampling errors. It is quite difficult to differentiate dysplastic nodules from cirrhotic nodules. Therefore, biopsy is not considered as the ultimate confirmatory test (Sangiovanni et al., 2010).

Immunohistochemical staining for glypican-3, heat shock protein-70, and glutamine synthetase may prove beneficial to be conclusive about the status of tumour. 30% of liver patients pose difficulty with this diagnostic method due to inability to locate tumour or bleeding risk (Sung et al., 2003). In that cases, physicians rely on contrast-based imaging techniques (McMahon et al., 2000). Computerized tomography and magnetic resonance imaging are used for intense contrast uptake in arterial phase followed by washout in venous phase.

US with contrast give false negative results and is not widely used. Due to high success results with CT and MRI, they are recommended to be used in nodules >10 mm. Even the imaging criteria have been documented for lesions>1cm. Lesion results may also be followed by biopsy which is still not 100% accurate (Yang et al., 2017).

Staging systems are defined to evaluate the progression of cancer followed by estimation of life expectancy and recommendation of a treatment. Several different staging systems are documented. One of the staging systems is Barcelona Clinic Liver Cancer (BCLC), developed in 1999, that successfully relates cancer stage with appropriate treatment (Bruix & Sherman, 2011).This staging system even includes cancerous lesions <2 cm originally identified in patients with no visible symptoms of HCC (Ramsey & Wu, 1995). This is defined as 'very early stage' or BCLC 0 which has opened the doors for treatments resulting in long-term cure. The other stages include BCLC A: Early (<3 cm nodules-still functioning liver), BCLC B: Intermediate (Large localized tumors-preserved liver function), BCLC C: Advanced (Widespread tumors-constitutional symptoms), and BCLC D: Terminal (Liver deterioration, Impaired liver function) (Huo et al., 2007). This staging system is by no means exhaustive and is subjected to patient's HCC status (Fleming, 2001).

2.8. Treatment

As for the treatment, great progress has been made considering the severity and pathogenic nature of HCC. The tumour stage of HCC largely determines the treatment options. There are over 10 cancer staging systems presented by the researchers for predicting HCC prognosis or selecting the optimal treatment regimen like Barcelona Clinic Liver Cancer (BCLC) staging system and Hong Kong Liver Cancer (HKLC) staging system (P.-H. Liu et al., 2016). BCLC is the most clinically used and widely recognized staging system and classifies HCC in five stages i.e., 0, A, B, C and D. These stages can further be divided as A1, A2, A3 and A4. BCLC staging system plays a key role in classification and prediction of prognosis mainly because the system's classification is based on tumor burden, patient performance status (PS) and liver dysfunction (Llovet, Brú, & Bruix, 1999). Based on BCLC staging system, different patients can be provided with different treatment methods. For instance, liver transplantation (LT) and other curative therapies are used for early-stage (BCLC 0/A) HCC patients while locoregional treatments like trans-arterial chemoembolization (TACE) are used for the treatment of intermediate (BCLC B) HCC. Currently pharmacological treatment is considered most effective for the treatment of patients with advanced HCC (BCLC C) and are mostly treated with Sorafenib (Cillo et al., 2004). Supportive care is usually given to stage D patients (J.-H. Wang et al., 2008). Globally, the treatment for HCC is not uniform because of different data collection instruments, availability of treatment facilities, and technical skills. Despite the existing strategies, the survival rates are overall very disappointing and there hence there is a need for more efficient therapies.

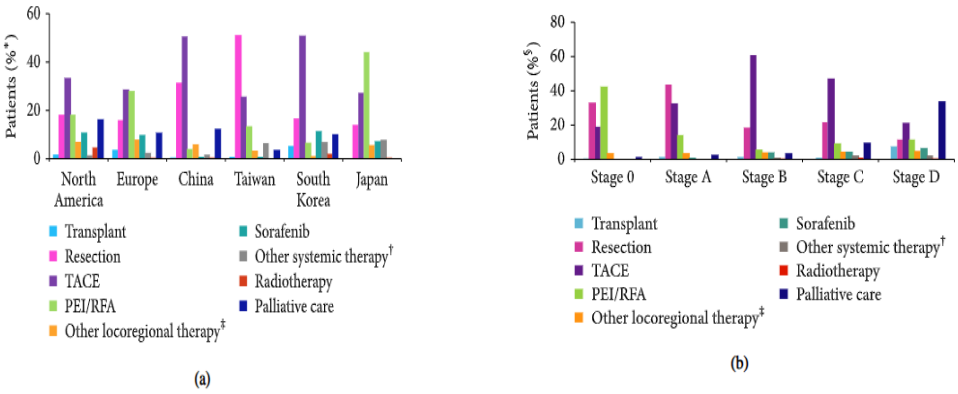

Figure 3. HCC treatment based on region and stage

In Pakistan, many HBV cases were reported during the period of 1970-1990. Later, with a rise in HCC cases due to HCV, a shift in HCC aetiology was observed (André, 2000). The most prevalent HCV genotype in Pakistan is genotype 3. Unfortunately, there is very less data available regarding the HCC treatment in Pakistan. Most of the HCC patients have nonresectable tumours. Some of the criterion available in selected areas include trans-arterial chemoembolization, radiofrequency ablation, resection and chemotherapy including sorafenib.

2.8.1. Hepatic Resection: This treatment is recommended for early-stage patients with a functional liver and single lesion of less than 5 cm or up to 3 lesions of less than 3 cm and with no extra hepatic manifestations or vascular invasion (Zhong et al., 2016). The limitation of this treatment method is that it increases the risk of hepatic decompensation in patients that have cirrhosis. There is approximately 70% five-year survival for patients who undergo this treatment method but there is a high risk of recurrence which is indicated by the presence of microscopic vascular invasion. Another limitation of this regimen is that it cannot be used for patients having multiple tumours (Barbier et al., 2013).

2.8.2. Trans-arterial Chemoembolization: TACE is mostly recommended for intermediate stage HCC patients. It improves the 2-year survival rate of HCC patients and hence is the best option for non-surgical treatment of HCC (W. Lu et al., 2017). It involves the injection of cytotoxic chemotherapeutic drugs and embolization particles into the tumour feeding artery which ultimately results in the ischemic necrosis of the tumour (Lencioni, de Baere, Soulen, Rilling, & Geschwind, 2016). There is an increased risk of complications associated with TACE in patients with poor residual liver function, portal vein thrombosis and those with extensive disease who may require non-selective embolization. Thus, the major problem is the lack of standardized therapy regimen for TACE. The absolute contraindications for TACE include decompensated cirrhosis, high tumour burden, large replacement of the liver lobe and tumour nodules greater than or equal to 10cm while the relative contraindications include bile duct obstruction and untreated high risk varicose veins (Facciorusso, 2018).

2.8.3. Radiofrequency Ablation: Radiofrequency ablation is recommended for patients with deteriorated liver function. The size of tumour for which RFA can be

safely and effectively used is still a matter of debate (Ahn & Kang, 2019). However, its efficiency decreases with the increasing tumour size or when the tumour lies near the main blood vessel (D. S. Lu et al., 2003).

2.8.4. Sorafenib: Multiple cellular kinases promote angiogenesis, cellular differentiation, survival, and proliferation and hence play an important role in the development and progression of HCC. Sorafenib is an oral multikinase inhibitor which inhibits the activity of multiple tyrosine kinases e.g., VEGFR 2/3, F1t3, c-Kit, platelet derived growth factor receptor (PDGFR) involved in tumour angiogenesis and progression. Raf kinases are also one of the targets of sorafenib which are involved in MAPK/ERK pathway (Cervello et al., 2012). It was approved by FDA since the year 2007 for the treatment of unresectable HCC (Hsu, Shen, Shao, Hsu, & Cheng, 2014). A consistent survival benefit can be obtained from an 800 mg/day dose of sorafenib (Faivre, de Gramont, & Raymond, 2016). In progressive HCC patients, it is recommended to be used as a first line systemic treatment (Cheng et al., 2009). The limitation associated with the use sorafenib is the observation of several toxicities in patients during the treatment e.g., gastrointestinal complications, anorexia, skin reactions and fatigue. A study showed that the use of sorafenib is permanently discontinued in about 28% of the patients due to the development of an overall 30% of grade 3-4 severity events (Lencioni et al., 2014). Moreover, it was observed that the survival benefit obtained from its use is not very sustainable and the patients continue to experience disease progression. The primary and acquired resistance also limit the use of sorafenib. The reason to why this occur remains unclear (Ezzoukhry et al., 2012).

After cardiovascular diseases, cancer is the primary cause of deaths worldwide. Although significant advancements have been made in the diagnosis, treatment, and prevention of cancer, there is still room left for improvements. Conventional treatments, such as chemotherapy, surgical resection, transcatheter arterial embolization, percutaneous ablation therapy, liver transplant, and target therapy, have not been found very successful for treating cancer patients. Moreover, chemotherapy adds little to the survival of patients because of less sensitivity towards cancer cells and development of drug resistance (Hassan, 2019; Liao et al., 2015). Researchers are now focusing on alternative medical treatments, including natural therapies with plant-derived compounds, for treating cancer with reduced adverse effects as compared to the traditional chemotherapy (Hassan, 2019). A combination of plant compounds, having anti-tumorigenic properties, has shown promising results in in-vitro studies. However, they have not been evaluated in humans yet (Desai et al., 2008; Hassan, 2019).

2.9. Flavonoids as Chemopreventive Agents

Herbal medicines have been used for years in Asia and Africa. According to World Health Organization (WHO), many countries still utilise herbal supplements for treatment purposes (Greenwell & Rahman, 2015). Plant extracts are non-toxic compounds with fewer side effects in cancer patients. This emerging trend of using natural compounds for cancer therapy is termed as chemoprevention. This therapy utilizes chemopreventive agents to inhibit, supress, or slow down the response of

carcinogens in cancer patients (Liao et al., 2015). Many phytochemicals and their analogues such as alkaloids, flavonoids, brassinosteroids, polyphenols, terpenes, taxanes, biomolecules and other secondary metabolites have shown anti-tumorigenic properties. These phytochemicals work by either inhibiting cancer cell activating proteins, enzymes and signalling pathways or by stimulating the synthesis of protective enzymes against tumour cells (Ali et al., 2016; Greenwell & Rahman, 2015; Iqbal et al., 2017).

2.9.1. Flavonoids: Being the most studied class of natural compounds, flavonoids are polyphenolic compounds with a large family of plant secondary metabolites. Different plant species, including fern species and litchi leaf, have been used in Chinese traditional medicine because of the presence of high flavonoid content. Flavonoids are mainly found in fruits, vegetables, roots, stems, flowers, and beans, as well as in foods that are ingested daily. Increased intake of flavonoids is associated with many health promoting properties (Cassidy et al., 2015; Liao et al., 2015; Weng, Patel, Panagiotidou, & Theoharides, 2015). High concentrations of flavonoids like anthocyanins, flavones, flavanols, and chalcones has been reported in various plant structures. Based on different molecular structures, flavonoids are classified into seven classes including flavonols, flavanones, isoflavones, flavonols flavonolignans, and anthocyanidins. Studies have indicated multiple anti-cancer properties of flavonoids against human cancers including breast cancer, cervical cancer, and hepatoma (Greenwell & Rahman, 2015). Flavonoids work as anti-cancerous agents by interacting with intrinsic and extrinsic signalling pathways associated with metabolism and survival of cancer cells (Greenwell & Rahman, 2015) (Batra & Sharma, 2013; Ren, Qiao, Wang, Zhu, & Zhang, 2003).

2.9.2. Role of Flavonoids in Hepatocellular Carcinoma (HCC)

Different flavonoids have exhibited anti-cancerous, anti-oxidative and anti-metastatic properties on liver cancer cells by inhibiting the tumour progression. Tetrahydroxyflavonone is an important flavonoid with a molecular formula $C_{15}H_{12}O_6$. Another synonym used for tetrahydroxyflavonone is dihydrofisetin (CID: 246330). It is mainly found in young fustic and lacquer tree. Different studies have indicated the biological roles of dihydrofisetin in the treatment of human diseases. A study conducted on carrageenan-induced paw oedema investigated the anti-inflammatory role of dihydrofistein and its underlying process. The results of the study indicated dihydrofisetin's role in reducing TNF-α and IL-6 levels in the oedema tissues (K. K. Li et al., 2018).

Fisetin, another important flavonoid, also known as 3,3′,4′,7-tetrahydroxyflavone, is found in various fruits and vegetables. Studies have described anti-proliferative, anti-oxidative, anti-tumorigenic and neuroprotective properties of fisetin due to which its being used in research against cancer nowadays (X.-F. Liu, Long, Miao, Liu, & Yao, 2017). A research has been conducted on liver cancer cells using mouse models to investigate the role of fisetin in relation to dopamine receptors. Dopamine receptors (DRs) are from the G-coupled family which are the targets for almost 50% of the

medicinal drugs. DRs consists of many interacting proteins that are involved in the transduction of intracellular signalling pathways. In this study, fisetin worked as a dopamine receptor 2 (DR2) agonist. The binding of fisetin with DR2 showed suppression in liver cancer cells proliferation, invasion, and migration. Furthermore, fisetin administration was followed by the inhibition of TGF-β1 which reduced EMT in fisetin-treated liver cancer cells (X.-F. Liu et al., 2017).

CHAPTER 3

METHODOLOGY

3.1. SNP Analysis

3.1.1. Data Mining

The ENSEMBL database was accessed to retrieve the pathogenic variants of TGFβ-1 (ENSG00000105329) and IL-6 (ENSG00000136244) genes. ENSEMBL uses the genome assembly GRCh38 to map variants to their genomic coordinates. From the review of literature, it was found that the IL-6 -174 G/C (rs1800795) variant is present in the intronic region, whereas the TGFβ-1 +29C/T variant is found in the coding region. Therefore, coordinates of variants found in promotor region, 3'UTR, 5'UTR were retrieved for IL-6 whereas variants found in coding region were retrieved for TGFβ-1 from ENSEMBL.

3.1.2. Evaluation of SNPs

Five computational tools (SIFT, Cadd, Revel, PolyPhen and MetaLr) were used to assess the potential pathogenicity of TGFβ-1 human missense variants. These tools predicted the deleteriousness of a variant in the form of scores which are available on ENSEMBL.

3.1.3. Functional Analysis of Variants

RegulomeDB (Boyle et al., 2012) was used for acquiring information about the regulatory function of variants through the variant gene coordinates, rank, and score. This information is derived through human genome sequencing studies.

3.1.4. Sequence of SNPs

The gene coordinates of the variants, obtained from RegulomeDB, were used for obtaining the sequence of the mutant IL-6 and TGFβ-1 genes through the NCBI genome data viewer (Rangwala et al., 2021).

3.1.5. Predicting the Transcription Factor Binding Sites of IL-6 -174 G/C

AliBaba2 (Grabe, 2002) was used for predicting the transcription factor binding sites in the mutant and wildtype IL-6 nucleotide sequence. The FASTA sequence of the wildtype and the IL-6 -174 G>C variant was submitted to this server.

3.1.6. Structural Analysis of TGFβ-1 +29 C/T

Project HOPE is an online tool used for analysing the structural differences caused by a point mutation in a protein sequence. This tool collects data from all the available databases and produces a comprehensive report with complete figures and information about the mutation (Yun, Kang, Lim, Oh, & Son, 2010). To understand the structural differences due to the +29 C/T mutation in the TGFβ-1 protein, the FASTA sequence

of the protein was inserted into the HOPE server, which produced a report on the mutated protein's structure and properties.

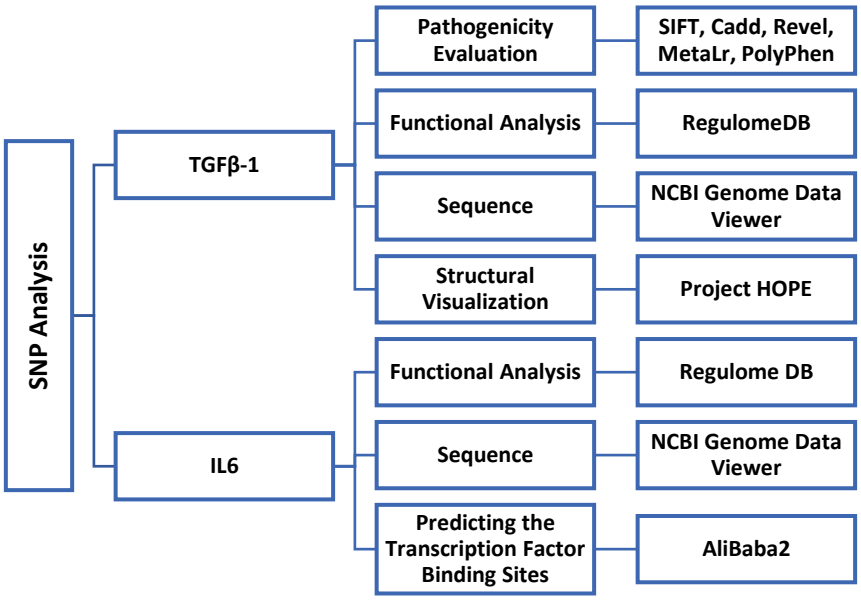

Figure 4. Flowchart of the methodology of TGF-β1 and IL-6 SNP analysis.

3.2. Protein-Ligand Docking

To assess the interaction of tetrahydroxyflavanone with TGFβ-1 and IL-6 proteins, a docking analysis was performed. The goal of molecular docking is to assess the non-covalent interactions between ligand and protein to predict their low-energy binding orientation and the highest binding affinity (Brooijmans & Kuntz, 2003). For this purpose, the 3D protein structures of TGFβ-1 having a PDB ID: 5VQP (Zhao, Xu, Dong, Lu, & Springer, 2018) and IL-6 with a PDB ID: 1IL-6 (Xu et al., 1997) were retrieved from the protein data bank (PDB) (Berman et al., 2000). Whereas the structure for tetrahydroxyflavanone (CID: 246330) was retrieved from PubChem (Kim et al., 2021).

3.2.1. Preparation of Proteins and Ligands

PyMol (Schrödinger, 2015), a source for molecular visualization, was used to visualize the protein structures. Water molecules were removed from PDB-downloaded structure using PyMol. ChemDraw (Mills, 2006), a molecular modelling software, was used to minimize the energy of the ligand i.e., tetrahydroxyflavanone, to achieve a stable configuration.

3.2.2. Protein-Ligand Docking

To understand the binding interactions of ligand and protein, CB Dock (Yang Liu et al., 2020) was used. Molecular docking between ligand and protein structures is studied through CB Dock that predicts the protein cavities, binding sites, centres, and sizes of

the docking boxes. Multiple structures are predicted by CB Dock, but the structure with the least energy is chosen for further analysis.

3.2.3. Schematic Representation of the Docking Structures

To visualize the docked structures, a PDB file of the docked structure was prepared in PyMol. This PDB file input gave a 2-dimentional representation of the ligand-protein complex in LigPlot (Wallace, Laskowski, & Thornton, 1995). For the 3-dimentional structure of the ligand-protein complex, the LigPlot file was visualized in PyMol.

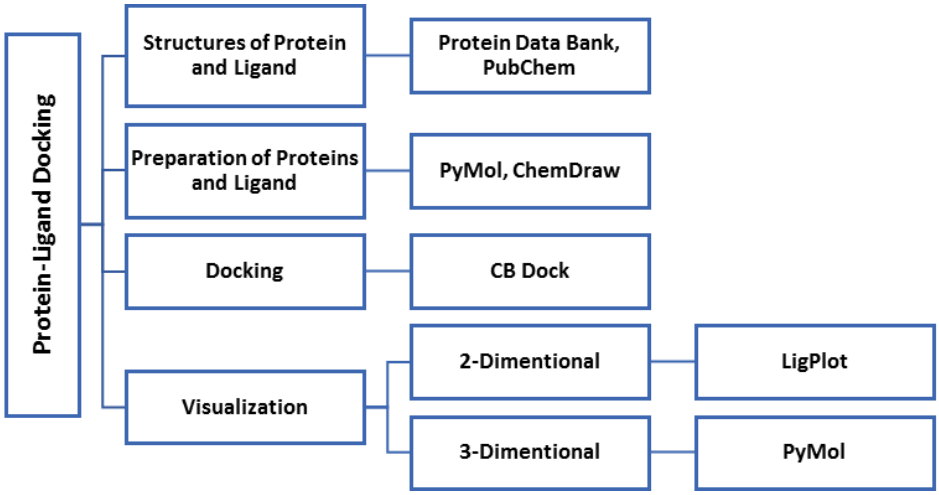

Figure 5.Flowchart of the methods used for protein and ligand docking analysis.

3.3. Single Nucleotide Polymorphism Profiling

3.3.1. Sample Collection and Storage

A total of 429 blood samples were collected from Combined Military Hospital, Rawalpindi (Table 1). The samples were stored at 4°C in EDTA tubes to prevent clotting. The samples were collected with the patient's consent via a consent form. The inclusion criterion was set as HCV infected HCC patients. All other HCC patients were excluded, and healthy subjects were used as control. The study was conducted under the laws of ethical committees and Atta-Ur-Rehman School of Applied Biosciences review board.

Table 1. Sample distribution of IL-6 and TGF-β1

IL-6		**TGFβ-1**	
Control	**HCV-infected HCC**	**Control**	**HCV-infected HCC**
216	213	216	213
Total Samples= 429		**Total Samples = 429**	

3.3.2. Genomic DNA Extraction

Table 2 shows the reagents needed for genomic DNA extraction whereas Table 3 shows the composition and the purpose of using different solution types in the extraction process (Sambrook & Russell, 2006).

Table 2. Reagents required for DNA extraction

	Reagents
1.	Blood sample
2.	Ethanol (70% or 100%)
3.	Proteinase K
4.	Salt solution (10M ammonium acetate, 8M LiCl, 5M NaCl, or 3M sodium acetate)
5.	10X TE buffer (pH 7.8)

Table 3. Composition and Purpose of using different solution types in genomic DNA extraction

Solution	**Composition**	**Amount in grams for 1 Liter**	**Purpose**
Solution-A	0.32M Sucrose	109.44 g	It helps in the release of DNA from cells by breaking the cell membrane.
	Tris (pH 7.5)	12.114 g	
	5mM Magnesium Chloride	0.476 g	
	1% V/V Triton X-100	10 ml	
Solution-B	Tris (pH 7.5)	12.114 g	It precipitates the DNA and helps in separating the proteins.
	400mM Sodium Chloride	23.37 g	
	2mM Ethylene Diamine Tetra Acetic Acid (EDTA)	0.58 g	
Solution-C	Phenol	As per the protocol	The aqueous and organic phases in the solution can be separated by using it.
Solution-D	24 volumes of Chloroform	48ml	It helps in the isolation of pure DNA by stabilizing the coagulation of proteins and reduces their foaming.
	1 volume of Isoamyl Alcohol	2ml	

3.3.2.1. Procedure

The phenol-chloroform method was used for DNA extraction which is a two-day process. Various steps which were performed during the process are written below.

- **Steps followed at Day 1**
  1. 750µl of blood sample was taken in an Eppendorf tube.
  2. 750µl of Solution-A was added to it followed by repeated shaking to facilitate the dissolving process.
  3. The tube was left at room temperature for 10 minutes.

4. After that, the tube was centrifuged at 13000 rpm for 10 minutes.
5. After centrifugation, the supernatant was discarded, and the nuclear pellet was resuspended in 400µl of Solution-B.
6. The tube was again centrifuged for 10 minutes at 13000 rpm.
7. The supernatant was discarded again following which 400µl of Solution-B, 12µl of 20% SDS and 5µl of Proteinase K was added into it.
8. The final solution was incubated overnight at 37°C.

- **Steps followed at Day 2**
    1. A 250µl of Solution-C and Solution-D were taken in separate tubes which were then added to the tube containing the sample (Incubated tube of Day 1)
    2. The tube was centrifuged for 10 minutes at 13000 rpm.
    3. The layer of DNA at the top was transferred to a separate tube whereas the remaining solution was discarded.
    4. The aqueous DNA layer was submerged in 55µl of 3M sodium acetate and 500µl iso-propanol followed by vigorous shaking to allow DNA precipitation.
    5. The tube was centrifuged at 13000rpm for 10 minutes.
    6. The supernatant was discarded, and the DNA pellet was resuspended in 200µl of 100% chilled ethanol.
    7. The tube was centrifuged again at 13000 rpm for 8 minutes and was air dried to evaporate the ethanol.
    8. The DNA was submerged in 200µl of PCR water or TE buffer after the drying step.

3.3.3. Agarose Gel Electrophoresis for DNA

Gel electrophoresis was performed for the qualitative analysis of the extracted DNA. Following steps were followed for this purpose:

a. 1% agarose gel was prepared in 1X TBE buffer by adding 0.5g of agarose in 50 ml of 1X TBE buffer.
b. A 6µl of DNA sample along with 2µl of 6X loading dye was loaded in the wells.
c. The gel with the sample was electrophoresed for 30 minutes at 55 volts.
d. After 30 minutes, the gel was visualized under the UV transilluminator. This confirmed the presence of DNA in the sample which was further subjected to ARMS-PCR.

3.3.4. ARMS-PCR for the Detection of Target SNPs

For validating IL-6 G/C and TGFβ-1 C/T variants, ARMS-PCR was performed with SNPs specific primers. These primers will allow the amplification of sample DNA only if the target allele is present.

3.3.4.1.Primer Designing

Primer1 (Collins & Ke 2012) was used for designing the allele-specific primer sets for IL-6 -174 G/C and TGFβ-1 +29 C/T genes. For IL-6 SNP analysis, the sequence of common primer was 5'-GAGCTTCTCTTTCGTTCC-3'. The sequences for G and C allele primers were 5'-CCCTAGTTGTGTCTTGCG-3' and 5'-CCCTAGTTGTGTCTTGCC-3', respectively. For SNP analysis of TGFβ-1, the sequence for common primer was 5'-GTTGTGGGTTTCCACCATTAG-3' and that for C and T alleles were 5'-CTCCGGGCTGCGGCTGCTGCC-3' and 5'-CTCCGGGCTGCGGCTGCTGCT-3', respectively. The sense and antisense internal control (β-globulin gene) primer sequences were 5'-ACACAACTGTGTTCACTAGC-3' and 5'-CAACTTCATCCACGTTCACC-3', respectively.

3.3.4.2.ARMS PCR for IL-6

- **Preparation of PCR tubes:** For IL-6, we prepare two tubes. The composition for both the tubes is shown in table 4 and 5.

Table 4. Composition of IL-6 PCR tube 1

Tube 1 composition	Amount (µl)
PCR water	4µl
Common Primer	1µl
G-allele Primer	1µl
2X Thermo Scientific DreamTaq Green PCR Master Mix	12µl
Sample	2µl

Table 5. Composition of IL-6 PCR tube 2

Tube 2 composition	Amount (µl)
PCR water	4µl
Common Primer	1µl
C-allele Primer	1µl
2X Thermo Scientific DreamTaq Green PCR Master Mix	12µl
Sample	2µl

3.3.4.3.ARMS PCR for TGFβ-1

- **Preparation of PCR tubes:** The composition of the two tubes that we prepared for TGFβ-1 are shown in table 6 and 7.

Table 6. Composition of TGF-β1 PCR tube 1

Tube 1 composition	Amount (µl)
PCR water	4µl
Common Primer	1µl
C-allele Primer	1µl
2X Thermo Scientific DreamTaq Green PCR Master Mix	12µl
Sample	2µl

Table 7. Composition of TGF-β1 PCR tube 2

Tube 2 composition	Amount (µl)
PCR water	4µl
Common Primer	1µl
T-allele Primer	1µl
2X Thermo Scientific DreamTaq Green PCR Master Mix	12µl
Sample	2µl

3.3.4.4.PCR Program

The series of steps in the PCR program for IL-6 and TGFβ-1 is illustrated in figure 6 and 7, respectively. The wet lab procedure was followed for 213 test and 216 control samples for both IL-6 (-174 G/C) and TGFβ-1 (+29 C/T) separately.

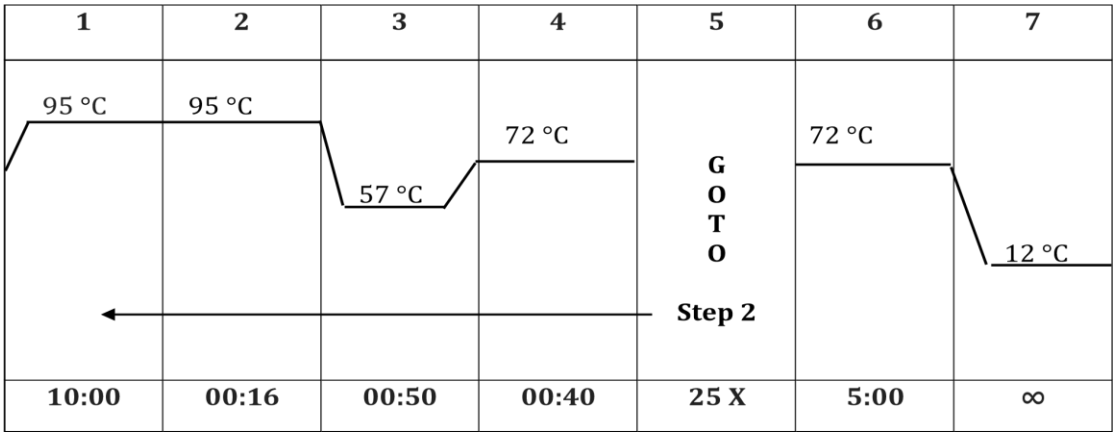

Figure 6. PCR program used for TGF-β1.

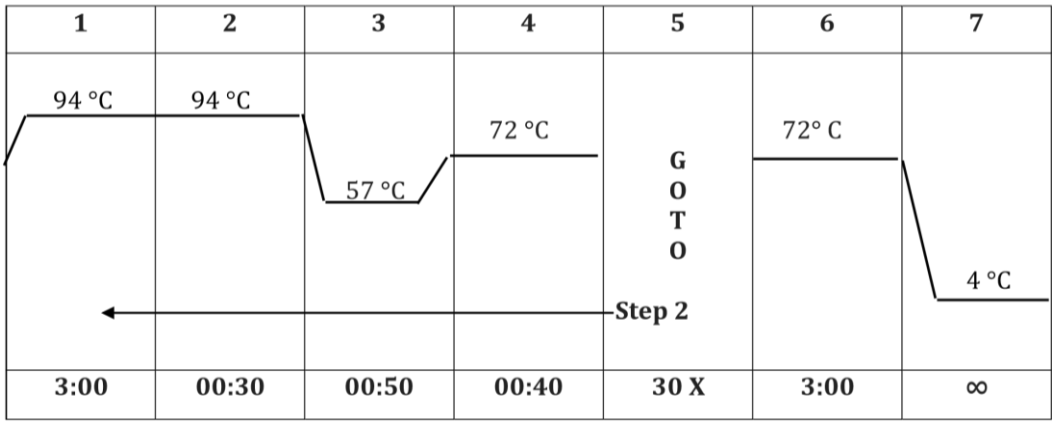

Figure 7. PCR program used for IL-6.

3.3.4.5.Gel Electrophoresis

The PCR product of ARMS-PCR was analysed in a 2% (w/v) agarose gel. Following steps are performed for gel electrophoresis.

1. 2% agarose gel was prepared adding 2g of agarose in 100ml of 1X TBE buffer.
2. 3µl of ethidium bromide was added in the cooled down solution.
3. The gel was poured in the gel casting tray with wells in it.
4. After the solidification of the gel, 6µl of DNA sample was loaded in the wells of the gel.
5. The sample was allowed to run at 55 volts for 30 minutes.
6. After 30 minutes, the gel was visualized in UV transilluminator and gel doc for the confirmation of SNP.

Note: No loading dye was added separately because Thermo Scientific DreamTaq Green already has the loading dye in it.

3.3.5. Statistical Analysis

For statistical analysis of 213 test and 216 control samples, we used Graphpad Prism which is a commercial scientific 2D graphing and statistics software (Swift, 1997). The IL-6 and TGFβ-1 genotypes in the test and control samples were divided into two sub-groups based on the gender and age of the subjects, respectively. Fisher's Exact Test and Chi Square Test were used to assess the statistical significance and association between various parameters involved in the study.

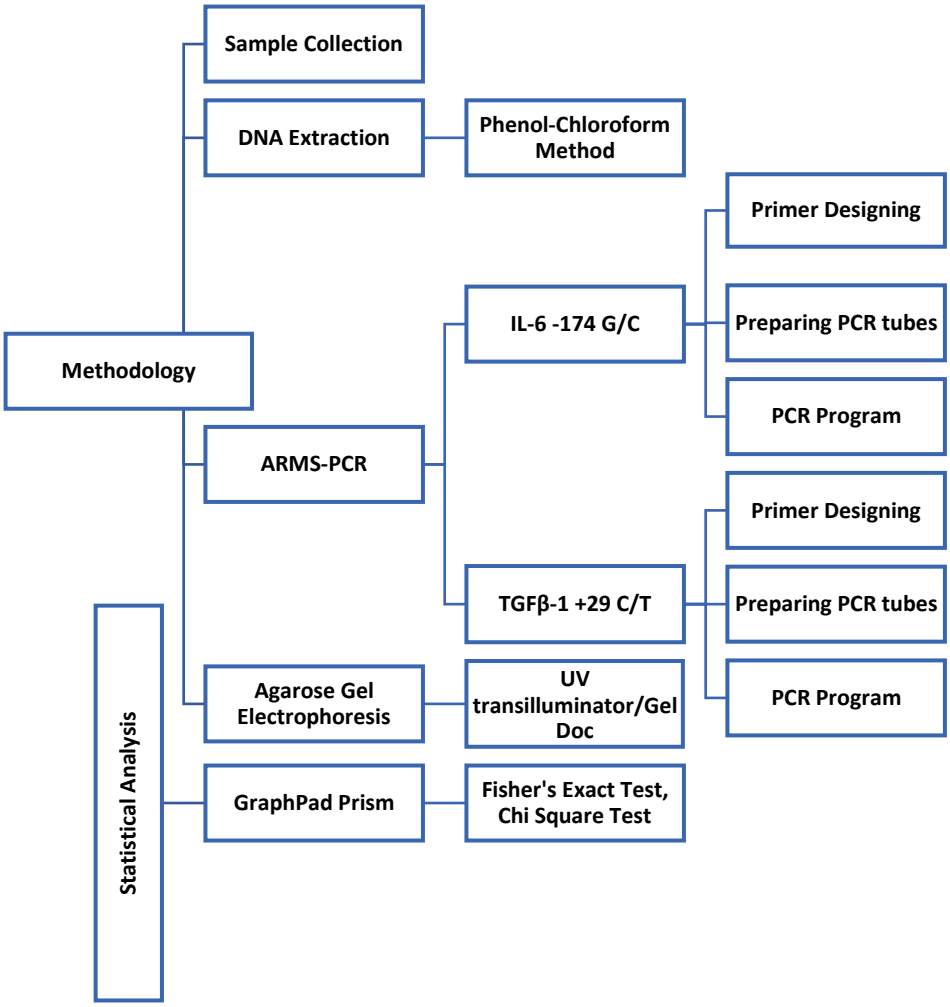

Figure 8. Summary for wet lab methodology.

CHAPTER 4

RESULTS

4.1. Insilico Prediction of Pathogenic Variants

4.1.1. SNP Datasets

Table 9 shows the missense, intronic and promotor region SNP datasets for IL-6 and TGFβ-1 genes, as reported by ENSEMBL.

Table 8. The missense, intron, 3'UTR and 5'UTR SNP datasets for IL-6 and TGF-β1 genes.

Type of variant	Number of variants	
	TGFβ-1	**IL-6**
Missense	863	974
Intron	32424	8365
3' UTR	181	433
5' UTR	321	375

Figure 9 shows the classification of 863 TGFβ-1 missense variants by SIFT, Cadd, Revel, PolyPhen and MetaLr tools. The PolyPhen tool classifies the pathogenic variants as possibly damaging and probably damaging. For the sake of simplicity, the collective number of the PolyPhen damaging variants has been represented in graph 1 (possibly damaging=170, probably damaging=261).

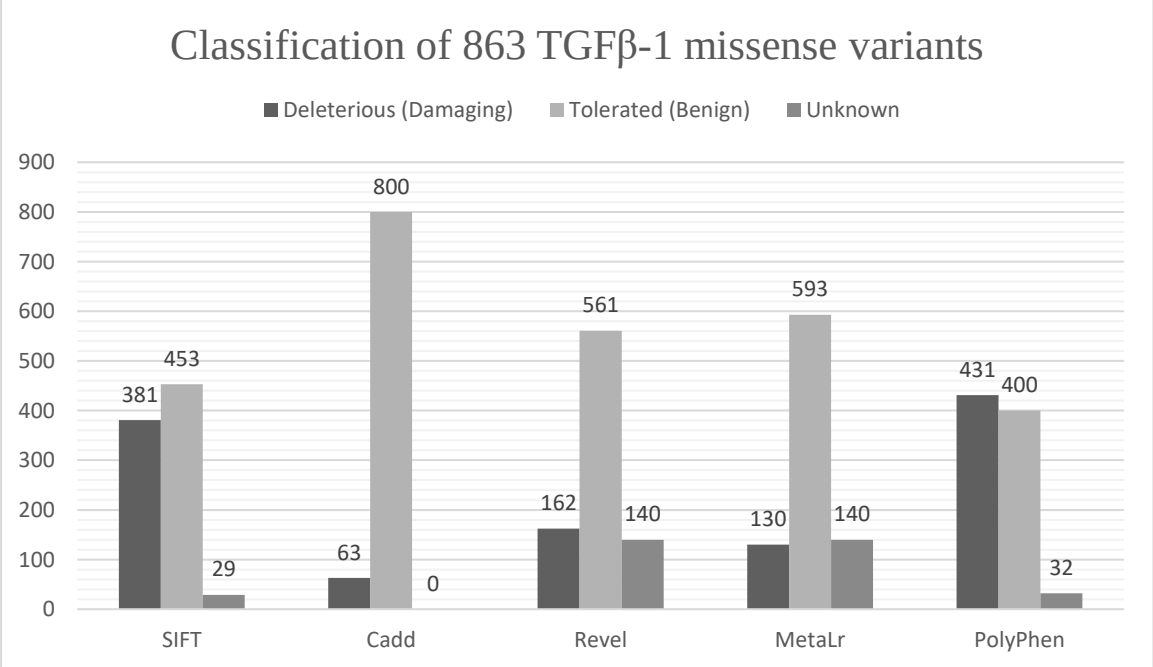

Figure 9. Classification of 863 missense variants of TGF-β1 gene by SIFT, Cadd, Revel, MetaLr and PolyPhen tools.

4.1.2. Prediction of Deleterious SNPs of TGFβ-1

The scores and deleteriousness of TGF-β1 +29C/T (rs1800470) as predicted by the SIFT, Cadd, Revel, PolyPhen and MetaLr tools can be viewed in Table 10.

Table 9. The scores of TGF-β1 +29C/T variant as predicted by SIFT, Cadd, Revel, PolyPhen and MetaLr tools.

Tool	Reference Value	Qualitative prediction	Obtained Score	Inference
SIFT	< 0.05	Deleterious	0.51	Tolerated
	≥ 0.05	Tolerated		
Cadd	> 30	Likely deleterious	18	Likely benign
	< 30	Likely benign		
Revel	> 0.5	Likely disease causing	0.03	Likely benign
	< 0.5	Likely benign		
Meta-Lr	Score between 0 & 1	Either are tolerated or damaging	0.034	Tolerated
PolyPhen	> 0.908	Probably damaging	0	Benign
	> 0.446 & ≤ 0.908	Possibly damaging		
	≤ 0.446	Benign		

4.2. Structural Analysis of TGFβ-1 +29 C/T

Project HOPE was used for identifying the changes in TGFβ-1 protein structure due to the +29 C/T point mutation. The software made use of UniProt-database and Reprof software for the analysis of mutations.

Figure 10.The structures of proline (left) and leucine (right). The backbone structure, coloured red, remains the same for both. Whereas the side chain (coloured black) is unique for each amino acid.

The +29 C/T polymorphism of the TGFβ-1 protein leads to an amino acid mutation of a proline to a leucine at position 10. Figure 3 shows the schematic representation of the wildtype (proline) and mutant (leucine) amino acid.

This mutation may have caused changes in the properties of the wildtype and the mutated TGFβ-1 protein. The report stated that the wildtype and mutant amino acids differ in sizes. The mutant residue is larger in size, which may cause bumps in the protein structure. As proline is known to have a rigid structure, essential for maintaining the protein backbone, its mutation may have caused serious changes in the structural confirmation of the TGFβ-1 protein. The HOPE server could not graphically represent the mutant protein due to the lack of its structural information.

4.3. Functional Analysis of SNPs

RegulomeDB gave annotation score and rank of the IL-6 (rs1800795) and TGFβ-1 (rs1800470) variants, which provided information about the functional impact of these

SNPs. The functional rank for both the variants was found to be 4, which suggests that these variants have limited functional significance. The variant classification system of RegulomeDB ranges from 1-6, which means that higher the rank, lower the functional significance of a variant. Rank 4 represents that these variants have minimal evidence of disrupting the binding site (Boyle et al., 2012). The probability score for both IL-6 and TGFβ-1 variants was predicted to be 0.60906. If the score is closer to 1, it reflects a regulatory function of the mutant (Boyle et al., 2012). Therefore, these IL-6 and TGFβ-1 variants are predicted to have a regulatory role. (Table 10)

Table 10. RegulomeDB analysis of IL-6 (rs1800796) and TGF-β1 (rs1800470) variants.

Variant ID	Functional Rank	Probability Score	Gene Coordinates
IL-6 (rs1800795)	4	0.60906	chr7:22766644-22766645
TGFβ-1 (rs1800470)	4	0.60906	chr19:41858920-41858921

4.4. Sequence of IL-6 -174 G/C and TGFβ-1 +29 C/T Polymorphisms

The sequence of the variants was retrieved from NCBI Genome Data Viewer, with reference to the 37-genome assembly. The sequence of the wildtype and mutant IL-6 and TGFβ-1 genes can be seen below.

Table 11. Sequences of wildtype and mutant IL-6 and TGF- β1 genes.

Sequence of wildtype IL-6	5'-CTAGTTGTGTCTTGCGATGCTAAAGGACGTCACATTGCACAATCTTAATAAGGT-3'
Sequence of mutant IL-6	5'-CTAGTTGTGTCTTGCCATGCTAAAGGACGTCACATTGCACAATCTTAATAAGGT-3'
Sequence of wildtype TGF-β1	5'-TAGCAGCAGCCGCAGCAGCCGCAGCCCGGAGGGCGGCATGGGGGAGGCGGCG-3'
Sequence of mutant TGF-β1	5'-TAGCAGCAGCTGCAGCAGCCGCAGCCCGGAGGGCGGCATGGGGGAGGCGGCG-3'

4.5. Prediction of Transcription Factor Binding sites

The wildtype and mutant sequences of IL-6 were separately inserted to AliBaba2. This software predicted different sites available for the binding of transcription factors in the sequences. AliBaba2 predicted 5 segments in wildtype IL-6, which were identified as potential binding sites for transcription factors. These binding sites include CREB, CRE-BP1, CPE_bind, c-FOS, and C/EBPbeta. (Table 12)

Table 12. AliBaba2 results of transcription factor binding sites for wildtype IL-6.

Segments	Nucleotide Range	Binding Sites
1. 1. 2. 0	24-33	CREB
1. 1. 1. 6	25-34	CRE-BP1
2. 3. 3. 0	25-34	CPE_bind
1. 1. 1. 2	29-38	c-Fos
1. 1. 3. 0	33-44	C/EBPbeta

In contrast to the wildtype IL-6 sequence, the mutant IL-6 sequence has an additional binding site of nuclear factor-1 (NF-1); thus, making a total of 6 segments which are predicted to be the potential binding sites for transcription factors. These include NF-1, CREB, CRE-BP1, CPE_bind, c-FOS and C/EBPbeta binding sites. (Table 13)

Table 13. AliBaba2 results tabulated to represent segments for the transcription factor binding sites and their nucleotide ranges in mutant IL-6 gene. The segment 9.9.539 (highlighted as yellow) is an additional binding site in the mutant gene as compared to its wildtype counterpart.

Segments	Nucleotide Range	Binding Sites
9. 9. 539	9-18	NF-1
1. 1. 2. 0	24-33	CREB
1. 1. 1. 6	25-34	CRE-BP1
2. 3. 3. 0	25-34	CPE_bind
1. 1. 1. 2	29-38	c-Fos
1. 1. 3. 0	33-44	C/EBP beta

4.6. Genotyping of the IL-6 -174 G/C and TGFβ-1 +29 C/T Polymorphisms

To further validate the *insilico* results, a case control study was performed and a total of 429 patients were enrolled in the study. Out of 429 entries, 213 were HCV-induced HCC experimental samples and 216 samples were taken from healthy individuals. The gender distribution of the collected samples was tried to be kept nearly equal to ensure fair representation of the population. (Table 14)

Table 14. Demographic characteristics of the study groups.

	Gender (n=213)	Age in years (Mean ± SD)	Age Groups (years)			
			1-20	21-40	41-60	61-80
HCC-infected Patients						
Male	91 (42.72%)	36.91 ± 12.88	4	57	26	4
Female	122 (57.28%)	34.56 ± 11.70	10	75	34	3
Control						
Male	103 (47.69%)	41.06 ± 13.99	4	51	36	11
Female	113 (52.31%)	39.11 ± 11.98	5	65	39	4

Demographics of study population can be seen in Table 14. Very fewer cases were reported in the 1-20 age group. Greater number of experimental samples fall in the 21-40 & 41-60 age groups which may suggest a higher disease burden in adults. The disease burden progressively decreases in the elderly population with the least number of cases being reported in 61-80 years of age. Control group samples were fairly collected to match the demographics of HCC patients to ensure an unbiased case-control study.

After ARMS-PCR, the electrophoresed gel was analyzed to assess the presence or absence of SNPs in control and experimental samples. In figure 13, a 100 bp ladder is presented by bands on the right side of the gel. The six lanes starting from immediate left are labelled for possible genotype combinations of C and T nucleotides of TGF-β1 gene. Lanes 1 & 2 are labelled as CT, lane 3 & 4 as CC, and lane 5 & 6 as TT genotype. (Figure 11)

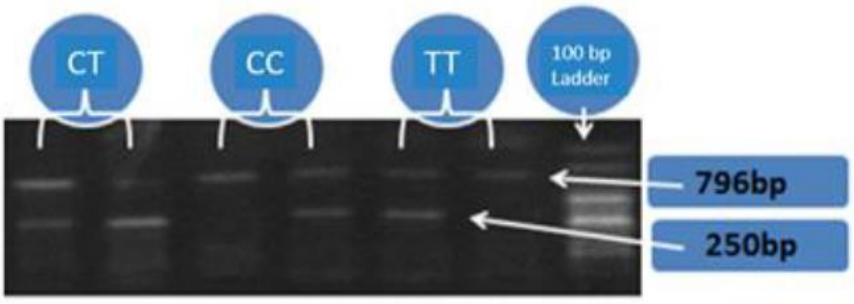

Figure 11. TGFβ-1 +29 C/T Polymorphism genotypes.

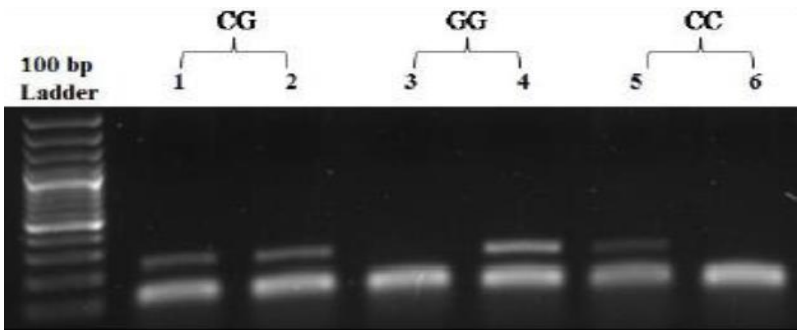

Figure 12. IL-6 -174 C/T polymorphism genotypes.

In Figure 12, a 100 bp ladder is presented by bands on left side of the gel. Remaining six lanes are labelled for various combination of G and C nucleotides of IL-6 gene. Lanes 1 & 2 are labelled as GC, lane 3 & 4 as GG, and lane 5 & 6 as CC genotype. (Figure 12)

4.6.1. Statistical Analysis

The results of ARMS-PCR were statistically analyzed with GraphPad Prism which applied the Fisher's Exact Test and Chi Square Test on them. As a result, the software calculated p-value, relative risk (RR), odds ratio (OR), and percentage frequency distribution of the IL-6 and TGFβ-1 genotypes in the test and control samples. The experimental and control data was analyzed in three different ways based on the overall genotype distribution, gender-dependent distribution, and age-dependent distribution. A p-value of <0.05 was considered statistically significant.

4.6.2. TGFβ-1 +29 C/T and IL-6 -174 G/C Polymorphic Distribution amongst Study Groups

Out of all three genotypes of TGFβ-1 +29 C/T, TT is the most prevalent (24.94%) and presents a positive association with the development of HCV-induced HCC (p<0.0001, OR=5.403, RR=2.062). The CT and CC genotypes have a relatively greater distribution in the control group (26.81% and 15.62%, respectively) as compared to the cancer group (15.85% and 8.86%, respectively). A negative correlation exists between cancer development and CT (p<0.0001, OR=0.4119, RR=0.6304) and CC (p=0.0016, OR=0.4829, RR=0.67) genotypes, respectively. (Table 15)

Table 15. Individual representation of TGFβ-1 +29 C/T and IL-6 -174 G/C genotypes amongst HCC and control groups.

	HCV-infected HCC Patients (%)	**Control (%)**	**OR**	**95% CI-OR**	**RR**	**95% CI-RR**	**p-value**
TGFβ-1							
TT	24.94%	7.93%	5.403	3.423 to 8.562	2.062	1.727 to 2.467	<0.0001
CT	15.85%	26.81%	0.4119	0.2789 to 0.6113	0.6304	0.5053 to 0.7771	<0.0001
CC	8.86%	15.62%	0.4829	0.3084 to 0.7521	0.67	0.5032 to 0.8666	0.0016
IL-6							
GG	9.09%	21.68%	0.2964	0.1919 to 0.4576	0.5043	0.3772 to 0.6586	<0.0001
GC	29.93%	19.72%	2.312	1.578 to 3.365	1.521	1.253 to 1.859	<0.0001
CC	10.47%	8.84%	1.247	0.7725 to 2.042	1.113	0.8743 to 1.373	0.3938

Amongst the IL-6 -174 G/C genotypes, GC has the maximum frequency distribution in the HCC group (29.6%). Moreover, GC genotype carriers have a positive correlation with HCC development (p<0.0001, OR=2.276, RR=1.512).

Subjects with GG genotype present a lower distribution in the HCC group (8.39%) and a negative association with cancer development (p<0.0001, OR=0.2456, RR=0.4685). No statistically significant difference was observed in the susceptibility of the disease amongst CC genotype carriers (p=0.3938). The genotypic distribution TGF-β1 and IL-6 genotypes is enclosed in Table 15.

4.6.3. Gender Dependent Distribution of TGFβ-1 +29 C/T and IL-6 -174 G/C Genotypes

In the gender dependent distribution of TGFβ-1 +29 C/T genotypes, TT is the predominant genotype in male (29.77%) and female (20.09%) genders. The results suggest a strong association of the TT genotype with the development of HCC in both male (p<0.0001, OR=6.531, RR=2.161) and female (p<0.0001, OR=4.304, RR=1.936) carriers. The CT genotype was found to be poorly distributed and negatively associated with the risk of developing cancer in patients (male: p=0.0004, 13.02%; female: p=0.0141, 18.69%). Similarly, only 9.77% men with HCC were CC genotype carriers (p=0.0124) and presented a negative association with HCC susceptibility. However, no statistically significant difference has been observed in the association of liver cancer development with the CC genotype in the female gender (p=0.0724). (See Table 16)

The distribution of IL-6 -174 G/C genotypes represents that GC is the most prevalent genotype amongst male and female cancer patients (male=27.46%, female=32.20%). The data suggests that there is a high risk of developing liver cancer in male (p=0.0003, OR=2.917, RR=1.772) and female (p=0.0185, OR=1.902, RR=1.370) carriers of GC genotype. Contrary to GC, the distribution of the GG genotype was lower in HCC patients as compared to their healthy counterparts in both male (8.29% vs 27.46%) and female (9.75% vs 16.95%) genders, respectively. The analysis of GG genotype indicates its protective role against liver carcinogenesis in both men (p<0.0001, OR=0.1972, RR=0.3834) and women (p=0.0053, OR=0.4298, RR=0.6380). The gender distribution of the IL-6 genotypes can be seen in Table 16.

Table 16. Gender-based distribution of TGFβ-1 +29 C/T and IL-6 -174 G/C genotypes in HCV-infected HCC and control populations. (M=Male, F=Female)

	HCC-infected Patients (%)	Control (%)	OR	95% CI-OR	RR	95% CI-RR	p-value
TGF-β1							
TT-M	29.77%	7.91%	6.531	3.449 to 12.53	2.161	1.692 to 2.793	<0.0001
CT-M	13.02%	23.26%	0.3426	0.1901 to 0.6166	0.5786	0.4118 to 0.7851	0.0004
CC-M	9.77%	16.28%	0.4370	0.2351 to 0.8300	0.6481	0.4403 to 0.9047	0.0124
TT-F	20.09%	7.94%	4.304	2.210 to 8.388	1.936	1.482 to 2.508	<0.0001
CT-F	18.69%	30.37%	0.5026	0.2885 to 0.8567	0.6921	0.5108 to 0.9262	0.0141
CC-F	7.94%	14.95%	0.5248	0.2641 to 1.010	0.6897	0.4449 to 1.006	0.0724
IL-6							
GG-M	8.29%	27.46%	0.1972	0.1051 to 0.3928	0.3834	0.2400 to 0.5857	<0.0001
GC-M	27.46%	16.58%	2.917	1.719 to 5.362	1.772	1.313 to 2.354	0.0003
CC-M	11.4%	8.81%	1.594	0.7820 to 3.158	1.259	0.8799 to 1.699	0.2127
GG-F	9.75%	16.95%	0.4298	0.2400 to 0.7669	0.6380	0.4402 to 0.8820	0.0053
GC-F	32.20%	22.46%	1.902	1.132 to 3.133	1.370	1.062 to 1.795	0.0185
CC-F	9.75%	8.90%	1.029	0.5213 to 1.936	1.014	0.7179 to 1.345	>0.9999

4.6.4. TGFβ-1 +29 C/T and IL-6 -174 G/C Polymorphic Distribution amongst different Age Groups of HCC and Control Patients

Through the analysis of age dependent distribution of TGFβ-1 +29 C/T genotypes in HCC and healthy patients, it has been found that the TT genotype is notably distributed in liver cancer patients aging from 21-40 (26.29%) and 41-60 (25.55%). The data suggests that there is a strong association of liver carcinogenesis with the presence of TT genotype in the 21-40 ($p<0.0001$, OR=4.591, RR=1.835) and 41-60 ($p<0.0001$, OR=10.02, RR=2.845) age groups. (Table 17)

On the other hand, subjects having the CT genotype present a relatively low distribution and a negative association with cancer development in the 21-40 (17.2%, $p=0.0064$) and 41-60 (10.95%, $p<0.0001$) age groups of HCC patients. The prevalence of CC genotype was lower in cancer patients aging from 21-40 years as compared to the prevalence of CT and TT genotypes within the same age bracket (10.36% vs 17.20%

and 26.29%, respectively). The presence of CC genotype in the 21-40 age group demonstrates a negative correlation with HCC development (p=0.0137, OR=0.4709, RR=0.6826). (See Table 17)

Table 17. The genotypic distribution of TGFβ-1 +29 C/T polymorphism in the 1-20, 21-40, 41-60 and 61-80 age groups of cancer and control patients.

	HCC-infected Patients (%)	**Control (%)**	**OR**	**95% CI-OR**	**RR**	**95% CI-RR**	**p-value**
1-20 TT	6.25%	12.50%	0.5833	0.0350 to 6.212	0.7222	0.1256 to 2.446	>0.9999
1-20 CT	31.25%	18.75%	5.000	0.6148 to 33.40	2.500	0.7820 to 9.391	0.3147
1-20 CC	6.25%	25.00%	0.2083	0.0149 to 2.546	0.3667	0.06323 to 1.474	0.3077
21-40 TT	26.29%	7.97%	4.591	2.554 to 8.314	1.835	1.480 to 2.280	<0.0001
21-40 CT	17.20%	22.80%	0.4756	0.2803 to 0.7914	0.7011	0.5351 to 0.8986	0.0064
21-40 CC	10.36%	15.54%	0.4709	0.2620 to 0.8498	0.6826	0.4848 to 0.9183	0.0137
41-60 TT	25.55%	6.57%	10.02	4.254 to 22.63	2.845	2.001 to 4.109	<0.0001
41-60 CT	10.95%	35.77%	0.1797	0.08889 to 0.3801	0.3719	0.2276 to 0.5833	<0.0001
41-60 CC	8.03%	13.14%	0.7089	0.3184 to 1.573	0.8193	0.4738 to 1.289	0.5289
61-80 TT	15.79%	15.79%	12.00	1.176 to 165.1	6.500	1.108 to 40.23	0.0709
61-80 CT	5.26%	31.58%	0.5000	0.0335 to 4.238	0.5714	0.08813 to 3.166	>0.9999
61-80 CC	0	21.05%	0.000	0.000 to 2.628	0.000	0.000 to 1.534	0.2554

Amongst the IL-6 -174 G/C genotypes, GC was observed to have high prevalence in the 21-40 (32.26%) and 41-60 (25.74%) age groups of HCC patients. The GC genotype carriers falling in these age groups were found to have a high association towards developing liver cancer (Table 18). Compared to the GC genotype, the GG genotype carriers in the 21-40 and 41-60 age groups had a lower frequency distribution in HCC patients (21-40 years= 8.91%, 41-60 years= 9.56%). The data suggests that there is a low risk of developing liver cancer in the GG genotype carriers falling in the 21-40 (p<0.0001, OR=0.3000, RR=0.5265) and 41-60 (p=0.0038, OR=0.3240, RR=0.5071) age groups. The results present no statistically significant difference in the development of HCC amongst the CC genotype carriers for all three age groups (p>0.05). The distribution of IL-6 genotypes in different age groups can be observed in Table 18.

Table 18. The genotypic distribution of IL-6 -174G/C polymorphism in the 1-20, 21-40, 41-60 and 61-80 age groups of cancer and control patients.

	HCC-infected Patients (%)	Control (%)	OR	95% CI-OR	RR	95% CI-RR	p-value
1-20 GG	8.7%	17.39%	0.2083	0.0341 to 1.398	0.4722	0.1335 to 1.132	0.1616
1-20 GC	43.48%	17.39%	3.125	0.5759 to 18.85	1.607	0.7973 to 3.954	0.3826
1-20 CC	8.7%	4.35%	1.333	0.1354 to 21.48	1.111	0.3332 to 2.605	>0.9999
21-40 GG	8.91%	18.62%	0.3000	0.1676 to 0.5493	0.5265	0.3593 to 0.7366	<0.0001
21-40 GC	32.26%	18.95%	2.259	1.374 to 3.794	1.466	1.154 to 1.885	0.0022
21-40 CC	12.10%	9.27%	1.189	0.6354 to 2.185	1.082	0.8048 to 1.388	0.6425
41-60 GG	9.56%	25.74%	0.3240	0.1533 to 0.7031	0.5071	0.3002 to 0.8099	0.0038
41-60 GC	25.74%	22.06%	2.147	1.103 to 4.333	1.529	1.044 to 2.270	0.0380
41-60 CC	8.82%	8.09%	1.477	0.6250 to 3.584	1.228	0.7451 to 1.820	0.4907
61-80 GG	9.09%	36.36%	0.35	0.0584 to 2.021	0.48	0.1214 to 1.678	0.3808
61-80 GC	18.18%	18.18%	3.667	0.6058 to 18.85	2.333	0.7152 to 7.592	0.3426
61-80 CC	4.55%	13.64%	0.6667	0.0446 to 5.462	0.75	0.1271 to 2.918	>0.9999

4.7. Docking Analysis of TGFβ-1 and IL-6 with Tetrahydroxyflavanone

The molecular interactions of tetrahydroxyflavanone (CID: 246330) with TGFβ-1 and IL-6 proteins were studied through in-silico docking methods to assess the inhibitory activity of tetrahydroxyflavanone against cytokine expression. The energy of the ligand was found to be 2.7018 kcal/mol through ChemDraw. CB Dock was used to analyse the molecular docking activity of TGF-β1 and IL-6 with tetrahydroxyflavanone, respectively (Figure 13 A, B).

Vina score	Cavity size	Center			Size		
		x	y	z	x	y	z
-7.7	1504	101	40	59	20	26	20
-6.6	191	97	54	52	20	20	20
-6.3	858	94	46	12	20	20	20
-5.8	211	97	29	44	20	20	20
-5.5	177	87	43	41	20	20	20

Vina score	Cavity size	Center			Size		
		x	y	z	x	y	z
-7.3	619	4	-1	-11	20	20	20
-7.1	814	4	-2	10	27	20	20
-7	375	-16	4	-4	20	20	20
-6.2	396	7	10	6	20	20	20
-6.1	164	-19	-3	8	20	20	20

Figure 13. (A) CB Dock results of TGFβ-1 interaction with tetrahydroxyflavanone. (B) CB Dock results of IL6 interaction with tetrahydroxyflavanone. The docking structure with the least energy (highlighted in green) was chosen.

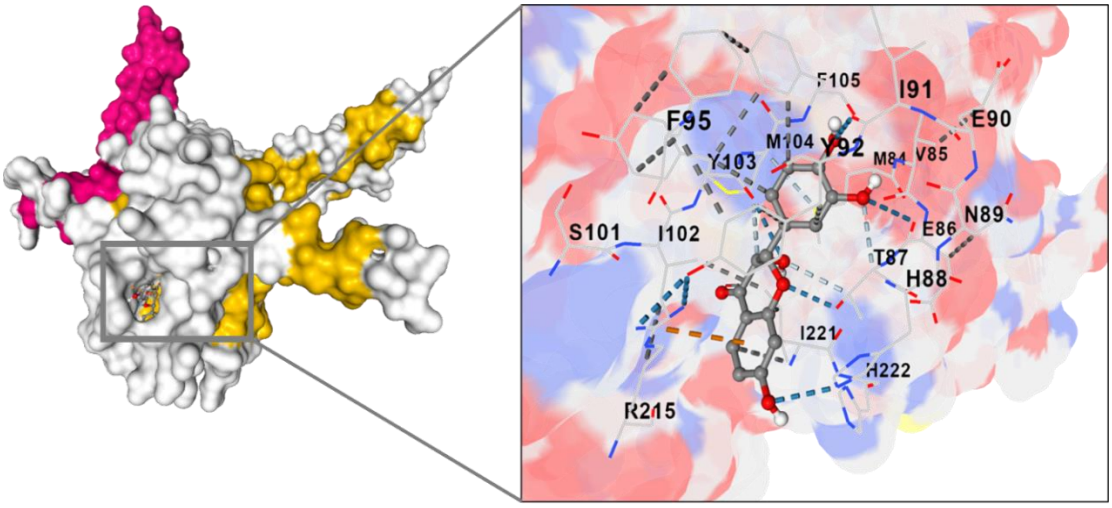

Figure 14. CB dock results of a docked structure between TGFβ-1 and tetrahydroxyflavanone.

The lesser the energy, the greater the stability and hence, greater will be the binding efficiency. The binding affinity between ligand and protein is estimated by vina score or docking energies (kJ/mol). Therefore, the docking structure with the least energy (represented by the vina score), -7.3 kJ/mol (for IL-6) and -7.7 kJ/mol (for TGF-β1), was chosen for further analysis (Figure 13A, 13B). Moreover, the IL-6 and TGF-β1 cavity sizes of 619 and 1504 Å represent a groove big enough to facilitate the binding of ligand with the protein. Figures 14 and 15 represent the docked structures of TGF-β1 and IL-6 with tetrahydroxyflavanone, respectively.

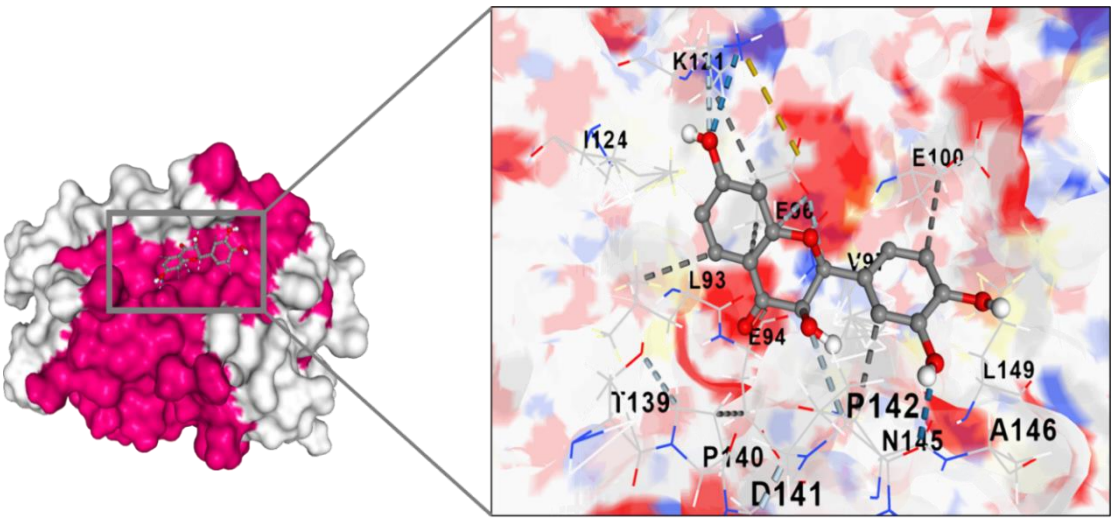

Figure 15. CB dock results of IL-6 docked structure with tetrahydroxyflavanone.

4.8. Post Docking Analysis

To analyse the docking interaction of IL-6 and TGFβ-1 with tetrahydroxyflavanone, LigPlot programme was used. LigPlot gave an analysis of the hydrogen bonds and hydrophobic interactions between amino acid residues of protein and ligand atoms within a range of 5 Å. A 2-dimentional molecular interaction of IL-6 and TGF-β1 with tetrahydroxyflavanone can be seen in figures 16A and 17A, respectively. Table 19 shows the amino acids interacting with the ligand to form hydrogen bonds and their respective bond lengths.

Table 19. The IL-6 and TGF-β1 amino acids interacting with the ligand to form hydrogen bonds and their respective bond lengths.

Ligand	Proteins	Amino Acids interacting through Hydrogen Bonding	H-Bond Length Å
Tetrahydroxyflavanone	TGF-β1	His88	2.99
		Thr87	2.57
		Asn89	2.91
	IL-6	Pro142	2.70
		Lys121	3.26

4.8.1. Domain Analysis of TGFβ-1

TGFβ-1 is forming three hydrogen bonds with the ligand. The amino acid residues His88, Thr87 and Asn89 are involved in making these hydrogen bonds with the ligand (Figure 16 A and B, and Table 19).

The non-ligand residues Arg215, Ile102, Ile 221, Tyr103, Phe105, Ile91 and Tyr92 (marked as red in Figure 16 A) are hydrophobically interacting with tetrahydroxyflavanone.

The LigPlot analysis also gives the respective bond lengths of the interacting amino acids and the ligand (Table 19). His88, Thr87 and Asn89 residues are forming hydrogen bonds with a bond length of 2.99, 2.57 and 2.91 Å, respectively. Figure 16B shows the 3-dimensional structure of TGF-β1 interacting with tetrahydroxyflavanone.

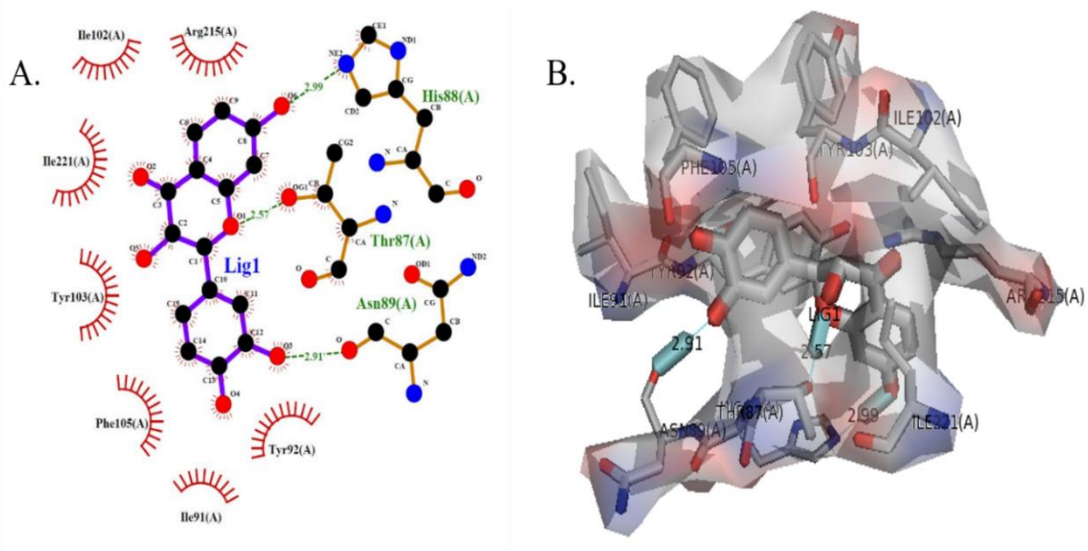

Figure 16. LigPlot analysis results: (A) A 2-Dimentional representation of TGFβ-1 interaction with tetrahydroxyflavanone. (B) The three-dimensional structure of TGF-β1 interacting with tetrahydroxyflavanone. The image is generated by PyMol.

Domain Analysis of IL-6

The molecular interactions of IL-6 protein with tetrahydroxyflavanone can be seen in figure 17 A and B. Figure 17A projects a 2-D structure generated by LigPlot, where IL-6 bonding to the ligand is shown through multiple hydrophobic and hydrogen bonding interactions.

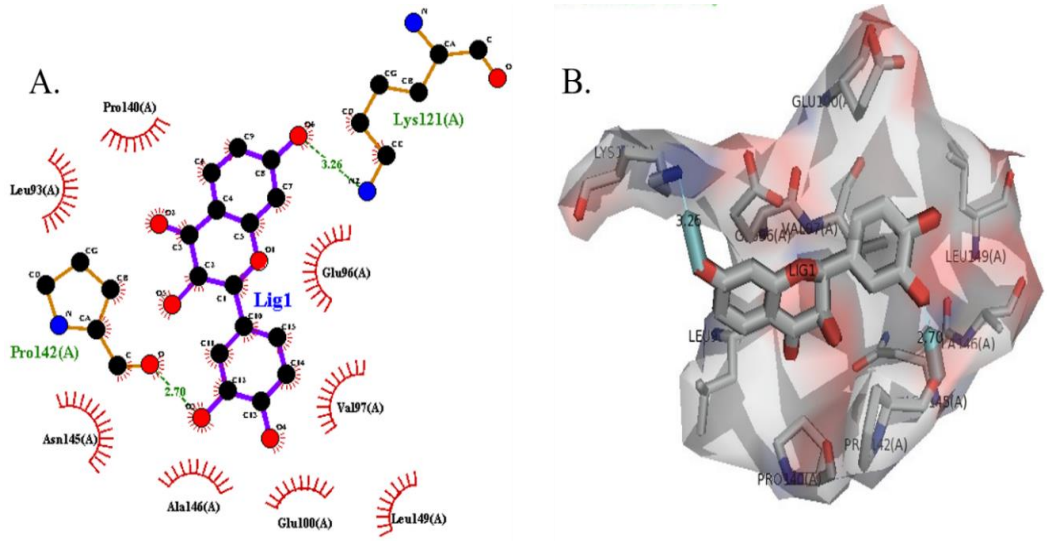

Figure 17. LigPlot analysis results: (A) A 2-Dimentional representation of IL-6 interaction with tetrahydroxyflavanone. (B) The 3-dimensional image of IL6 interacting with tetrahydroxyflavanone. This image is generated by PyMol.

Pro142 and Lys121 are forming hydrogen bonds with the ligand. The hydrophobic interactions with ligand are established through Ala146, Glu100, Leu149, Va197, Glu96, Pro 140, Leu93, and Asn145. The bond lengths of Pro 142 and Lys 121 bonding with ligand are 2.70 Å and 3.26 Å, respectively (as predicted by Ligplot).

CHAPTER 5

DISCUSSION

HCC is marked as the most common liver malignancy, especially in HBV and HCV-infected patients. The life expectancy of HCC depends on the progression of disease and stage of cancer. When diagnosed at an early stage, the treatment may be effective. However, at an advanced stage the therapy does not produce effective results (Tunissiolli, Castanhole-Nunes, Biselli-Chicote, Pavarino, & da Silva, 2017). Multiple studies have reported an association of -174 G/C IL-6 polymorphism with the pathogenesis of liver diseases including HCC derived from HCV(Aleagha, Oltulu, & Sadeghi, 2020; Giannitrapani et al., 2013). Moreover, the IL-6 G/C polymorphism is also found to be associated with the prevalence of breast, ovarian, prostatic, cervical, colorectal, and oral cancer (Berek et al., 1991; DeMichele et al., 2003; Landi et al., 2003; Singh et al., 2015; Slattery, Curtin, et al., 2007; Yeh, Li, Hsieh, Chen, & Tang, 2010). Studies have also identified the possible role of TGFβ-1+29 C/T polymorphism in the pathogenesis of osteoarthritis (Čengić et al., 2015),breast cancer (Gautam et al., 2015) urinary bladder cancer (Gaussin et al., 2012), development of cervical lesions (Trugilo et al., 2019), sarcopenia (Fuku et al., 2012) and chronic periodontitis (Heidari, Mahmoudzadeh-Sagheb, Hashemi, & Rigi-Ladiz, 2014). To our knowledge, studies on the assocation of IL-6 -174 G/C and TGFβ-1 +29 C/T polymorphisms with HCV-induced HCC are not widely available. This study aims to assess the risk of developing hepatic cancer in HCV-infected patients due to the presence of IL-6 -174 G/C and TGFβ-1 +29 C/T polymorphisms.

Despite the reported deleterious nature of this variant, the bioinformatics analysis classified the clinical significance of TGFβ-1 +29 C/T polymorphism as benign which suggests that this missense variant has little functional significance. According to RegulomeDB analysis, both the -174 G/C IL-6 and +29 C/T TGFβ-1 polymorphisms are predicted to have small functional and regulatory roles as their probability scores (0.60906) are closer to 1.

An additional nuclear factor-1 (NF-1) binding site, based on 9 nucleotides, was identified in the IL-6 variant as compared to the wildtype sequence. NF-1, also known as the CAAT box-binding transcription factor (CTF), is a wide family of DNA-binding proteins that recognize and bind to the CAAT box (GCCAAT sequence) in the promotor region. The CTF/NF-1 proteins regulate the gene expression by acting as transcription factors for RNA polymerase II. Moreover, the CTF/NF-1 proteins also function as initiation factors for recruiting DNA polymerase for adenovirus DNA replication (Gaussin et al., 2012; Santoro, Mermod, Andrews, & Tjian, 1988). The binding sites for CTF/NF1 have been reported in the promotor, enhancer, and silencer regions of a gene. The alternative splicing generates multiple variants of NF-1 protein that function as activators or repressors of transcription (Gronostajski, 2000). Some studies have formed an association of increased IL-6 serum levels with the presence of IL-6 -174G/C polymorphism (Woo & Humphries, 2013; Wypasek et al., 2010). Our

finding of NF-1 binding site in the IL-6 variant may suggest that it could cause either upregulation or downregulation of IL-6 gene transcription. Unfortunately, in this study we were not able to evaluate the serum IL-6 levels in the samples. Thus, more studies are required to assess the impact of NF-1 binding site on the IL-6 gene expression.

According to the structural analysis of TGFβ-1 variant, there is a point mutation of wildtype proline into mutant leucine at amino acid residue 10. Proline has a side chain that is covalently bonded to the peptide backbone to form a cyclized structure (Karvonen et al., 1998; Morgan & Rubenstein, 2013). This cyclic structure confers rigidity to the protein (Biedermannova, Riley, Berka, Hobza, & Vondrasek, 2008; Deber, Brodsky, & Rath, 2010). Proline inhibits the secondary and tertiary confirmation of protein because it does not allow the formation of alpha-helix or beta-sheet structure. Instead, it introduces breaks and kinks into the alpha-helical part of the protein backbone (Karvonen et al., 1998; Morgan & Rubenstein, 2013). On the other hand, leucine has a hydrophobic aliphatic side chain which favours the formation of alpha-helical structure (Karvonen et al., 1998). This suggests that the Pro10Leu mutation may have caused changes in the binding pattern and rigidity of the TGFβ-1 variant as compared to its wildtype counterpart. Moreover, leucine is larger in size than proline. The Pro10Leu mutation may have caused the formation of bumps in mutant TGFβ-1 protein structure, as predicted by Project HOPE.

TGFβ-1 protein has a dimeric structure. Its sequence is based on 390 amino acids. TGFβ-1 is synthesized as a precursor protein having three distinct parts: the N terminal signal peptide (1-29 amino acids), the prodomain or latency-associated peptide (LAP: 30-278 amino acids) and the C terminal growth factor or mature peptide (mature TGFβ-1: 279-390 amino acids) (Gentry, Lioubin, Purchio, & Marquardt, 1988). The Pro10Leu polymorphism lies in the signal peptide domain of the TGFβ-1 protein. A signal peptide directs a newly synthesised protein to the ER for further processing (Verner & Schatz, 1988). Based on the chemical differences in proline and leucine, a study has previously hypothesized that the Pro10Leu mutation may influence the binding of the nascent protein to the TGFβ-1 protein, thus impacting the peptide export efficiency of the TGF-β1 (Ma et al., 2017). This may influence the availability and the maturation of the nascent protein. However, more studies are required to validate this.

Previous studies have shown that flavonoids can downregulate the TGFβ-1/SMAD signalling pathway (Yifan Zhang et al., 2019; YF Zhang et al., 2016). Furthermore, flavonoids have also been reported to exhibit anti-inflammatory effects against inflammatory cytokines and antioxidant pathways in in-vitro and in-vivo studies. They significantly reduce the production of many cytokines including IL-6 (Leyva-López, Gutierrez-Grijalva, Ambriz-Perez, & Heredia, 2016). This study has evaluated the therapeutic potential of tetrahydroxyflavanone against TGFβ-1and IL-6 proteins.

As the propeptide or LAP domain of TGFβ-1 ranges from 30 to 278 amino acids (Gentry et al., 1988), all the residues forming hydrogen bonds and hydrophobic interactions with the ligand (figure 18) fall in the LAP domain of TGFβ-1 protein. The TGF-β isoforms undergo intracellular cleavage by an enzyme called endopeptidase

furin which cleaves the C-terminal from the N-terminal part of the protein. This produces two products that individually assemble to form dimeric structures. The N-terminal dimer protein is called LAP and the C-terminal dimer is called the mature or active TGFβ-1. Despite the cleavage, LAP becomes non-covalently associated with mature TGFβ-1. This association of LAP with mature TGFβ-1 renders it biologically inactive (Khalil, 1999). The LigPlot results suggest that tetrahydroxyflavanone is directly interacting with the LAP domain of TGFβ-1, which could possibly alter the interaction of LAP with mature TGFβ-1. Consequently, the mature TGFβ-1 may bind to its receptors and stimulate either tumor promotion or tumor suppression. Further studies are required for determining the exact mechanism.

The IL-6 structure consists of four alpha-helical chains. There are three sites within the IL-6 structure that are important for IL-6 binding to IL-6R and gp130 receptors. Site 1 comprises of the C terminal portion of helix D and C terminal region of AB loop/N terminal part of Helix B (Manfredini et al., 2003; Simpson et al., 1997). Site 2 is composed of Helix A and C (Manfredini et al., 2003; Paonessa et al., 1995). Site 3 is comprised of the N terminal residues of helix D/CD loop and some residues of initial part of AB loop (Manfredini et al., 2003; Paonessa et al., 1995; Simpson et al., 1997; Somers et al., 1997). Helix E is not included in any of the site, however its positioning within the CD loop might indicate some sort of disruption in the gp130 binding activity of Site 3 (Gelinas et al., 2014). According to the LigPlot analysis, only Lys121 and Pro142 are forming hydrogen bonds. Whereas the Ala146, Glu100, Leu149, Va197, Glu96, Pro 140, Leu93, and Asn145 residues are not able to form hydrogen bonds with the ligand. All the hydrophobic and hydrophilic amino acid residues were studied for their presence in the IL-6 protein structure. The Leu93 residue is present in the AB loop and is believed to be involved in site 1 activity of binding to IL-6R (Simpson et al., 1997; Somers et al., 1997). Glu96, Val97, and Glu100 residues are also present within helix B but are not involved in binding (Simpson et al., 1997; Somers et al., 1997). Lys121 falls in Helix C and is possibly involved in Site 2 activity of binding to gp130 (Simpson et al., 1997). The presence of Asn145, Ala146, Leu149, and Pro142 residues within the E helix in the Site 3 might play a role in binding to gp130 (Gelinas et al., 2014; Simpson et al., 1997; Somers et al., 1997). Each site of IL-6 acts as a target for monoclonal antibodies. These three sites in IL-6 structure have been previously targeted to inhibit the IL-6 signalling pathway (McElvaney, Curley, Rose-John, & McElvaney, 2021). In this study, since tetrahydroxyflavanone is interacting with all three sites of IL-6, it is most likely to inhibit the binding of IL-6 to IL-6R and gp130 receptors, which may consequently inhibit the IL-6-driven inflammatory signalling pathway.

Studies performed on breast cancer, and colo-rectal cancer identified a positive association of IL-6 -174 G/C polymorphisms with HCC development (Hefler et al., 2005; S. Wang, Ding, Tang, & Li, 2018). A meta-analysis study has also confirmed a significant association of IL-6 -174 G/C polymorphism with HCC susceptibility (Ying Liu, Gao, Du, & Wang, 2014). Some groups have studied the association of SNPs in IL-6 gene with the development of HCC in Pakistani population (Adnan et al., 2020; Badshah et al., 2018), however, none of these studies focused on the -174G/C

polymorphism in HCV induced HCC. Badshah et al. studied IL-6-174G/C polymorphisms in association with the pathogenesis and prognosis of HCV infection. The study reported a high prevalence of homozygous genotypes (GG and CC) but the heterozygous GC genotype was identified to be protective against HCV infection (Badshah et al., 2018). On the other hand, the results of our study suggest that the GC genotype holds a positive association, and the GG genotype holds a negative association against the development of HCC. The findings of the above-mentioned study contradict with our reported results. However, it creates room for further investigation of the association of IL-6 -174G/C polymorphisms with HCV infection in both HCC and non-HCC cases to strengthen the validity of reported results.

A study on the colon cancer patients has also identified the negative association of GG genotype (of -174 G/C) with cancer which is suggestive of its protective impact against the disease (Slattery, Wolff, et al., 2007). The positive or negative association of the CC genotype with disease prevalence has not been reported in the abovementioned studies, which is also consistent with our findings. The GC genotype was found to have a higher prevalence in male patients as compared to female patients, which could be a possible explanation of the higher disease burden of HCC in men.

To our understanding, the association of TGFβ-1 +29 C/T polymorphism with HCV-induced HCC has not been studied in Pakistani population before. This is the first time that a study has been conducted to establish an association of this polymorphism with the pathogenesis of HCC in HCV-infected patients. A research performed on bladder cancer patients in Indian population has reported the TT homozygous genotype to be disease-causing (Gautam et al., 2015). Another group studying +29 C/T polymorphism in breast cancer patients in India also identified TT to be a pathogenic genotype (Pooja et al., 2013). This is consistent with our finding of TT genotype having a pathogenic association with HCC development in both male and female genders. No data has been reported previously on the possible association of CC or CT genotypes with HCC pathogenesis. T allele has been reported to be a disease associated allele whereas no such association has been established for the C allele (Pooja et al., 2013). This suggests that the CC genotype might hold a protective role against HCC development. This is in accordance with our findings of poor association of CC genotype with cancer prevalence. Another research on investigating the role of +29C in prostate cancer also found no significant association of CC genotype with the cancer development (Ewart-Toland, Chan, Yuan, Balmain, & Ma, 2004).

The association of TGFβ-1 TT genotype and IL-6 GC genotype with HCC is predominated in 21-40 and 41-60 age groups. Although no data has been reported on age-dependent distribution of IL-6 -174 G/C and TGFβ-1 +29 C/T polymorphisms, yet we can infer from our findings that the adult age group infected with HCV (21-60 years) carries the highest disease burden of HCC.

CONCLUSION

To summarize, this study reports that the TGFβ-1 +29 C/T and IL-6 -174 G/C polymorphisms are involved in the carcinogenesis of HCC in HCV-infected population. The TT genotype of TGFβ-1 gene and the GC genotype of IL-6 gene are found to be deleterious due to their contribution towards the development of HCC in patients. These findings propose that an early diagnosis of HCC may be possible through genotyping of HCV patients. The molecular docking analysis revealed significant binding interactions of tetrahyrdoxyflavanone with the amino acids in the IL-6 and TGFβ-1 protein structures. This is suggestive of the promising therapeutic role of tetrahydroxyflavanone against the pathogenesis of liver cancer.

FUTURE PROSPECTS

The limitations of this study included our inability to perform a sequencing analysis of the IL-6 -174 G/C and TGFβ-1 +29 C/T polymorphisms and that we could not measure the serum levels of IL-6 and TGFβ-1 proteins in the samples. Thus, more detailed investigations are required with larger population cohorts to assess the pathogenic genotypes and correlate their expression with the incidence of HCC. Sequencing analysis is required to validate our findings of ARMS-PCR. The effect of pathogenic genotypes on the serum concentrations of cytokines and growth factors needs to be investigated. As this study has evaluated the risk of developing HCC in patients, it opens a new window to investigate the real-time expression of IL-6 and TGFβ-1 genes and their impact on the progression of HCV-induced liver carcinogenesis. This would require sampling at different stages of cancer.

Furthermore, extensive invitro and invivo studies are required to explore the therapeutic activity of tetrahydroxyflavanone in the inhibition of cytokine and growth factor-mediated pathways.

REFERENCES

Adnan, F., Khan, N. U., Iqbal, A., Ali, I., Petruzziello, A., Sabatino, R., . . . Khan, S. (2020). Interleukin-6 polymorphisms in HCC patients chronically infected with HCV. *Infectious agents and cancer, 15*, 1-7.

Ahn, K. S., & Kang, K. J. (2019). Appropriate treatment modality for solitary small hepatocellular carcinoma: Radiofrequency ablation vs. resection vs. transplantation? *Clinical and molecular hepatology, 25*(4), 354.

Aleagha, O. E., Oltulu, P., & Sadeghi, M. (2020). Association between interleukin 6 polymorphisms (rs1800796, rs1800795, rs2069837, rs17147230, and rs1800797) and hepatocellular carcinoma susceptibility: a meta-analysis. *Clinical and experimental hepatology, 6*(4), 359.

Ali, S., Nasreen, S., Safeer, S., Andleeb, S., Ejaz, M., Bano, S., & Shakir, H. A. (2016). Medicinal plants as therapeutic agents for cancer treatment. *Punjab univJ Zool, 31*, 295-305.

Alisi, A., Giambartolomei, S., Cupelli, F., Merlo, P., Fontemaggi, G., Spaziani, A., & Balsano, C. (2003). Physical and functional interaction between HCV core protein and the different p73 isoforms. *Oncogene, 22*(17), 2573-2580.

André, F. (2000). Hepatitis B epidemiology in Asia, the middle East and Africa. *Vaccine, 18*, S20-S22.

Arbuthnot, P., & Kew, M. (2001). Hepatitis B virus and hepatocellular carcinoma. *International journal of experimental pathology, 82*(2), 77-100.

Axley, P., Ahmed, Z., Ravi, S., & Singal, A. K. (2018). Hepatitis C virus and hepatocellular carcinoma: a narrative review. *Journal of clinical and translational hepatology, 6*(1), 79.

Badshah, Y., Arshad, N., Khan, M., Jannat, A., & Hayat, H. (2018). Association of interleukin-4 (-590 C/T) and interleukin-6 (-174 G/C) polymorphism in pathogenesis and prognosis of hepatitis C virus infection. *Journal of Interferon & Cytokine Research, 38*(12), 559-565.

Balogh, J., Victor III, D., Asham, E. H., Burroughs, S. G., Boktour, M., Saharia, A., . . . Monsour Jr, H. P. (2016). Hepatocellular carcinoma: a review. *Journal of hepatocellular carcinoma, 3*, 41.

Barbier, L., Fuks, D., Pessaux, P., Muscari, F., Le Treut, Y.-P., Faivre, S., & Belghiti, J. (2013). Safety of liver resection for hepatocellular carcinoma after sorafenib therapy: a multicenter case-matched study. *Annals of surgical oncology, 20*(11), 3603-3609.

Batra, P., & Sharma, A. K. (2013). Anti-cancer potential of flavonoids: recent trends and future perspectives. *3 Biotech, 3*(6), 439-459.

Berek, J. S., Chung, C., Kaldi, K., Watson, J. M., Knox, R. M., & Martínez-Maza, O. (1991). Serum interleukin-6 levels correlate with disease status in patients with epithelial ovarian cancer. *American journal of obstetrics and gynecology, 164*(4), 1038-1043.

Bergmann, J., Müller, M., Baumann, N., Reichert, M., Heneweer, C., Bolik, J., . . . Boretius, S. (2017). IL-6 trans-signaling is essential for the development of hepatocellular carcinoma in mice. *Hepatology, 65*(1), 89-103.

Berman, H. M., Westbrook, J., Feng, Z., Gilliland, G., Bhat, T. N., Weissig, H., . . . Bourne, P. E. (2000). The protein data bank. *Nucleic acids research, 28*(1), 235-242.

Biedermannova, L., Riley, K. E., Berka, K., Hobza, P., & Vondrasek, J. (2008). Another role of proline: stabilization interactions in proteins and protein complexes concerning proline and tryptophane. *Physical Chemistry Chemical Physics, 10*(42), 6350-6359.

Bosetti, C., Turati, F., & La Vecchia, C. (2014). Hepatocellular carcinoma epidemiology. *Best practice & research Clinical gastroenterology, 28*(5), 753-770.

Boyle, A. P., Hong, E. L., Hariharan, M., Cheng, Y., Schaub, M. A., Kasowski, M., . . . Weng, S. (2012). Annotation of functional variation in personal genomes using RegulomeDB. *Genome research, 22*(9), 1790-1797.

Brooijmans, N., & Kuntz, I. D. (2003). Molecular recognition and docking algorithms. *Annual review of biophysics and biomolecular structure, 32*(1), 335-373.

Bruix, J., Gores, G. J., & Mazzaferro, V. (2014). Hepatocellular carcinoma: clinical frontiers and perspectives. *Gut, 63*(5), 844-855.

Bruix, J., & Sherman, M. (2011). Management of hepatocellular carcinoma: an update. *Hepatology, 53*(3), 1020-1022.

But, D. Y.-K., Lai, C.-L., & Yuen, M.-F. (2008). Natural history of hepatitis-related hepatocellular carcinoma. *World journal of gastroenterology: WJG, 14*(11), 1652.

Cassidy, A., Rogers, G., Peterson, J. J., Dwyer, J. T., Lin, H., & Jacques, P. F. (2015). Higher dietary anthocyanin and flavonol intakes are associated with anti-inflammatory effects in a population of US adults. *The American journal of clinical nutrition, 102*(1), 172-181.

Castillejo, A., Rothman, N., Murta-Nascimento, C., Malats, N., García-Closas, M., Gómez-Martínez, A., . . . García-Closas, R. (2009). TGFB1 and TGFBR1 polymorphic variants in relationship to bladder cancer risk and prognosis. *International journal of cancer, 124*(3), 608-613.

Čengić, T., Trkulja, V., Pavelić, S. K., Ratkaj, I., Markova-Car, E., Mikolaučić, M., & Kolundžić, R. (2015). Association of TGFB1 29C/T and IL-6-572G/C polymorphisms with developmental hip dysplasia: a case–control study in adults with severe osteoarthritis. *International orthopaedics, 39*(4), 793-798.

Cervello, M., Bachvarov, D., Lampiasi, N., Cusimano, A., Azzolina, A., McCubrey, J. A., & Montalto, G. (2012). Molecular mechanisms of sorafenib action in liver cancer cells. *Cell cycle, 11*(15), 2843-2855.

Chen, S. L., & Morgan, T. R. (2006). The natural history of hepatitis C virus (HCV) infection. *International journal of medical sciences, 3*(2), 47.

Cheng, A.-L., Kang, Y.-K., Chen, Z., Tsao, C.-J., Qin, S., Kim, J. S., . . . Yang, T.-S. (2009). Efficacy and safety of sorafenib in patients in the Asia-Pacific region

with advanced hepatocellular carcinoma: a phase III randomised, double-blind, placebo-controlled trial. *The lancet oncology*, *10*(1), 25-34.

Cillo, U., Bassanello, M., Vitale, A., Grigoletto, F. A., Burra, P., Fagiuoli, S., . . . Brolese, A. (2004). The critical issue of hepatocellular carcinoma prognostic classification: which is the best tool available? *Journal of hepatology*, *40*(1), 124-131.

Costanza, B., Umelo, I. A., Bellier, J., Castronovo, V., & Turtoi, A. (2017). Stromal modulators of TGF-β in cancer. *Journal of clinical medicine*, *6*(1), 7.

Dash, S., Aydin, Y., Widmer, K. E., & Nayak, L. (2020). Hepatocellular carcinoma mechanisms associated with chronic HCV infection and the impact of direct-acting antiviral treatment. *Journal of hepatocellular carcinoma*, *7*, 45.

Deber, C. M., Brodsky, B., & Rath, A. (2010). Proline residues in proteins. *eLS*.

DeMichele, A., Martin, A.-M., Mick, R., Gor, P., Wray, L., Klein-Cabral, M., . . . Weber, B. (2003). Interleukin-6− 174G→ C polymorphism is associated with improved outcome in high-risk breast cancer. *Cancer research*, *63*(22), 8051-8056.

Desai, A. G., Qazi, G. N., Ganju, R. K., El-Tamer, M., Singh, J., Saxena, A. K., . . . Bhat, H. K. (2008). Medicinal plants and cancer chemoprevention. *Current drug metabolism*, *9*(7), 581-591.

Ewart-Toland, A., Chan, J. M., Yuan, J., Balmain, A., & Ma, J. (2004). A gain of function TGFB1 polymorphism may be associated with late stage prostate cancer. *Cancer Epidemiology and Prevention Biomarkers*, *13*(5), 759-764.

Ezzoukhry, Z., Louandre, C., Trécherel, E., Godin, C., Chauffert, B., Dupont, S., . . . Galmiche, A. (2012). EGFR activation is a potential determinant of primary resistance of hepatocellular carcinoma cells to sorafenib. *International journal of cancer*, *131*(12), 2961-2969.

Facciorusso, A. (2018). Drug-eluting beads transarterial chemoembolization for hepatocellular carcinoma: current state of the art. *World journal of gastroenterology*, *24*(2), 161.

Faivre, S., de Gramont, A., & Raymond, E. (2016). Learning from 7 years of experience with sorafenib in advanced HCC: sorafenib better than sorafenib? *Targeted oncology*, *11*(4), 565-567.

Fleming, I. D. (2001). AJCC/TNM cancer staging, present and future: Wiley Online Library.

Fuku, N., Mori, S., Murakami, H., Gando, Y., Zhou, H., Ito, H., . . . Miyachi, M. (2012). Association of 29C> T polymorphism in the transforming growth factor-β1 gene with lean body mass in community-dwelling Japanese population. *Geriatrics & gerontology international*, *12*(2), 292-297.

García, E. R., Gutierrez, E. A., Melo, F. C. S. A. d., Novaes, R. D., & Gonçalves, R. V. (2018). Flavonoids effects on hepatocellular carcinoma in murine models: A systematic review. *Evidence-Based Complementary and Alternative Medicine*, *2018*.

Gaussin, A., Modlich, U., Bauche, C., Niederländer, N., Schambach, A., Duros, C., . . . Mermod, N. (2012). CTF/NF1 transcription factors act as potent genetic insulators for integrating gene transfer vectors. *Gene therapy, 19*(1), 15-24.

Gautam, K. A., Pooja, S., Sankhwar, S. N., Sankhwar, P. L., Goel, A., & Rajender, S. (2015). c. 29C> T polymorphism in the transforming growth factor-β1 (TGFB1) gene correlates with increased risk of urinary bladder cancer. *Cytokine, 75*(2), 344-348.

Gelinas, A. D., Davies, D. R., Edwards, T. E., Rohloff, J. C., Carter, J. D., Zhang, C., . . . Nakaishi, Y. (2014). Crystal structure of interleukin-6 in complex with a modified nucleic acid ligand. *Journal of Biological Chemistry, 289*(12), 8720-8734.

Gentry, L. E., Lioubin, M., Purchio, A. F., & Marquardt, H. (1988). Molecular events in the processing of recombinant type 1 pre-pro-transforming growth factor beta to the mature polypeptide. *Molecular and cellular biology, 8*(10), 4162-4168.

Giannitrapani, L., Soresi, M., Balasus, D., Licata, A., & Montalto, G. (2013). Genetic association of interleukin-6 polymorphism (-174 G/C) with chronic liver diseases and hepatocellular carcinoma. *World journal of gastroenterology: WJG, 19*(16), 2449.

Goossens, N., & Hoshida, Y. (2015). Hepatitis C virus-induced hepatocellular carcinoma. *Clinical and molecular hepatology, 21*(2), 105.

Goumans, M.-J., Liu, Z., & Ten Dijke, P. (2009). TGF-β signaling in vascular biology and dysfunction. *Cell research, 19*(1), 116-127.

Grabe, N. (2002). AliBaba2: context specific identification of transcription factor binding sites. *In silico biology, 2*(1), S1-S15.

Greenwell, M., & Rahman, P. (2015). Medicinal plants: their use in anticancer treatment. *International journal of pharmaceutical sciences and research, 6*(10), 4103.

Gronostajski, R. M. (2000). Roles of the NFI/CTF gene family in transcription and development. *Gene, 249*(1-2), 31-45.

Guerrero, R. B., & Roberts, L. R. (2005). The role of hepatitis B virus integrations in the pathogenesis of human hepatocellular carcinoma. *Journal of hepatology, 42*(5), 760-777.

Hassan, B. (2019). Plants and Cancer Treatment. *Med. Plants Use Prev. Treat. Dis*, 1-11.

Hefler, L. A., Grimm, C., Lantzsch, T., Lampe, D., Leodolter, S., Koelbl, H., . . . Tempfer, C. (2005). Interleukin-1 and interleukin-6 gene polymorphisms and the risk of breast cancer in caucasian women. *Clinical cancer research, 11*(16), 5718-5721.

Heidari, Z., Mahmoudzadeh-Sagheb, H., Hashemi, M., & Rigi-Ladiz, M. A. (2014). Quantitative Analysis of Interdental Gingiva in Patients With Chronic Periodontitis and Transforming Growth Factor-β1 29C/T Gene Polymorphisms. *Journal of periodontology, 85*(2), 281-289.

Hoshida, Y., Fuchs, B. C., Bardeesy, N., Baumert, T. F., & Chung, R. T. (2014). Pathogenesis and prevention of hepatitis C virus-induced hepatocellular carcinoma. *Journal of hepatology, 61*(1), S79-S90.

Hsu, C.-H., Shen, Y.-C., Shao, Y.-Y., Hsu, C., & Cheng, A.-L. (2014). Sorafenib in advanced hepatocellular carcinoma: current status and future perspectives. *Journal of hepatocellular carcinoma, 1*, 85.

Hui, L., Zatloukal, K., Scheuch, H., Stepniak, E., & Wagner, E. F. (2008). Proliferation of human HCC cells and chemically induced mouse liver cancers requires JNK1-dependent p21 downregulation. *The Journal of clinical investigation, 118*(12), 3943-3953.

Huo, T.-I., Lin, H.-C., Hsia, C.-Y., Wu, J.-C., Lee, P.-C., Chi, C.-W., & Lee, S.-D. (2007). The model for end-stage liver disease based cancer staging systems are better prognostic models for hepatocellular carcinoma: a prospective sequential survey. *American Journal of Gastroenterology, 102*(9), 1920-1930.

Informatics, P. (2021). ChemDraw.

Iqbal, J., Abbasi, B. A., Mahmood, T., Kanwal, S., Ali, B., Shah, S. A., & Khalil, A. T. (2017). Plant-derived anticancer agents: A green anticancer approach. *Asian Pacific Journal of Tropical Biomedicine, 7*(12), 1129-1150.

Ito, N., Kawata, S., Tamura, S., Takaishi, K., Yabuuchi, I., Matsuda, Y., . . . Tarui, S. (1990). Expression of Transforming Growth Factor-β1 mRNA in Human Hepatocellular Carcinoma. *Japanese journal of cancer research, 81*(12), 1202-1205.

Jones, S. A., Scheller, J., & Rose-John, S. (2011). Therapeutic strategies for the clinical blockade of IL-6/gp130 signaling. *The Journal of clinical investigation, 121*(9), 3375-3383.

Kao, C.-F., Chen, S.-Y., Chen, J.-Y., & Lee, Y.-H. W. (2004). Modulation of p53 transcription regulatory activity and post-translational modification by hepatitis C virus core protein. *Oncogene, 23*(14), 2472-2483.

Karvonen, M. K., Pesonen, U., Koulu, M., Niskanen, L., Laakso, M., Rissanen, A., . . . Uusitupa, M. I. (1998). Association of a leucine (7)-to-proline (7) polymorphism in the signal peptide of neuropeptide Y with high serum cholesterol and LDL cholesterol levels. *Nature medicine, 4*(12), 1434-1437.

Kew, M. C. (2015). Whither α-FP in the diagnosis of hepatocellular carcinoma? *Journal of hepatocellular carcinoma, 2*, 119.

Khalil, N. (1999). TGF-β: from latent to active. *Microbes and infection, 1*(15), 1255-1263.

Kim, S., Chen, J., Cheng, T., Gindulyte, A., He, J., He, S., . . . Yu, B. (2021). PubChem in 2021: new data content and improved web interfaces. *Nucleic Acids Research, 49*(D1), D1388-D1395.

Koike, K. (2009). Steatosis, liver injury, and hepatocarcinogenesis in hepatitis C viral infection. *Journal of gastroenterology, 44*(19), 82-88.

Landi, S., Moreno, V., Gioia-Patricola, L., Guino, E., Navarro, M., de Oca, J., . . . Canzian, F. (2003). Association of common polymorphisms in inflammatory genes interleukin (IL) 6, IL8, tumor necrosis factor α, NFKB1, and peroxisome

proliferator-activated receptor γ with colorectal cancer. *Cancer research, 63*(13), 3560-3566.

Lemon, S. M., & McGivern, D. R. (2012). Is hepatitis C virus carcinogenic? *Gastroenterology, 142*(6), 1274-1278.

Lencioni, R., de Baere, T., Soulen, M. C., Rilling, W. S., & Geschwind, J. F. H. (2016). Lipiodol transarterial chemoembolization for hepatocellular carcinoma: a systematic review of efficacy and safety data. *Hepatology, 64*(1), 106-116.

Lencioni, R., Kudo, M., Ye, S. L., Bronowicki, J. P., Chen, X. P., Dagher, L., . . . Papandreou, C. (2014). GIDEON (Global Investigation of therapeutic DE cisions in hepatocellular carcinoma and Of its treatment with sorafeNib): second interim analysis. *International journal of clinical practice, 68*(5), 609-617.

Leyva-López, N., Gutierrez-Grijalva, E. P., Ambriz-Perez, D. L., & Heredia, J. B. (2016). Flavonoids as cytokine modulators: a possible therapy for inflammation-related diseases. *International journal of molecular sciences, 17*(6), 921.

Li, K. K., Shen, S. S., Deng, X., Shiu, H. T., Siu, W. S., Leung, P. C., . . . Cheng, B. H. (2018). Dihydrofisetin exerts its anti-inflammatory effects associated with suppressing ERK/p38 MAPK and heme oxygenase-1 activation in lipopolysaccharide-stimulated RAW 264.7 macrophages and carrageenan-induced mice paw edema. *International immunopharmacology, 54*, 366-374.

Li, W., Wu, G., & Wan, Y. (2007). The dual effects of Cdh1/APC in myogenesis. *The FASEB Journal, 21*(13), 3606-3617.

Liao, C.-Y., Lee, C.-C., Tsai, C.-c., Hsueh, C.-W., Wang, C.-C., Chen, I., . . . Su, K.-J. (2015). Novel investigations of flavonoids as chemopreventive agents for hepatocellular carcinoma. *BioMed research international, 2015*.

Lin, W., Tsai, W. L., Shao, R. X., Wu, G., Peng, L. F., Barlow, L. L., . . . Jang, J. Y. (2010). Hepatitis C virus regulates transforming growth factor β1 production through the generation of reactive oxygen species in a nuclear factor κB–dependent manner. *Gastroenterology, 138*(7), 2509-2518. e2501.

Liu, P.-H., Hsu, C.-Y., Hsia, C.-Y., Lee, Y.-H., Su, C.-W., Huang, Y.-H., . . . Huo, T.-I. (2016). Prognosis of hepatocellular carcinoma: assessment of eleven staging systems. *Journal of hepatology, 64*(3), 601-608.

Liu, S., Chen, S., & Zeng, J. (2018). TGF-β signaling: A complex role in tumorigenesis. *Molecular medicine reports, 17*(1), 699-704.

Liu, X.-F., Long, H.-J., Miao, X.-Y., Liu, G.-L., & Yao, H.-L. (2017). Fisetin inhibits liver cancer growth in a mouse model: Relation to dopamine receptor. *Oncology reports, 38*(1), 53-62.

Liu, Y., Gao, S.-j., Du, B.-x., & Wang, J.-j. (2014). Association of IL-6 polymorphisms with hepatocellular carcinoma risk: evidences from a meta-analysis. *Tumor Biology, 35*(4), 3551-3561.

Liu, Y., Grimm, M., Dai, W.-t., Hou, M.-c., Xiao, Z.-X., & Cao, Y. (2020). CB-Dock: a web server for cavity detection-guided protein–ligand blind docking. *Acta Pharmacologica Sinica, 41*(1), 138-144.

Llovet, J. M., Brú, C., & Bruix, J. (1999). *Prognosis of hepatocellular carcinoma: the BCLC staging classification.* Paper presented at the Seminars in liver disease.

Lu, D. S., Raman, S. S., Limanond, P., Aziz, D., Economou, J., Busuttil, R., & Sayre, J. (2003). Influence of large peritumoral vessels on outcome of radiofrequency ablation of liver tumors. *Journal of vascular and interventional radiology, 14*(10), 1267-1274.

Lu, W., Jin, X.-L., Yang, C., Du, P., Jiang, F.-Q., Ma, J.-P., . . . Zhang, Z. (2017). Comparison of efficacy between TACE combined with apatinib and TACE alone in the treatment of intermediate and advanced hepatocellular carcinoma: A single-center randomized controlled trial. *Cancer biology & therapy, 18*(6), 433-438.

Ma, W., Zha, Z., Chen, K., Chen, H., Wu, Y., Ma, J., . . . Yao, S. (2017). Genetic association study of common variants in TGFB1 and IL-6 with developmental dysplasia of the hip in Han Chinese population. *Scientific reports, 7*(1), 1-7.

Machida, K., Liu, J.-C., McNamara, G., Levine, A., Duan, L., & Lai, M. M. (2009). Hepatitis C virus causes uncoupling of mitotic checkpoint and chromosomal polyploidy through the Rb pathway. *Journal of virology, 83*(23), 12590-12600.

Malaguarnera, G., Giordano, M., Paladina, I., Berretta, M., Cappellani, A., & Malaguarnera, M. (2010). Serum markers of hepatocellular carcinoma. *Digestive diseases and sciences, 55*(10), 2744-2755.

Manfredini, R., Tenedini, E., Siena, M., Tagliafico, E., Montanari, M., Grande, A., . . . Gemelli, C. (2003). Development of an IL-6 antagonist peptide that induces apoptosis in 7TD1 cells. *Peptides, 24*(8), 1207-1220.

Masjedi, A., Hashemi, V., Hojjat-Farsangi, M., Ghalamfarsa, G., Azizi, G., Yousefi, M., & Jadidi-Niaragh, F. (2018). The significant role of interleukin-6 and its signaling pathway in the immunopathogenesis and treatment of breast cancer. *Biomedicine & Pharmacotherapy, 108*, 1415-1424.

McElvaney, O. J., Curley, G. F., Rose-John, S., & McElvaney, N. G. (2021). Interleukin-6: obstacles to targeting a complex cytokine in critical illness. *The Lancet Respiratory Medicine*.

McGlynn, K. A., & London, W. T. (2005). Epidemiology and natural history of hepatocellular carcinoma. *Best practice & research Clinical gastroenterology, 19*(1), 3-23.

McGlynn, K. A., & London, W. T. (2011). The global epidemiology of hepatocellular carcinoma: present and future. *Clinics in liver disease, 15*(2), 223-243.

McGlynn, K. A., Petrick, J. L., & London, W. T. (2015). Global epidemiology of hepatocellular carcinoma: an emphasis on demographic and regional variability. *Clinics in liver disease, 19*(2), 223-238.

McMahon, B. J., Bulkow, L., Harpster, A., Snowball, M., Lanier, A., Sacco, F., . . . Williams, J. (2000). Screening for hepatocellular carcinoma in Alaska natives infected with chronic hepatitis B: a 16-year population-based study. *Hepatology, 32*(4), 842-846.

Mills, N. (2006). ChemDraw Ultra 10.0 CambridgeSoft, 100 CambridgePark Drive, Cambridge, MA 02140. www. cambridgesoft. com. Commercial Price:

1910fordownload, 2150 for CD-ROM; Academic Price: 710fordownload, 800 for CD-ROM: ACS Publications.

Mittal, S., & El-Serag, H. B. (2013). Epidemiology of HCC: consider the population. *Journal of clinical gastroenterology, 47*, S2.

Morgan, A. A., & Rubenstein, E. (2013). Proline: the distribution, frequency, positioning, and common functional roles of proline and polyproline sequences in the human proteome. *PloS one, 8*(1), e53785.

Nault, J. C., Mallet, M., Pilati, C., Calderaro, J., Bioulac-Sage, P., Laurent, C., . . . Zucman-Rossi, J. (2013). High frequency of telomerase reverse-transcriptase promoter somatic mutations in hepatocellular carcinoma and preneoplastic lesions. *Nature communications, 4*(1), 1-7.

Okuda, K. (2002). Hepatocellular carcinoma—history, current status and perspectives. *Digestive and Liver Disease, 34*(9), 613-616.

Paonessa, G., Graziani, R., De Serio, A., Savino, R., Ciapponi, L., Lahm, A., . . . Ciliberto, G. (1995). Two distinct and independent sites on IL-6 trigger gp 130 dimer formation and signalling. *The EMBO Journal, 14*(9), 1942-1951.

Peng, X., Shi, J., Sun, W., Ruan, X., Guo, Y., Zhao, L., . . . Li, B. (2018). Genetic polymorphisms of IL-6 promoter in cancer susceptibility and prognosis: a meta-analysis. *Oncotarget, 9*(15), 12351.

Perlemuter, G., Sabile, A., Letteron, P., Vona, G., Topilco, A., Chrétien, Y., . . . Barba, G. (2002). Hepatitis C virus core protein inhibits microsomal triglyceride transfer protein activity and very low density lipoprotein secretion: a model of viral-related steatosis. *The FASEB journal, 16*(2), 185-194.

Pooja, S., Francis, A., Rajender, S., Tamang, R., Rajkumar, R., Saini, K. S., . . . Rao, D. R. (2013). Strong impact of TGF-β1 gene polymorphisms on breast cancer risk in Indian women: a case-control and population-based study. *PLoS One, 8*(10), e75979.

Ramsey, W. H., & Wu, G. Y. (1995). Hepatocellular carcinoma: update on diagnosis and treatment. *Digestive Diseases, 13*(2), 81-91.

Rangwala, S. H., Kuznetsov, A., Ananiev, V., Asztalos, A., Borodin, E., Evgeniev, V., . . . Rudnev, D. (2021). Accessing NCBI data using the NCBI Sequence Viewer and Genome Data Viewer (GDV). *Genome Research, 31*(1), 159-169.

Ren, W., Qiao, Z., Wang, H., Zhu, L., & Zhang, L. (2003). Flavonoids: promising anticancer agents. *Medicinal research reviews, 23*(4), 519-534.

Sambrook, J., & Russell, D. W. (2006). Purification of nucleic acids by extraction with phenol: chloroform. *Cold Spring Harbor Protocols, 2006*(1), pdb. prot4455.

Sangiovanni, A., Manini, M. A., Iavarone, M., Romeo, R., Forzenigo, L. V., Fraquelli, M., . . . Rumi, M. G. (2010). The diagnostic and economic impact of contrast imaging techniques in the diagnosis of small hepatocellular carcinoma in cirrhosis. *Gut, 59*(5), 638-644.

Santoro, C., Mermod, N., Andrews, P. C., & Tjian, R. (1988). A family of human CCAAT-box-binding proteins active in transcription and DNA replication: cloning and expression of multiple cDNAs. *Nature, 334*(6179), 218-224.

Schett, G. (2018). Physiological effects of modulating the interleukin-6 axis. *Rheumatology*, *57*(suppl_2), ii43-ii50.

Schmidt-Arras, D., & Rose-John, S. (2016). IL-6 pathway in the liver: From physiopathology to therapy. *Journal of hepatology*, *64*(6), 1403-1415.

Schrödinger, L. (2015). The PyMOL molecular graphics system, version 1.8: November.

Schulze-Krebs, A., Preimel, D., Popov, Y., Bartenschlager, R., Lohmann, V., Pinzani, M., & Schuppan, D. (2005). Hepatitis C virus-replicating hepatocytes induce fibrogenic activation of hepatic stellate cells. *Gastroenterology*, *129*(1), 246-258.

Selimovic, D., El-Khattouti, A., Ghozlan, H., Haikel, Y., Abdelkader, O., & Hassan, M. (2012). Hepatitis C virus-related hepatocellular carcinoma: An insight into molecular mechanisms and therapeutic strategies. *World journal of hepatology*, *4*(12), 342.

Silver, J., & Hunter, C. (2010). gp130 at the nexus of inflammation, autoimmunity, and cancer. *Journal of leukocyte biology*, *88*(6), 1145-1156.

Simpson, R. J., Hammacher, A., Smith, D. K., Matthews, J. M., & Ward, L. D. (1997). Interleukin-6: Structure-function relationships. *Protein Science*, *6*(5), 929-955.

Singh, P. K., Chandra, G., Bogra, J., Gupta, R., Kumar, V., Jain, A., . . . Ahmad, M. K. (2015). Association of interleukin-6 genetic polymorphisms with risk of OSCC in Indian population. *Meta Gene*, *4*, 142-151.

Slattery, M. L., Curtin, K., Baumgartner, R., Sweeney, C., Byers, T., Giuliano, A. R., . . . Wolff, R. R. (2007). IL-6, aspirin, nonsteroidal anti-inflammatory drugs, and breast cancer risk in women living in the southwestern United States. *Cancer Epidemiology and Prevention Biomarkers*, *16*(4), 747-755.

Slattery, M. L., Wolff, R. K., Herrick, J. S., Caan, B. J., & Potter, J. D. (2007). IL-6 genotypes and colon and rectal cancer. *Cancer Causes & Control*, *18*(10), 1095-1105.

Somers, W., Stahl, M., & Seehra, J. S. (1997). 1.9 Å crystal structure of interleukin 6: implications for a novel mode of receptor dimerization and signaling. *The EMBO journal*, *16*(5), 989-997.

Sung, Y. K., Hwang, S. Y., Park, M. K., Farooq, M., Han, I. S., Bae, H. I., . . . Kim, M. (2003). Glypican-3 is overexpressed in human hepatocellular carcinoma. *Cancer science*, *94*(3), 259-262.

Swift, M. L. (1997). GraphPad prism, data analysis, and scientific graphing. *Journal of chemical information and computer sciences*, *37*(2), 411-412.

Tanaka, H. (2020). Current role of ultrasound in the diagnosis of hepatocellular carcinoma. *Journal of Medical Ultrasonics*, *47*(2), 239-255.

Tanaka, N., Moriya, K., Kiyosawa, K., Koike, K., Gonzalez, F. J., & Aoyama, T. (2008). PPARα activation is essential for HCV core protein–induced hepatic steatosis and hepatocellular carcinoma in mice. *The Journal of clinical investigation*, *118*(2), 683-694.

Tarocchi, M., Polvani, S., Marroncini, G., & Galli, A. (2014). Molecular mechanism of hepatitis B virus-induced hepatocarcinogenesis. *World Journal of Gastroenterology: WJG, 20*(33), 11630.

Trugilo, K. P., Cebinelli, G. C. M., Berti, F. C. B., Okuyama, N. C. M., Cezar-dos-Santos, F., Sena, M. M., . . . de Oliveira, K. B. (2019). Polymorphisms in the TGFB1 signal peptide influence human papillomavirus infection and development of cervical lesions. *Medical microbiology and immunology, 208*(1), 49-58.

Tu, Y., Ortiz, A., Gollub, R. L., Cao, J., Gerber, J., Lang, C., . . . Chan, S.-T. (2019). Multivariate resting-state functional connectivity predicts responses to real and sham acupuncture treatment in chronic low back pain. *NeuroImage: Clinical, 23*, 101885.

Tunissiolli, N. M., Castanhole-Nunes, M. M. U., Biselli-Chicote, P. M., Pavarino, É. C., & da Silva, R. F. (2017). Hepatocellular carcinoma: a comprehensive review of biomarkers, clinical aspects, and therapy. *Asian Pacific journal of cancer prevention: APJCP, 18*(4), 863.

Verner, K., & Schatz, G. (1988). Protein translocation across membranes. *Science, 241*(4871), 1307-1313.

Veverka, V., Baker, T., Redpath, N. T., Carrington, B., Muskett, F. W., Taylor, R. J., . . . Carr, M. D. (2012). Conservation of functional sites on interleukin-6 and implications for evolution of signaling complex assembly and therapeutic intervention. *Journal of Biological Chemistry, 287*(47), 40043-40050.

Volk, M. L., Hernandez, J. C., Su, G. L., Lok, A. S., & Marrero, J. A. (2007). Risk factors for hepatocellular carcinoma may impair the performance of biomarkers: A comparison of AFP, DCP, and AFP-L3 1. *Cancer Biomarkers, 3*(2), 79-87.

Wallace, A. C., Laskowski, R. A., & Thornton, J. M. (1995). LIGPLOT: a program to generate schematic diagrams of protein-ligand interactions. *Protein engineering, design and selection, 8*(2), 127-134.

Wang, J.-H., Changchien, C.-S., Hu, T.-H., Lee, C.-M., Kee, K.-M., Lin, C.-Y., . . . Lu, S.-N. (2008). The efficacy of treatment schedules according to Barcelona Clinic Liver Cancer staging for hepatocellular carcinoma–Survival analysis of 3892 patients. *European journal of cancer, 44*(7), 1000-1006.

Wang, S., Ding, Z., Tang, J., & Li, G. (2018). The association of interleukin-6 gene polymorphism and risk of colorectal cancer in Chinese patients. *TRANSLATIONAL CANCER RESEARCH, 7*(2), 401-410.

Wang, Y., Deng, B., Tang, W., Liu, T., & Shen, X. (2013). TGF-β1 secreted by hepatocellular carcinoma induces the expression of the Foxp3 gene and suppresses antitumor immunity in the tumor microenvironment. *Digestive diseases and sciences, 58*(6), 1644-1652.

Weng, Z., Patel, A. B., Panagiotidou, S., & Theoharides, T. C. (2015). The novel flavone tetramethoxyluteolin is a potent inhibitor of human mast cells. *Journal of Allergy and Clinical Immunology, 135*(4), 1044-1052. e1045.

Woo, P., & Humphries, S. E. (2013). IL-6 polymorphisms: a useful genetic tool for inflammation research? *The Journal of clinical investigation, 123*(4), 1413-1414.

Wormald, S., Zhang, J.-G., Krebs, D. L., Mielke, L. A., Silver, J., Alexander, W. S., . . . Hilton, D. J. (2006). The comparative roles of SOCS1 and SOCS3 in the inhibition and desensitization of cytokine signaling. *Journal of Biological Chemistry*.

Wypasek, E., Undas, A., Sniezek-Maciejewska, M., Kapelak, B., Plicner, D., Stępień, E., & Sadowski, J. (2010). The increased plasma C-reactive protein and interleukin-6 levels in patients undergoing coronary artery bypass grafting surgery are associated with the interleukin-6−174G > C gene polymorphism. *Annals of Clinical Biochemistry, 47*(4), 343-349. doi: 10.1258/acb.2010.090305

Xie, L., Law, B. K., Chytil, A. M., Brown, K. A., Aakre, M. E., & Moses, H. L. (2004). Activation of the Erk pathway is required for TGF-β1-induced EMT in vitro. *Neoplasia, 6*(5), 603-610.

Xu, G.-Y., Yu, H.-A., Hong, J., Stahl, M., McDonagh, T., Kay, L. E., & Cumming, D. A. (1997). Solution structure of recombinant human interleukin-6. *Journal of molecular biology, 268*(2), 468-481.

Yang, J. D., Dai, J., Singal, A. G., Gopal, P., Addissie, B. D., Nguyen, M. H., . . . Harnois, D. M. (2017). Improved performance of serum alpha-fetoprotein for hepatocellular carcinoma diagnosis in HCV cirrhosis with normal alanine transaminase. *Cancer Epidemiology and Prevention Biomarkers, 26*(7), 1085-1092.

Yates, A. D., Achuthan, P., Akanni, W., Allen, J., Allen, J., Alvarez-Jarreta, J., . . . Flicek, P. (2019). *Ensembl 2020.* 48. Retrieved from https://doi.org/10.1093/nar/gkz966 (D1)

Yeh, K.-Y., Li, Y.-Y., Hsieh, L.-L., Chen, J.-R., & Tang, R.-P. (2010). The− 174 G/C polymorphism in interleukin-6 (IL-6) promoter region is associated with serum IL-6 and carcinoembryonic antigen levels in patients with colorectal cancers in Taiwan. *Journal of clinical immunology, 30*(1), 53-59.

Yu, Y., Wang, W., Zhai, S., Dang, S., & Sun, M. (2012). IL-6 gene polymorphisms and susceptibility to colorectal cancer: a meta-analysis and review. *Molecular biology reports, 39*(8), 8457-8463.

Yun, E. H., Kang, Y. H., Lim, M. K., Oh, J.-K., & Son, J. M. (2010). The role of social support and social networks in smoking behavior among middle and older aged people in rural areas of South Korea: a cross-sectional study. *BMC Public Health, 10*(1), 1-8.

Zhang, Y., Wang, J., Zhou, S., Xie, Z., Wang, C., Gao, Y., . . . Li, Q. (2019). Flavones hydroxylated at 5, 7, 3′ and 4′ ameliorate skin fibrosis via inhibiting activin receptor-like kinase 5 kinase activity. *Cell death & disease, 10*(2), 1-13.

Zhang, Y., Zhou, S., Cheng, X., Yi, B., Shan, S., Wang, J., & Li, Q. (2016). Baicalein attenuates hypertrophic scar formation via inhibition of the transforming growth factor-β/Smad2/3 signalling pathway. *British Journal of Dermatology, 174*(1), 120-130.

Zhao, B., Xu, S., Dong, X., Lu, C., & Springer, T. A. (2018). Prodomain–growth factor swapping in the structure of pro-TGF-β1. *Journal of Biological Chemistry, 293*(5), 1579-1589.

Zheng, X., Han, C., Shan, R., Zhang, H., Zheng, Z., Liu, Y., & Wang, A. (2015). Association of interleukin-6 polymorphisms with susceptibility to hepatocellular carcinoma. *International journal of clinical and experimental medicine, 8*(4), 6252.

Zhong, J.-H., Torzilli, G., Xing, H., Li, C., Han, J., Liang, L., . . . Shen, F. (2016). Controversies and evidence of hepatic resection for hepatocellular carcinoma. *BBA clinical, 6*, 125-130.