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Transcriptomic Analysis on Hepatocellular Carcinoma Model in Response to Safranal Treatment

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TRANSCRIPTOMIC ANALYSIS ON HEPATOCELLULAR CARCINOMA MODEL IN RESPONSE TO SAFRANAL TREATMENT

Badriya Mahmood Ahmed Baig

This thesis is submitted in partial fulfilment of the requirements for the degree of
Master of Science in Molecular Biology and Biotechnology

Under the Supervision of Professor Amr Amin

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Declaration of Original Work

I, Badriya Mahmood Ahmed Baig, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this thesis entitled "*Transcriptomic Analysis on Hepatocellular Carcinoma Model in Response to Saffron Treatment*", hereby, solemnly declare that this thesis is my own original research work that has been done and prepared by me under the supervision of Professor Amr Amin, in the College of Science at UAEU. This work has not previously been presented or published, or formed the basis for the award of any academic degree, diploma or a similar title at this or any other university. Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my thesis have been properly cited and acknowledged in accordance with appropriate academic conventions. I further declare that there is no potential conflict of interest with respect to the research, data collection, authorship, presentation and/or publication of this thesis.

Student's Signature: _____ Date: 23/4/2018

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Declaration of Original Work

I, Badriya Mahmood Ahmed Baig, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this thesis entitled "*Transcriptomic Analysis on Hepatocellular Carcinoma Model in Response to Saffron Treatment*", hereby, solemnly declare that this thesis is my own original research work that has been done and prepared by me under the supervision of Professor Amr Amin, in the College of Science at UAEU. This work has not previously been presented or published, or formed the basis for the award of any academic degree, diploma or a similar title at this or any other university. Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my thesis have been properly cited and acknowledged in accordance with appropriate academic conventions. I further declare that there is no potential conflict of interest with respect to the research, data collection, authorship, presentation and/or publication of this thesis.

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Abstract

Unsolved riddle of cancer remains a major global health concern particularly in hepatocellular carcinoma (HCC) which is among the leading causes of cancer-related death worldwide. Saffron (*Crocus sativus* L.) and its bioactive phytochemicals possess promising anti-cancer activities. One of these phytochemicals, safranal, has demonstrated potent anti-cancer activities against different types of cancers. Yet, its association with liver cancer remains under documented. This study investigated HCC response to safranal's treatment via transcriptomic approach. Safranal was found to be involved in mitochondrial dysfunction, induced oxidative stress and increased reactive oxygen species (ROS) production leading to cell death. Interestingly, safranal has seemingly interfered with mitogen-activated protein kinase (MAPK) signaling pathway that is responsible for cell growth, proliferation, differentiation and survival. Through ingenuity pathway analysis (IPA), 45 genes were associated with HCC inhibition. To further pinpoint specific genes, IPA re-analysis revealed 8 genes to be associated with decrease in tumor's incidence and angiogenesity. Taken together, this study unravel safranal's novel potential as a natural therapeutic agent and a much needed adjuvant against HCC.

Keywords: Hepatocellular carcinoma, RNA sequencing, Saffron, Safranal, Transcriptomic analysis.

Title and Abstract (in Arabic)

تحليل ترنسكربيتومي لنموذج سرطان الكبد طبقا لإستجابته لعلاج الزعفران

الملخص

يبقى مرض السرطان، المرض العضال، أحد أبرز المشكلات الصحية على مستوى العالم و لا سيما سرطان الكبد (HCC) و الذي يعتبر من ضمن الأسباب الرئيسية المرتبطة بوفيات السرطان عالمياَ. و تستفيد هذه الدراسة من خلال الزعفران (*Crocus sativus* L.) و ما يشمله من مواد كيميائية نباتية تحمل خصائص مضادة لبعض الأنشطة السرطانية. إن أحد هذه المواد الكيميائية (Safranal) يتمتع بخصائص مضادة عالية لمختلف أنواع السرطانات وعلى الرغم من ذلك تبقى علاقته بسرطان الكبد محل تساؤل و تستلزم المزيد من البحث و الدراسة. و تتناول الدراسة التي بين أيدينا استجابة سرطان الكبد (HCC) للعلاج بـ (Safranal) من منظور ترانسكبتومي (Transciptomic).

وجد أن الـ (Safranal) تسبب في إختلال الميتوكوندريا، و قد زاد أيضاَ من إنتاج أنواع الأكسجين التفاعلية (ROS) مما جعله يصل إلى مرحلة يصبح فيها موت الخلية إلزامياَ. و قد تتدخل (Safranal) أيضا بصورة مدهشة بـ ألية (MAPK) و هي المسؤولة عن النمو، تكاثر، تمايز الخلوي و بقاء الخلية على قيد الحياة.

و عند إجراء تحليل (IPA) ، عُرف أن 45 من الجينات الوراثية تمت بصلة لإحباط سرطان الكبد (HCC)، وللمزيد من الدقة تم إعادة إجراء تحليل (IPA) للجينات السابقة، مما ساعد على إيجاد 8 من الجينات الوراثية التي لها علاقة بتقليل نسب حدوث السرطان و نمو الأوعية الدموية الجديدة. و بهذا، فإن هذه الدراسة تقدم هذه الخصائص الفريدة للـ (Safranal) كمنتج علاجي طبيعي و مادة مساعدة ضد سرطان الكبد (HCC).

مفاهيم البحث الرئيسية: سرطان الكبد، تقنية تسلسل الحمض النووي الرايبوزي، الزعفران، التحليل الترنسكربيتومي.

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My deepest gratitude goes to my childhood friend, Asma Al-Hammadi, who have witnessed my nervous breakdowns and my joyful moments throughout my journey.

Last but not least, my everlasting gratitude goes to my beloved parents. No words can acknowledge the sacrifices they have made for my sake and no words would be enough to convey my appreciation. I also must mention my sisters and brother for providing me with strength to persist through the hardships. Truthfully, I am blessed for being born into a loving family.

Dedication

To my soul, beloved parents, family and close friends.

To the unfading memory.

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List of Abbreviations

2D	Two Dimensional
ACOX1	Acyl-CoA Oxidase 1
BiNGO	Biological Networks Gene Ontology
BME	β-Mercaptoethanol
CAA	Cholangiocarcinoma
CAGE	Cap Analysis Gene Expression
CDH1	E-cadherin
cDNA	Complementary DNA
CTNNB1	Catenin Beta 1
DEGs	Differential Expressed Genes
DEN	Diethylnitrosamine
DIC	Disseminated Intravascular Coagulation
ER	Endoplasmic Reticulum
EST	Expressed Sequence Tag
FDA	Food and Drug Administration
FDR	False Discovery Rate
GO	Gene Ontology
HBV	Hepatitis B Virus
HCC	Hepatocellular Carcinoma
HCV	Hepatitis C Virus
HDV	Hepatitis D Virus
IPA	Ingenuity Pathway Analysis
MAPK	Mitogen-Activated Protein Kinase

mROS	Mitochondrial Reactive Oxygen Species
NAFLD	Nonalcoholic Fatty Liver Disease
NASH	Nonalcoholic Steatohepatitis
P450s	Cytochromes P450
PCA	Principle Component Analysis
PDGFR-β	Platelet-Derived Growth Factor Receptor β
PEI	Percutaneous Ethanol Injection
qPCR	Quantitative Polymerase Chain Reaction
RFA	Radiofrequency Ablation
RGD	Rat Genomic Database
RIN	RNA Integrity Number
RNA-seq	RNA Sequencing
ROS	Reactive Oxygen Species
RTK	Receptor Tyrosine Kinase
SAGE	Serial Analysis of Gene Expression
SERPINC1	Serpin family C member 1
STRING	Search Tool for The Retrieval of Interacting Genes
TACE	Trans-Arterial Chemoembolization
VEGFRs	Vascular Endothelial Growth Factor Receptors
VLCFA	Very Long Chain Fatty Acids

Chapter 1: Introduction

1.1 Cancer

Cancer riddle remains unresolved and stubbornly lingers as major global health concern. Cancer is a disease that is characterized by uncontrolled growth and division of genetically unstable cells. The accumulation of genomic alterations varies among different subpopulations of cells and accounts for the heterogeneity of cancer (Shen, 2011; Ferguson et al., 2015). The terms tumor and cancer may often be used interchangeably, however, that is not always the case. Tumor refers to the abnormal growth of mass and might be either benign or malignant growth. A benign tumor is an abnormal growth confined to its original place and it can be removed surgically. A malignant tumor is able to invade surrounding tissues and spread out to distant body sites by means of the circulatory or lymphatic systems hence referred as metastasis process. Only malignant tumors are denoted as cancer reinforcing the fact of their ability to metastasize (Cooper, 2000).

Cancer cells acquire distinct 6 biological features from their normal counterparts. These biological features were first introduced in 2000 and were defined as hallmarks of cancer consist of sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis and activating invasion and metastasis. In 2011, additional 4 hallmarks of cancer were introduced which were deregulating cellular energetics, avoiding immune destruction, genomic instability and mutation and tumor promoting inflammation (Hanahan and Weinberg, 2011).

Cancer is the second leading cause of death worldwide accounting for 8.8 million deaths in 2015; that is 1 in every 6 deaths is due to cancer (World Health Organization, 2018). Top cancer mortality were attributed to lung cancer by 1.69 million deaths, liver cancer by 788000 deaths, colorectal cancer by 774000 deaths, stomach cancer by 754000 deaths and breast cancer by 571000 deaths. It is estimated that in the next two decades, the number of new cases of cancer could mark a 70% increase revealing an overwhelming cancer expansion.

According to Authority of Health of United Arab Emirates in Abu Dhabi, cancer is the third leading cause of death accounting for total 16% deaths that comes after road trauma injuries and cardiovascular diseases (Health Authority of Abu Dhabi, 2018). Records of around 427 death cases were documented due to cancer in 2015 with 56% male and 44% female. Top cancer mortality were attributed to lung cancer, colorectal cancer, liver cancer, leukaemia and pancreas cancer in men.

There are almost 200 types of cancer, their classification depends on the site of origin or the cell type originated from. Cancer is mostly classified into three large groups. Carcinomas are malignancies of epithelial cells and are considered the most common of human cancers such as squamous cell carcinoma, adenocarcinoma, melanoma and basal cell carcinoma. Sarcomas are solid tumors of connective tissues, such as muscle, bone, cartilage, and fibrous tissue and are considered rare in humans. Lastly, leukemias and lymphomas are malignancies rose from the blood cells and immune system cells, respectively (Cooper, 2000; Kirkham and Shepherd, 2001; Song et al., 2015).

1.1.1 Liver cancer

Liver is one of the vital organs that might become susceptible to cancer development. Liver importance rise from its wide range of functions such as metabolism, digestion, and detoxification. It is the only known human internal organ that is capable of regeneration (Laursen, 2014). Liver cancer is found to be fairly problematic to treat, and quite lethal in case of late diagnosis. In fact, thanks to its poor diagnosis, liver cancer is now the second leading cause of global cancer-related death with more prevalence in males rather than females (Torre et al., 2015). Primary liver cancer can normally be distinguished into several different forms of cancer including hepatocellular carcinoma (HCC), cholangiocarcinoma (CCA); also known as Bile-duct cancer, and hepatoblastoma. HCC is the most common primary liver cancer by approximant 78% of incidence. CCA accounts for 15% of incidence while hepatoblastoma and other various liver sarcomas and carcinomas accounts for 7% of incidence (Laursen, 2014).

1.1.2 Hepatocellular carcinoma

Due to the wide prevalence of HCC over all other forms of liver cancer, its aggressiveness, high rate malignancy and poor prognosis, HCC is the main contributor to the global health burden of liver cancer (Tanaka and Arii, 2012). HCC originate from adult hepatocytes upon genomic insult thereby dedifferentiate into precursor cells that eventually transform into HCC cells (Sia et al., 2017). HCC is rather strongly associated with numerous risk factors, including cirrhosis, chronic hepatitis B virus (HBV), chronic hepatitis C virus (HCV), hepatitis D virus (HDV) infections, alcohol abuse, nonalcoholic fatty liver disease (NAFLD) and Nonalcoholic steatohepatitis (NASH) (Ghouri et al., 2017).

Although the overall incidence of HCC varies based on etiology, ethnicity, gender, age, and geographic region, still that does not overpower the most common existence of fibrosis in HCC (Zhang and Friedman, 2012). In fact, around 90% of HCC occurrence take place under cirrhosis settings (Seitz and Stickel, 2006). Hepatotropic viruses, HBV, HCV and HDV, and their association with HCC development explains the higher rate of HCC in certain geographical region. For instance, 10-20% of Eastern Asia and Central Africa population are infected with HBV, the incidence of HCC is 30-120 in every 100,000 residents while in North and South America, and Europe, where less HBV infections exist, the incidence of HCC is only 5-10 in every 100,000 residents (Simon et al., 2012). There is a close relationship almost synergistic between the hepatotropic viruses; namely HBV and HCV infections, and alcohol intake to increase the possibility of HCC development. Yet, even without HBV or HCV infections, the effects of alcohol intake on HCC development was evident (Donato et al., 2002). NAFLD is chronic liver disease with wide spectrum stretching from simple hepatic steatosis to a more serious liver injury and inflammation recognized as NASH (Cholankeril et al., 2017). In reality, 15-20% of patients of NAFLD have developed NASH (Zezos and Renner, 2014). Exponential increase of HCC development due to NASH risk factor is highly anticipated as the prevalence of NASH is expected to escalate thanks to the growing epidemic of obesity and diabetes (Cholankeril et al., 2017).

1.1.3 Hepatocellular carcinoma diagnoses

In general, early diagnoses dramatically enhance the survival rate of cancer patients as the tumor mass is still restrained within the organ of origin (Kakushadze et al, 2017). This is ineligible to HCC as the lack of clear symptoms and coexistence of

HCC with other related diseases often hinders the process of distinguishing and detecting HCC. Only 25% of the total HCC patients were diagnosed within early stage of HCC, that is when a single node of HCC is smaller than 5 cm, or three nodes of HCC each smaller than 3 cm (Farinati et al., 2009; Tsuchiya et al., 2015). It is often recommended for patients with liver cirrhosis or chronic liver diseases to have regular screening tests to take advantage of early HCC diagnosis. Yet, gaining the benefit of early HCC diagnosis is only possible if the liver function is still compensated. Sadly, this does not work in patients having impaired liver function (Plentz and Malek, 2015).

1.1.4 Hepatocellular carcinoma treatment

Several factors should be taken into consideration when HCC therapeutic strategies in question. These factors are tumor size and location, extrahepatic spread, HCC stage, patient's performance status and underlying liver function (Crissien and Frenette, 2014). The standard curative treatments of HCC is represented by either surgical resection or liver transplantation. Yet, those surgical managements are only applicable for early stage HCC achieving 5 years' survival with approximate of 50% overall survival rates (Pang and Lam, 2014). Intermediate stage HCC might be eligible for resection therapy as their only option for curative treatment. Other therapeutic options can be locoregional therapy including percutaneous ethanol injection (PEI), trans-arterial chemoembolization (TACE) and radiofrequency ablation (RFA). Sorafenib is the first line treatment of advanced stage HCC which is also considered as palliative treatment. Whilst, terminal stage HCC receives symptomatic treatment as the only possible approach out there (Bruix and Sherman, 2011; Raza and Sood, 2014).

In advanced stage HCC, sorafenib (Nexavar) was the only chemotherapeutic regimen approved by Food and Drug Administration (FDA). Most recently

regorafenib (Stivarga) was another chemotherapeutic FDA approved as second line treatment to be administrated to patients who have been previously treated with sorafenib (Rimassa et al., 2017; Ziogas and Tsoulfas, 2017). Regorafenib is structurally similar to Sorafenib but with different biochemical profile (Mirantes et al., 2016). Sorafenib mechanism of action is expressed through its ability to inhibit multiple molecular targets mainly kinases involved in tumor proliferation and angiogenesis. These targets are serine-threonine kinases CRAF and BRAF involved in RAF/MEK/ERK signaling cascade, RET, FLT-3, tyrosine kinase activity of vascular endothelial growth factor receptors (VEGFRs) 1, 2 and 3 and platelet-derived growth factor receptor β (PDGFR-β) (Le Grazie et al., 2017). Despite the positive effect of sorafenib on advance stage HCC patients, the average overall survival is only prolonged by nearly 3 months (Llovet et al., 2008). Moreover, sorafenib exhibited some adverse effects such as skin-related toxicities, hypertension, proteinuria, diarrhea, and cytopenias. Other serious life-threatening complications have been witnessed as well including thromboembolism, bleeding, and bowel perforation. This has led 20-30% of patients to discontinue sorafenib's use (Crissien and Frenette, 2014). In addition, the initial responses might seems promising but over time loss of efficacy become evident which most probably due to drug resistance. Hence, new treatments of HCC are desperately needed.

1.2 Herbal medicine

Form the ancient times and throughout mankind civilization, herbal/traditional medicine was used on a regular basis to cure human diseases. Gradually but steadily, modern medical practices replaced herbal medicine although the later still holds a solid ground in many parts of the world especially the developing countries largely due to

its cultural acceptance and less adverse effects (Rana and Rana, 2014). The therapeutic value of a specific herb can be gained from the biologically active extracts or from the intact plant which display drug-like properties. Thus different methodologies of extraction, molecular characterization, standardization and some possible slight modification on the biological active components could enhance the drug-like activity. Within the last decade, improving extraction methods promoted herbal medicine among the scientific communities (Ansari et al., 2012). As matter of fact, 25% of worldwide drugs are actually derived from plants and 121 biological active extract are in use (Rates, 2001).

1.2.1 Saffron

Saffron is the dried stigma of the plant *Crocus sativus* L.; a member of Iridaceae family. The flower of *C. sativa* is light purple with three thread-like reddish-colored stigmas that are recognized as saffron spice and as a natural colorant. Saffron is known as “red gold” in the producing countries such as Iran, India and Greece. It has uphold its reputation as the most expensive cultivated spice herb in the world due to cultivation labor, harvesting, and handling since it has to be handpicked. About 70,000 flowers are needed obtain over 200000 dried stigmas to yield enough for 500 g pure saffron (Srivastava et al., 2010; Gohari et al., 2013; Hosseinzadeh, 2014).

Saffron has been traditionally used to treat cramps, asthma and bronchospasms, menstruation disorders, liver disease and pain. It was claimed to have a soothing and tonifying effect on the gastrointestinal tract. Also it has been used as stimulant and antidepressant (Schmidt et al., 2007). Recently, other medical properties have been associated with saffron including anti-tumor effects, anti-inflammatory effects and

cardioprotective effect (Abdullaev, 2002; Mehdizadeh et al., 2013; Amin and Hosseinzadeh, 2015).

Around 150 volatile and non-volatile compounds are present in saffron, however, less than 50 constituents have been identified. Among the volatiles, the main component is safranal whilst the non-volatile component are crocins, crocetin, picrocrocin and flavonoids (quercetin and kaempferol). Crocin structurally is glucosyl esters of crocetin and is found to be water-soluble carotenoids that is responsible for saffron's characteristic red color. Picrocrocin is structurally glycoside of safranal and is responsible for the bitter taste of the spice. Safranal is the main component of the distilled essential oil and is a monoterpene aldehyde that is responsible for saffron's characteristic aroma. Safranal, crocin and crocetin are considered to be the main constituents of saffron and are heavily investigated for their biological activates (Pitsikas, 2016).

1.2.2 Safranal

Amongst its biological activities, safranal was found to exert potent anticonvulsant activity (Hosseinzadeh and Sadeghnia, 2007), anti-oxidant activities (Hosseinzadeh et al., 2005; Hosseinzadeh and Sadeghnia, 2005; Hosseinzadeh et al., 2009), anti-inflammatory activities (Hazman and Bozkurt, 2015) and anti-tumor activities (Shabestari and Samarghandian, 2013; Farahzad et al., 2014; Zhang et al., 2017). The molecular basis of safranal's profound anti-cancer effects remains unclear.

1.3 Transcriptome profiling

Transcriptome is the complete set of RNA transcripts and their quantity in a given cell under certain developmental stage or physiological condition. Investigating

the transcriptome may explain the functional elements of the genome and the underlying mechanisms of development or disease (Wang et al., 2009; Yang and Kim, 2015). Transcriptome started in 1991 with a partial human transcriptome covering a mere of 609 mRNA sequences. In 2008, two human transcriptomes consisting of millions of transcript covering 16,000 genes. Transcriptomes of hundreds of individuals can now be analyzed just like any other routine laboratory procedure. This cascade of transcriptomes evolution clearly reflects the drastic development of new transcriptome technologies (Lowe et al., 2017).

Gene expression studies at first were conducted using northern blots and quantitative polymerase chain reaction (qPCR) both considered as low-throughput methods allowing few number of transcripts measurement. Then, a sequence-based technology was developed where generated expressed sequence tag (EST) libraries by Sanger sequencing of complementary DNA (cDNA) was used in gene expression studies, yet transcript quantification was not practical. Several tag-based methods were also developed to yield a higher throughput and precise quantification of expression levels such as serial analysis of gene expression (SAGE) and cap analysis gene expression (CAGE). For high-throughput method, hybridization-based microarray technologies could be performed but with notable limitations. For instance, priori sequence information is required and cross-hybridization artifacts may interfere with the correct interpretation. Methodologies described here are insensitive toward splice isoforms and are not set to identify novel genes (Shendure, 2008; Kukurba and Montgomery, 2015; Hrdlickova et al., 2016).

1.3.1 RNA sequencing

RNA sequencing (RNA-seq) is high-throughput sequencing method. Unlike, Sanger sequencing and microarray-based methods, RNA-Seq provides better coverage and superior overview of transcriptomic dynamic nature. Not only gene expression quantification is generated but also novel transcripts are discovered, alternatively spliced genes are identified and allele-specific expression are detected. (Kukurba and Montgomery, 2015).

RNA-seq standard workflow is shown in (Fig. 1). It starts with the experimental set-up where the purpose of the study is outlined and statistical design is taken into consideration. Tissue preparation and library generation, herein, involve samples collection, RNA extraction and quality assessment, rRNA depletion or poly-A enrichment, cDNA synthesis, library preparation, polymerase chain reaction (PCR) reaction and library normalization. Then comes the high-throughput sequencing where the sequencing platform is chosen, sequence coverage is decided. Then comes the bioinformatics processing part, where critical needs should be met such as computing resources and programing skills to aid in transcriptome reconstruction, mapping strategy, gene expression quantification. Afterword, the biological inference involves normalization, differential gene expression, gene function and interaction. Lastly, experimental validation is needed where quantitative polymerase chain reaction (qPCR) quantification becomes handy.

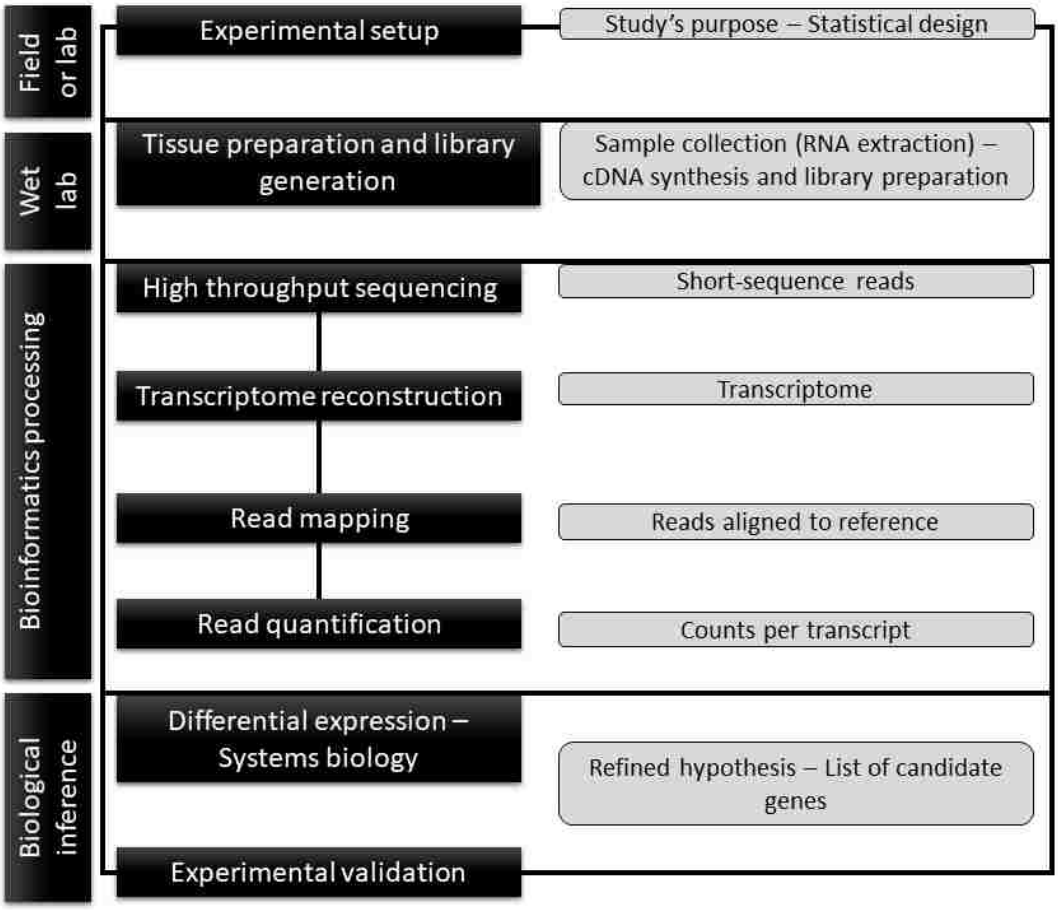

Figure 1: A standard RNA-seq workflow (adapted from Wolf, 2013)

1.4 Hypothesis to be tasted

The hypothesis of this study is considering saffron's reputation as potent anti-cancer natural based product, will safranal holdup the same anti-cancer effect against HCC? And what are some of its underlying mechanism?

1.5 Aims of the study

The overall aim of this study is to investigate safranal's therapeutic effect on HCC through transcriptomic approach.

The specific objectives are:

1. Transcriptomic analysis to figure out significant candidate genes involved in HCC due to safranal treatment.
2. Verification of the result obtained from RNA seq using qPCR quantification.

Chapter 2: Methods

2.1 *In vivo* Study

2.1.1 Animal

Wister male rats were obtained from the animal facility of the College of Medicine and Health Sciences, UAEU. Animals were housed under a 12 hr light/dark cycle at 24 – 26°C. They were maintained on standard laboratory animal diet with food and water ad libitum. All animal studies were carried out in accordance with, and after approval of the Animal Research Ethics Committee of the College of Medicine and Health Sciences, UAEU (Approval No. A8-15).

2.1.2 Experimental design

The experimental protocol of HCC development was adapted and followed in our lab as per described by DePeralta et al. (2016) and Pacheco-Rivera et al. (2016) with some modifications and adjustments. This study was performed with an average weighing 170 g male Wistar rats. Wistar rats were randomly divided into 8 groups (n=6). The first 4 groups were labelled as control groups subdivided into control water (C W), control safranal (C SF), control sorafenib (C SB) and control safranal and sorafenib (C SF SB). Whereas the remaining 4 groups labelled as experimental groups subdivided into HCC (H), HCC treated with safranal (H SF), HCC treated with sorafenib (H SB) and HCC treated with both safranal and sorafenib (H SF SB).

Control groups were subjected to the following treatments: Group 1 (C W): Rats were treated with distilled water alone throughout the experimental period. Group 2 (C SF): Rats were administered orally 200 mg/kg of safranal 5 days a week for 3 weeks, total of 15 oral gavage doses. This dose was selected based on a preventive study of safranal on nephrotoxicity

(Karafakıoğlu et al., 2017). Group 3 (C SB): Rats were administered orally 10 mg/kg of sorafenib 5 days a week for 3 weeks, total of 15 oral gavage doses. This dose was selected based on earlier studies of sorafenib on a rat HCC model (Alsaied et al., 2014; Wang et al., 2014; El-Ashmawy et al., 2016). Group 4 (C SF SB): Rats were administered orally both doses of 200 mg/kg of safranal and 10 mg/kg of sorafenib 5 days a week for 3 weeks, total of 15 oral gavage doses.

Whereas the experimental groups were subjected to the following treatments: Group 5 (H): Rats were injected intraperitoneally with 50 mg/kg of diethylnitrosamine (DEN) once a week for 15 week, total of 15 injections. Group 6 (H SF): Rats were injected intraperitoneally with 50 mg/kg of DEN once a week for 15 week, total of 15 injections and then followed by orally administered 200 mg/kg of safranal 5 days a week for 3 weeks, total of 15 oral gavage doses. Group 7 (H SB): Rats were injected intraperitoneally with 50 mg/kg of DEN once a week for 15 week, total of 15 injections and then followed by orally administered 10 mg/kg of sorafenib 5 days a week for 3 weeks, total of 15 oral gavage doses. Group 8 (H SF SB): Rats were injected intraperitoneally with 50 mg/kg of DEN once a week for 15 week, total of 15 injections and then followed by orally administered both doses of 200 mg/kg of safranal and 10 mg/kg of sorafenib 5 days a week for 3 weeks, total of 15 oral gavage doses.

At the end of experimental period and 24 hrs after the last treatment, rats were anesthetized by mild Diethyl ether and dissected (Fig. 2). The whole liver was removed and immediately flash frozen for further analysis. This experimental model has been developed and HCC development has been histopathologically confirmed and well characterized in our lab (AlMansoori ongoing MSc Thesis).

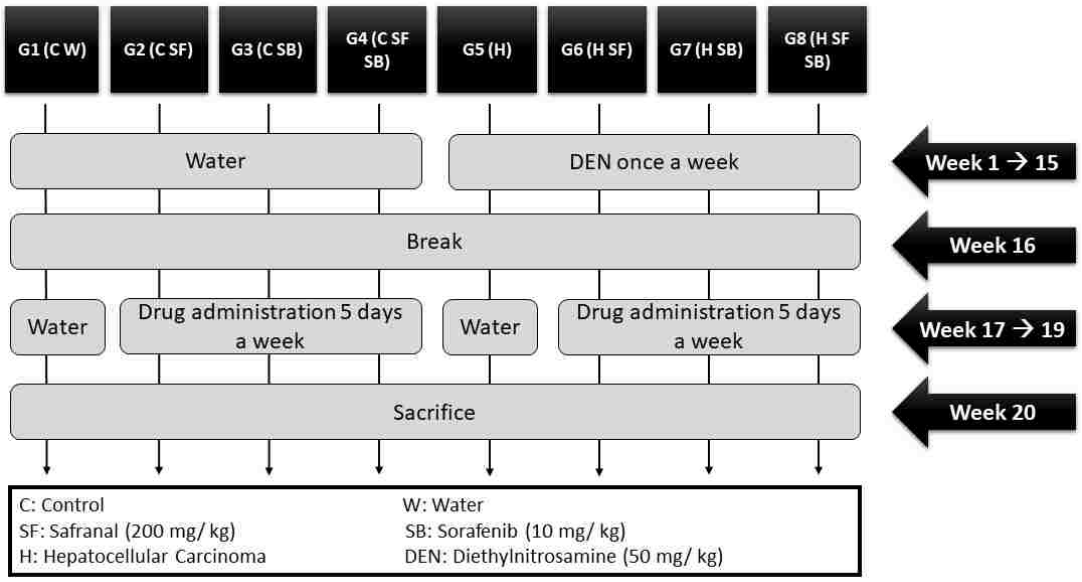

Figure 2: Schematic illustration of the *in vivo* experimental design (adapted from AlMansoori ongoing MSc Thesis)

2.2 Transcriptomic analysis

2.2.1 RNA extraction

Total RNA was extracted from flash frozen liver tissues using Maxwell 16 System along with Maxwell 16 Total RNA Purification Kit (Promega, US), according to the manufacturer's instructions with slight modifications. Briefly, 50 mg of liver tissue immersed in 200 µl lysis buffer with 2 µl of β-mercaptoethanol (BME) was mechanically homogenized until no visible tissue fragments is seen. The homogenized sample was incubated for 10 min on ice for a complete lysis. Microcentrifuge was used for a quick spin and the sample lysate was transferred into a new 1.5 ml microcentrifuge tube. 167 µl of RNA dilution buffer was added to the sample lysate along with 125 µl of a completely resuspend the clearing agent. The sample was vortexed for 30 sec then it was placed in 70°C heat block for 3 min. After heating the

sample was vortexed again for 30 sec and incubated for 5 min at room temperature. After that the sample was transferred into clearing column assembly and centrifuged at 12,000 × *g* for 2 min.

Afterward, the collected flowthrough from the collection tube was transferred into first wall of maxwell 16 RNA cartridge while a plunger was placed into the last wall of the cartridge. A 200 µl of nuclease-free water was placed in a separate elution tube. Maxwell 16 Instrument was programmed according to the manufacturer's instructions. Upon the completion of the RNA purification, the elution tube was removed from the platform and placed into magnetic elution tube rack for 10 min. the eluted sample of total RNA was then transferred into a storage tube. Total RNA concentration was measured using NanoDrop1000 spectrophotometer (Thermo Fisher Scientific Inc.) while RNA quality, RNA integrity number (RIN) was verified using Bioanalyzer 2100 and RNA 6000 Nano chips according to the manufacturer's instructions (Agilent Technologies, CA). Total RNA concentration and RIN value per sample are listed in (Table 1).

Table 1: Extracted total RNA measurement

Sample code	**Conc. ng/µl**	**RIN**	**Sample code**	**Conc. ng/µl**	**RIN**
G1 (C W) - 1	192.1	9.3	**G5 (H) - 1**	306.6	N/A*
G1 (C W) - 2	121.8	9.4	**G5 (H) - 2**	435.1	8.6
G1 (C W) - 3	191.9	9.5	**G5 (H) - 3**	199	9.1
G2 (C SF) - 1	242.8	9.3	**G6 (H SF) - 1**	530.4	9.0
G2 (C SF) - 2	165.1	9.1	**G6 (H SF) - 2**	328.4	9.3
G2 (C SF) - 3	258.8	9.1	**G6 (H SF) - 3**	296.3	9.2
G3 (C SB) - 1	259.4	9.1	**G7 (H SB) - 1**	139.6	9.2
G3 (C SB) - 2	226.9	8.9	**G7 (H SB) - 2**	204.9	9.0
G3 (C SB) - 3	265.6	9.4	**G7 (H SB) - 3**	190.1	9.6
G4 (C SF SB) - 1	211.7	9.1	**G8 (H SF SB) - 1**	209.7	9.2
G4 (C SF SB) - 2	233.4	8.9	**G8 (H SF SB) - 2**	255.9	9.1
G4 (C SF SB) - 3	205.8	9.0	**G8 (H SF SB) - 3**	223.3	9.2

Note: (*) not available due to disturbance in the fast region estimated to be 9.2.

2.2.2 RNA-seq libraries construction, sequencing and analysis

Brigham Young University DNA Sequencing Center (Provo, Utah, USA) provided the service of library preparation and sequencing. Briefly, RNA-seq libraries were prepared using KAPA Stranded mRNA-Seq kit Illumina Platforms (KAPA Biosystems, US) following the manufacturer's instructions. The kit is based on poly-A mRNA enrichment. The produced libraries were quantified using a Kapa library quantification kit Illumina Platforms (KAPA Biosystems, US) based on SYBR green chemistry. Libraries were sequenced on an Illumina HiSeq 2500 sequencing system (Illumina, Inc.) to a minimum of 20 million reads per sample.

Bioinformatics Core at New York University Abu Dhabi (NYUAB) have processed the generated data through their standard RNA-seq analysis pipeline. In short, alignments were performed using tophat2 v2.1.0 (Kim et al., 2013). Following the tophat2 alignment stage, read counts were generated using HTseq count (Anders et al., 2014).

2.2.3 Hierarchical cluster and principle component analyses

Both hierarchical cluster analysis principle component analysis (PCA) were constructed by uploading the raw count into ClustVis, web tool for visualizing clustering of multivariate data (Metsalu and Vilo, 2015).

2.2.4 Differential gene expression analysis

To generate differential expressed genes (DEGs) and their volcano plots, raw counts were analyzed using RNA-seq 2G (http://52.90.192.24:3838/rnaseq2g/) an online interface, user friendly, with over 25 statistical methods used to perform two-group analysis of differential gene expression. DESeq2 statistical method was

employed and significant genes were identified using cutoff values of false discovery rate (FDR) < 0.05, and log 2 (fold change).Venn diagrams were constructed using InteractiVenn, web-based tool (Heberle et al., 2015). Gene expected function was extracted using Rat Genomic Database (RGD) (Shimoyama et al., 2015).

2.2.5 Gene ontology enrichment analysis

Gene set enrichment analysis of DEGs was performed using the Biological Networks Gene Ontology tool (BiNGO) (Maere et al., 2005), plug-in for Cytoscape software (Shannon et al., 2003). BiNGO found the relevant biological process annotations. This enrichment test is based on hypergeometric test and corrected multiple testing using Benjamini and Hochberg FDR correction ≤ 0.05.

2.2.6 Pathway analysis

DEGs were analyzed through Ingenuity Pathway Analysis (IPA) (QIAGEN Inc., https://www.qiagenbioinformatics.com/products/ingenuitypathway-analysis) (Krämer et al., 2014). To allow more stringent analysis, the cutoff value of FDR was set to 0.01. Core analysis were implicated with default settings, canonical pathways and associated toxicity functions were reviewed. 45 genes that were associated with HCC were re-analyzed (core analysis) for further toxicity function association.

2.2.7 Network analysis

Network analysis were performed using Search Tool for The Retrieval of Interacting Genes (STRING) version 10.5 (http://string-db.org/) (Szklarczyk et al., 2017). STRING, an online genes/proteins association database curated from literature, high-throughput experimental data, and predicted associations from systemic genome

comparisons. *Rattus norvegicus* databases were utilized to generate association network for two sets of genes of interest that showed significant fold change in expression.

2.2.8 qPCR quantification

Previously extracted RNA was used for cDNA synthesis corresponding to 1000 ng of total RNA using GoScript™ Reverse Transcription System (Promega US) according to manufacturer's instructions. Quantitative PCR reactions were performed on an QuantStudio 5 Real-Time PCR System (Thermo scientific,US) using GoTaq® qPCR Master Mix kit (Promega US) with gene-specific primers as per manufacturer's protocol . Data were normalized relative to 18S rRNA gene values, which exhibited stable expression levels between the two groups of G5 (H) and G6 (H SF) unlike ACTB and GAPDH. Relative expression level of each gene was calculated using $2^{-\Delta\Delta Ct}$ method. All reactions were run in triplicate. The primers for the qPCR reactions are listed in (Table 2).

Table 2: qPCR primers sequences

Gene	Forward primer (5'→3')	Reverse primer (5'→3')	Source
BTG1	CGTTGTATTCGCATCAACC	AGCCATCCTCTCCAATCC	Li et al., 2009
PAWR	GCAGGACAGAAGGAACGGAA	TGGGGCACTGATTGTGCTTT	NCBI-Primer designing tool (Ye et al., 2012)
BMP4	GGGATCTTTACCGGCTCCAG	TCTCCAGATGTTCTTCGTGATGG	NCBI-Primer designing tool (Ye et al., 2012)
DNAJB9	GGGCGCACGGGTTATTAGA	CTCTGAGGCTGACTTTGGCA	NCBI-Primer designing tool (Ye et al., 2012)
CIDEA	TCCTCGGCTGTCTCAATGTC	GAACCGCAGCAGACTCCTTA	NCBI-Primer designing tool (Ye et al., 2012)
18s RRNA	GCAATTATTCCCCATGAACG	GGCCTCACTAAACCATCCAA	Asanoma et al., 2011
GAPDH	AGACAGCCGCATCTTCTTGT	CTTGCCGTGGGTAGAGTCAT	Li et al., 2015
ACTB	AGACCTTCAACACCCCAGC	GTCACGCACGATTTCCCT	Wang et al., 2014

Chapter 3: Results

3.1 Overview of gene expression profiles

Out of whole data set of 32884 sequenced genes, randomly 2400 genes were selected to view an overall expression pattern among the different groups through hierarchical cluster analysis (Fig. 3). This analysis displayed visibly distinguishable patterns across the groups as it separated the experimental groups from the control groups with a twist. Cancer induced groups/experimental groups clustered together except for one; G5 (H) is clustered among G7 (H SB) and G8 (H SF SB) whereas G6 (H SF) is closely clustered with control groups G1 (C W) and then G3 (C SB). And the rest of control groups are clustered together; G2 (C SF) and G (C SF SB). It might be obvious that the experimental cluster together but based on this analysis, G6 (H SF) is shown gene expression pattern closer to the control groups.

3.2 Principle component analysis

Principle component analysis (PCA) is another methodology used for dimension reduction, simplifying data into 2 dimensional (2D) plot where PCA axes emphasize data variation. The whole data set of 32884 sequenced genes was used in this analysis (Fig. 4). Although a defined variance mostly on PC1 is shown among the different control groups still does not overweight the variance shown between the experimental groups. Clearly, G5 (H) is separated from the rest of the experimental groups. Interestingly, both G7 (H SB) and G8 (H SF SB) are located closely to each other also they fall within the prediction ellipse of the control groups. Whereas G6 (H SF) showed no variance on PC1 compared to the nearest control groups while a small variance could be seen on PC2 implying an overall small variance.

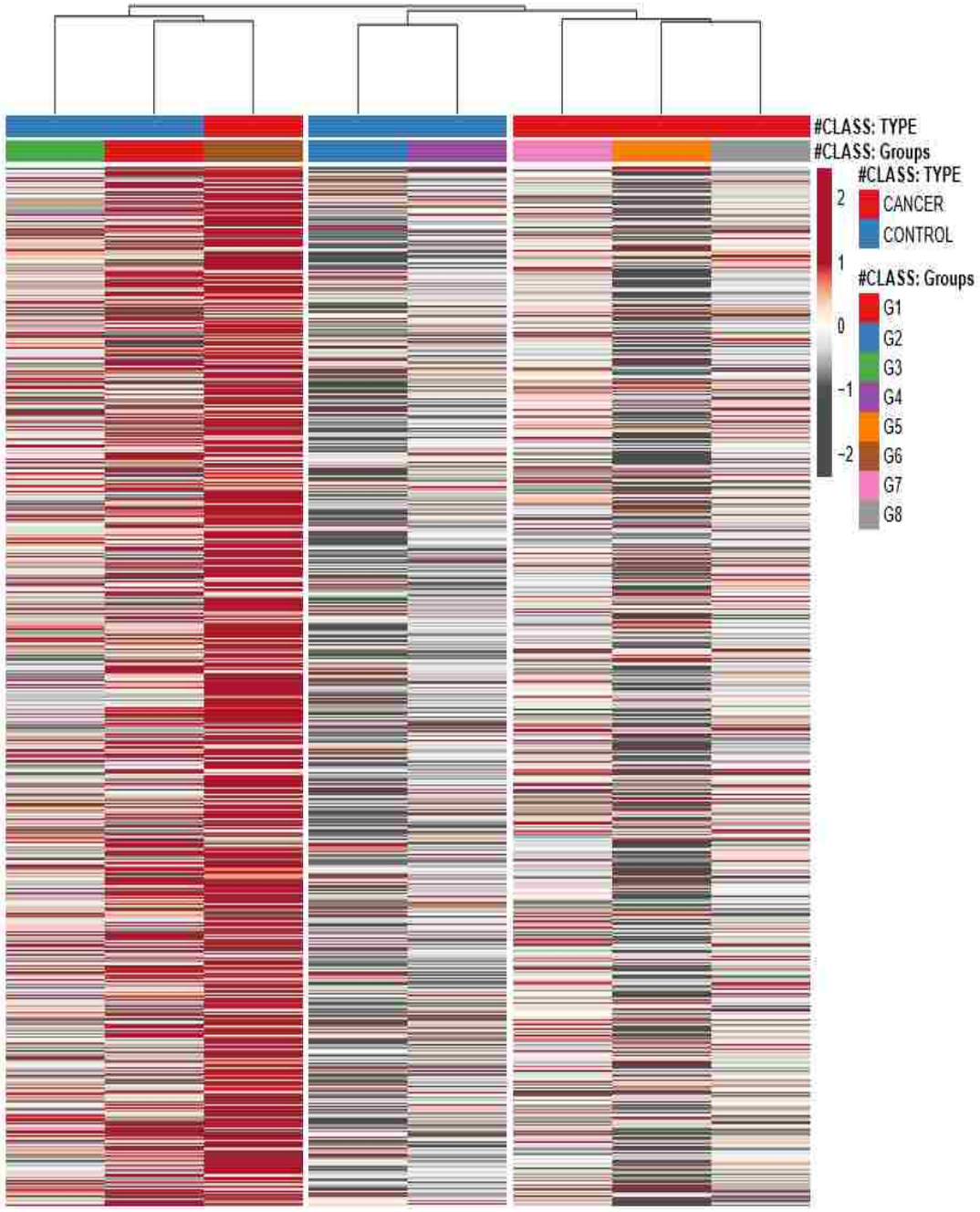

Figure 3: Heatmap of gene expression profiles for all the groups. Hierarchical cluster analysis represents the mean of the triplicate for each group. Raw were centered and unit variance scaling was applied. Groups were clustered by maximum distance and complete linkage. Expression levels are coloured red for high expression and grey for low expression.

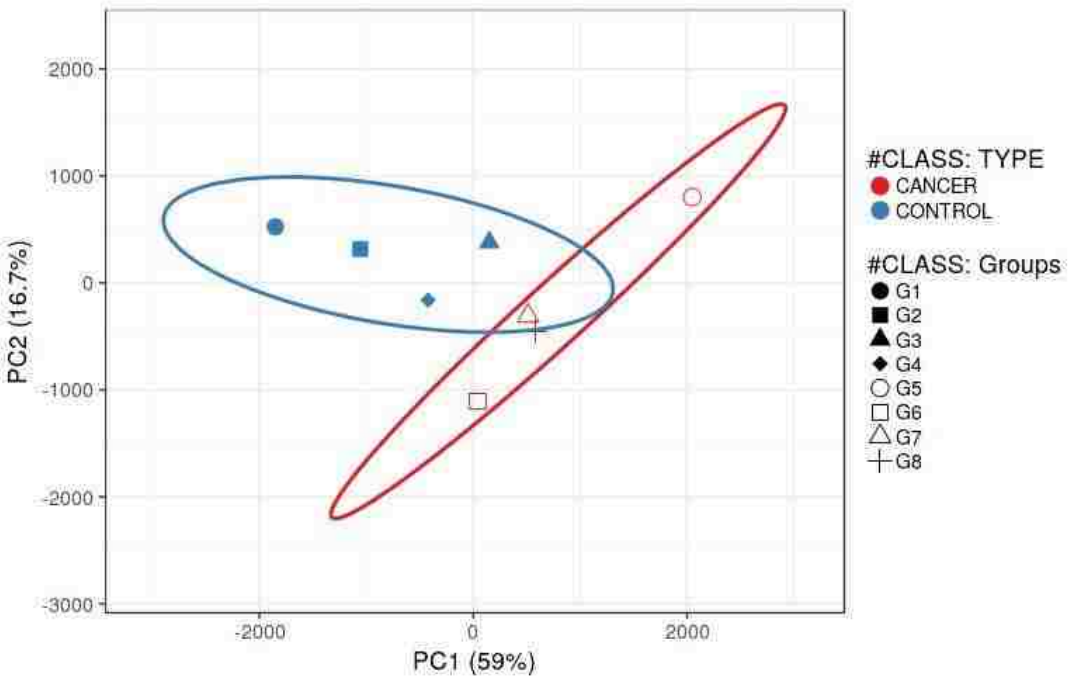

Figure 4: Principle component analysis (2D) plot. Each mark represents the mean of the triplicate for each group. Pareto scaling were applied to rows and SVD with imputation was used to calculate principal components. PC1 and PC2 explain 59% and 16.7% of the total variance, respectively along with 95% of prediction ellipses.

3.3 Differential gene expression analysis

Differentially expressed genes (DEGs) were obtained for the following experimental groups, G6 (H SF), G7 (H SB) and G8 (H SF SB). To define DEGs, a threshold was set on two categories, 0.05 false discovery rate (FDR), which was calculated based on *p* value using the Benjamini-Hochberg method and log 2 (fold change). Firstly, a total of 830 genes were differentially expressed in G6 (H SF) where 484 genes were up regulated and 346 genes were down regulated. Secondly, a total of 733 genes were differentially expressed in G7 (H SB) where 486 genes were up regulated and 247 genes were down regulated. Lastly, a total of 401 genes were differentially expressed in G8 (H SF SB) where 285 genes were up regulated and 116

genes were down regulated. A volcano plot is used as visualization for the fold change compared to the usual statistical significance of *p* values for each of the three experimental groups instead of FDR (Fig. 5A-C). Top 5 up regulated and down regulated DEGs of these groups are shown in (Table 3) and (Table 4), respectively. DEGs were found to be uniquely expressed within each respective group as well as other common genes were found overlapping between the three experimental groups. DEGs with total of 138 genes were up regulated between the three experimental groups while total of 47 genes were down regulated (Fig. 5D-E).

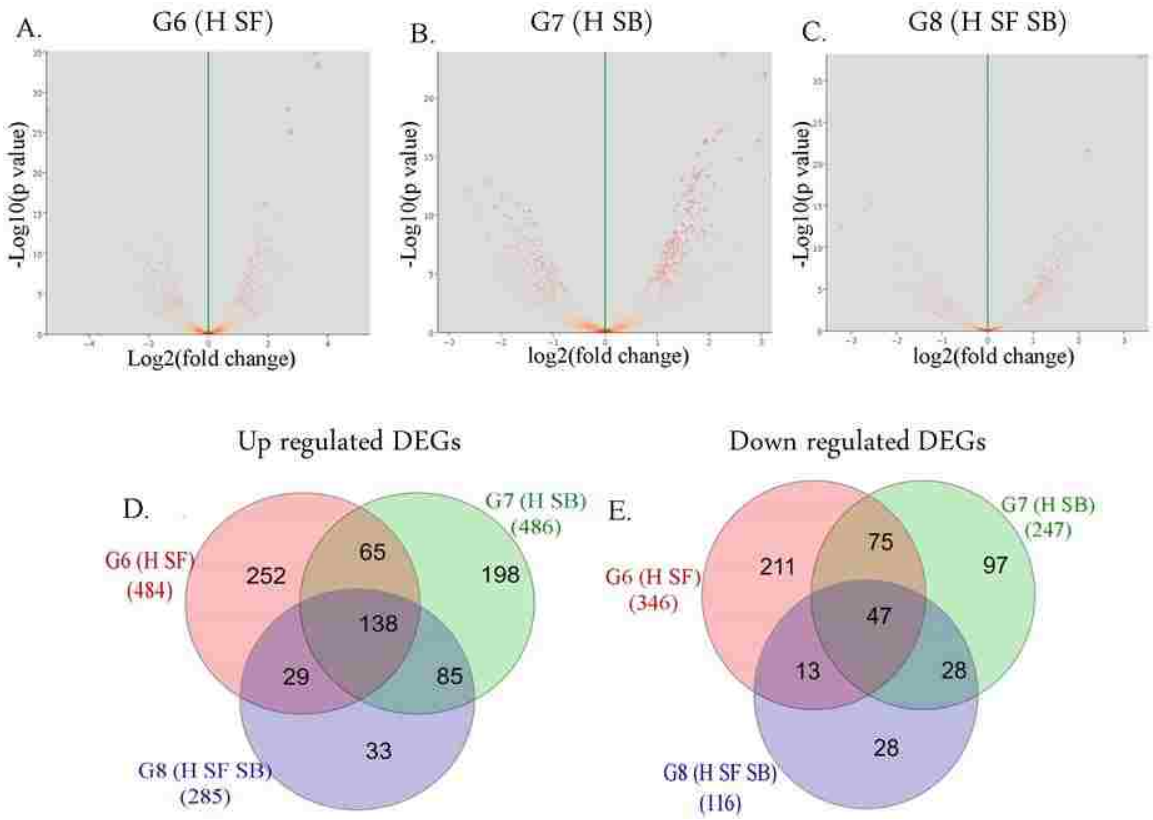

Figure 5: Differential gene expression analysis. (A-C) Volcano plot for DEGs for each of the three experimental groups (A) G6 (H SF). (B) G7 (H SB). (C) G8 (H SF SB). Illustrating log2 (fold change) against statistical significance of –log10 (*p* values). (D-E) Venn diagrams of DEGs of the three experimental groups. (D) Distribution of the up regulated DEGs. (E) Distribution of the down regulated DEGs.

Table 3: Top up regulated DEGs in the three experimental groups

Gene ID	Description	Fold change	FDR	Expected Function
G6 (H SF)				
Cyp2b2	Cytochrome P450, family 2, subfamily b, polypeptide 2	12.80434	3.59E-30	Aging; drug metabolic process; nicotine metabolic process
Cyp2b1	Cytochrome P450, family 2, subfamily b, polypeptide 1	12.10023	1.80E-31	Drug metabolic process; response to calcium ion; response to drug
Ces2a	Carboxylesterase 2A	6.758936	2.96E-22	Retinoid metabolic process
Vnn1	Vanin 1	5.862473	3.18E-06	Acute inflammatory response ; cell-cell adhesion; chronic inflammatory response
Acaa2	Acetyl-coa acyltransferase 2	5.01974	2.33E-11	Acetyl-coa metabolic process; fatty acid beta-oxidation; cellular response to hypoxia
G7 (H SB)				
Cpa1	Carboxypeptidase A1	9.202453	3.68E-11	Proteolysis
Aldh3a1	Aldehyde dehydrogenase 3 family, member A1	8.399899	9.81E-19	Aging; positive regulation of cell proliferation; response to camp
Akr1b8	Aldo-keto reductase family 1, member B8	6.098191	3.06E-12	Oxidation-reduction process
Nefl	Neurofilament, light polypeptide	5.641934	3.37E-06	Cerebral cortex development; hippocampus development; intermediate filament polymerization or depolymerization
Slc10a4	Solute carrier family 10, member 4	4.828877	0.000127	Adult behavior; response to drug
G8 (H SF SB)				
Cyp2b1	Cytochrome P450, family 2, subfamily b, polypeptide 1	11.55794	9.21E-30	Drug metabolic process; response to calcium ion; response to drug
Cyp2b2	Cytochrome P450, family 2, subfamily b, polypeptide 2	10.36073	9.21E-30	Aging; drug metabolic process; nicotine metabolic process
Akr1b8	Aldo-keto reductase family 1, member B8	4.650745	1.61E-18	Oxidation-reduction process
Cpa1	Carboxypeptidase A1	4.281811	0.000209	Proteolysis
Acaa2	Acetyl-coa acyltransferase 2	3.832358	1.51E-08	Acetyl-coa metabolic process; fatty acid beta-oxidation; cellular response to hypoxia

Table 4: Top down regulated DEGs in the three experimental groups

Gene ID	Description	Fold change	FDR	Expected Function
G6 (H SF)				
Arntl2	Aryl hydrocarbon receptor nuclear translocator-like 2	-4.64973	0.000397	Circadian rhythm; positive regulation of circadian rhythm; positive regulation of transcription by RNA polymerase II
LOC102549948	Uncharacterized LOC102549948	-4.34139	0.002859	Unkown
Hcn3	Hyperpolarization-activated cyclic nucleotide-gated potassium channel 3	-4.32149	0.001026	Cellular response to dopamine; response to cisplatin; potassium ion transmembrane transport
Npas2	Neuronal PAS domain protein 2	-4.2387	7.08E-07	Circadian regulation of gene expression; circadian rhythm;
ND2	NADH dehydrogenase subunit 2	-4.20382	3.88E-05	Reactive oxygen species metabolic process
G7 (H SB)				
Ptx3	Pentraxin 3	-6.06793	6.68E-10	Negative regulation by host of viral exo-alpha-sialidase activity; negative regulation by host of viral glycoprotein metabolic process
Fos	FBJ osteosarcoma oncogene	-4.86634	1.24E-10	Aging; cellular response to hormone stimulus; conditioned taste aversion
Pcdh17	Protocadherin 17	-4.71013	1.53E-06	Adult behavior; negative regulation of synaptic transmission; presynaptic active zone assembly
Opcml	Opioid binding protein/cell adhesion molecule-like	-4.36873	0.000109	Cell adhesion
Lrrtm2	Leucine rich repeat transmembrane neuronal 2	-4.02803	6.18E-05	Synapse organization; long-term synaptic potentiation; negative regulation of receptor internalization;
G8 (H SF SB)				
Fos	FBJ osteosarcoma oncogene	-5.8923	1.68E-12	Aging; cellular response to hormone stimulus; conditioned taste aversion
Ptx3	Pentraxin 3	-3.80319	6.69E-06	Negative regulation by host of viral exo-alpha-sialidase activity; negative regulation by host of viral glycoprotein metabolic process
Inhba	Inhibin beta A subunit	-3.73907	4.18E-08	Cellular response to cholesterol; cellular response to follicle-stimulating hormone stimulus; activin receptor signaling pathway
Ngp	Neutrophilic granule protein	-3.59586	0.001207	Defense response; negative regulation of endopeptidase activity
Tlr12	Toll-like receptor 12	-3.46918	5.02E-05	Signal transduction

3.4 Gene ontology enrichment analysis

In order to investigate the biological processes associated with DEGs, gene ontology (GO) enrichment analysis was performed using BiNGO, a known Cytoscape software plugin. This analysis was carried out on the up regulated and the down regulated genes for the three experimental groups G6 (H SF), G7 (H SB) and G8 (H SF SB) in addition to the up regulated and the down regulated common genes mentioned previously (Table 5).

Table 5: List of relevant GO enrichment analysis

Term name	Genes number	FDR	Term name	Genes number	FDR
G6 (H SF) Up regulated			**G6 (H SF) Down regulated**		
Regulation of programmed cell death	36	2.85E-03	Cellular respiration	8	2.14E-03
Response to organic substance	80	9.15E-14	Regulation of biological process	89	2.40E-02
Response to stress	71	9.63E-06	Negative regulation of biosynthetic process	17	2.59E-02
G7 (H SB) Up regulated			**G7 (H SB) Down regulated**		
Response to drug	26	7.92E-04	Electron transport chain	6	2.30E-02
Response to oxidative stress	19	1.15E-04	Cellular respiration	5	3.83E-02
Regulation of cell projection size	2	3.82E-02	Negative regulation of cellular process	29	3.72E-02
G8 (H SF SB) Up regulated			**G8 (H SF SB) Down regulated**		
Response to drug	25	4.32E-07	Regulation of epinephrine secretion	2	1.96E-02
Regulation of lipid metabolic process	11	1.41E-04	Cyclooxygenase pathway	2	1.66E-02
Regulation of tumor necrosis factor production	4	1.33E-02	Regulation of macrophage differentiation	2	3.70E-02
Common genes - Up regulated			**Common genes - Down regulated**		
Response to drug	12	2.73E-03	Cyclooxygenase pathway	2	5.57E-03
Regulation of response to stress	7	4.51E-02	Cellular respiration	3	2.06E-02
Inflammatory response	6	4.51E-02	Electron transport chain	3	2.09E-02

Some repetitive GO enriched terms can be seen among the different gene sets most notably: response to drug, response to stress, cellular respiration and electron transport chain. Moreover, unique GO enriched terms have been found. For example, in G6 (H SF) regulation of programmed cell death, G8 (H SF SB) regulation of tumor necrosis factor production. And inflammatory response in the common genes.

3.5 Pathway analysis

Next, pathway analysis was explored only in regards to G6 (H SF) as it is the focus of this study. Pathway analysis were performed using ingenuity pathway analysis (IPA) software. For more stringent analysis, FDR threshold was changed to 0.01 instead of 0.05, leaving total of 529 DEGs where 308 genes were up regulated and 221 genes were down regulated. The most significant canonical pathways are presented in (Table 6). Other canonical pathway of interest presented were oxidative phosphorylation and unfolded protein response.

Table 6: List of top significant canonical pathway associated with G6 (H SF)

Pathway	***p* value**	**Overlap percentage**	**Genes**
Superpathway of cholesterol biosynthesis	5.13E-07	29.60%	*ACAT2, EBP, FDPS, HADHB, HMGCR, HMGCS2, MSM01, PMVK*
Superpathway of geranylgeranyl diphosphate biosynthesis 1 (via mevalonate)	1.94E-06	40%	*ACAT2, FDPS, HADHB, HMGCR, HMGCS2, PMVK*
Mevalonate pathway I	1.16E-05	41.70%	*ACAT2, HADHB, HMGCR, HMGCS2, PMVK*
Sirtuin signaling pathway	1.97E-05	8.50%	*ACADL, APP, ATP5F1A, CDH1, CPT1A, CPT1B, EPAS1, GABARAPL2, GADD45A, HMGCS2, IDH2,PGK1, SDHC, SLC25A5, SLC2A1, TIMM17A, TOMM40L,UQCRFS1, XPC*
Mitochondrial dysfunction	3.32E-05	10.80%	*APP, ATP5F1A, ATP5MC3, ATP5MG, COX5A, CPT1A,CPT1B, HSD17B10, PINK1, PRDX3,SDHC, UQCRC1, UQCRFS1*

Furthermore, IPA offers toxicity functions linked to the DEGs provided and manually curated IPA knowledge base supporting clinical pathology endpoints. Among them “Hepatotoxicity” showed five top enriched toxicity functions which were liver steatosis, HCC, liver hyperplasia / hyperproliferation, liver cholestasis and liver damage. Focusing on our target HCC, IPA predicted an inhibition of HCC with *p* value of 8.27E-05 and total of 45 genes effecting HCC (Fig. 6). One of these 45 genes, *HMGCR* found to be inconsistent with the aim of this analysis while *ACOX1* and *CTNNB1* have a direct inhibition relationship. Leaving the rest of 42 genes with unpredicted effect. These 45 genes were reinvestigated in both hierarchical cluster analysis as it displayed the 45 gene expression profiles among the other experimental groups (Fig. 7) and BiNGO for GO enrichment analysis shown in (Table 7).

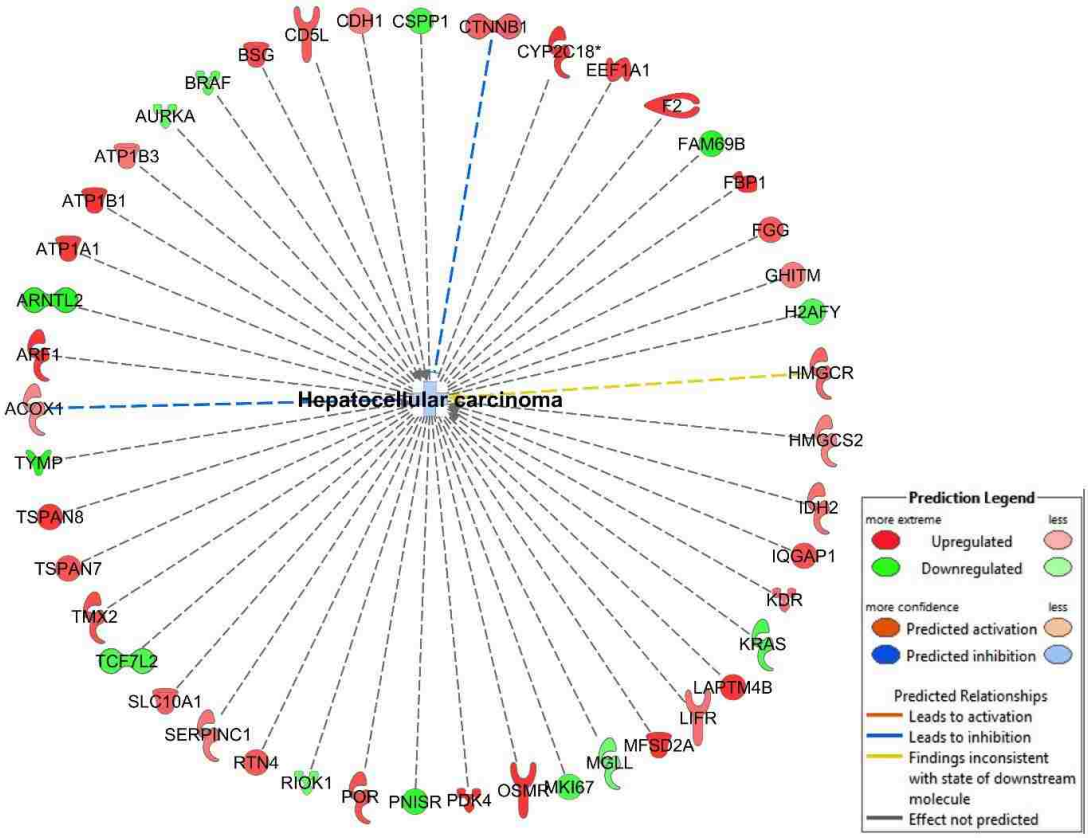

Figure 6: Inhibition of HCC in G6 (H SF)

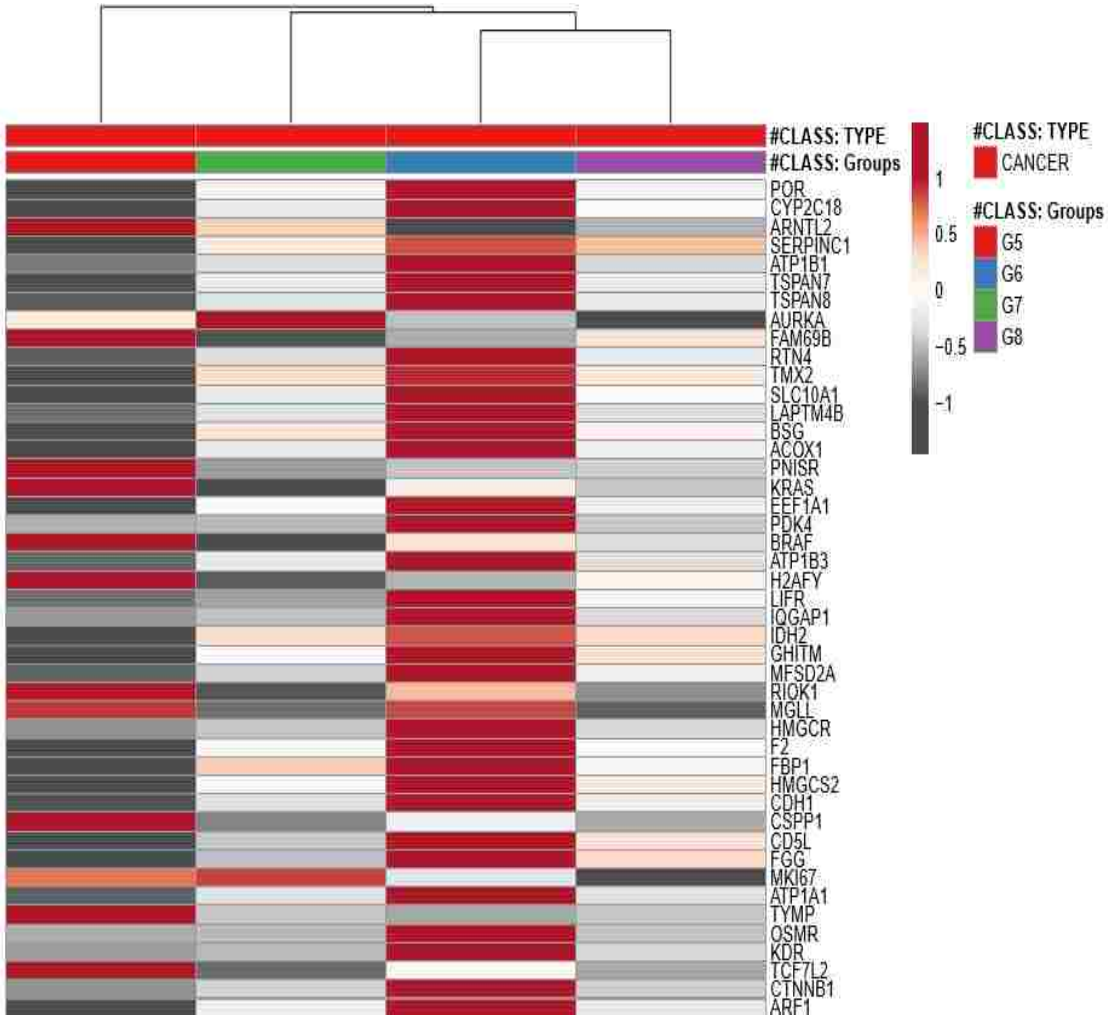

Figure 7: Heatmap of the 45 genes involved in HCC inhibition. Hierarchical cluster analysis represents the mean of the triplicate for each group. Raw were centered and unit variance scaling was applied. Groups were clustered by maximum distance and complete linkage. Expression levels are coloured red for high expression and grey for low expression.

Table 7: GO enrichment analysis of the 45 genes involved in HCC inhibition

Term name	Genes number	FDR	Term name	Genes number	FDR
Up regulated genes			**Down regulated genes**		
Negative regulation of cell development	2	4.77E-02	Negative regulation of apoptosis	3	2.95E-02
Regulation of wound healing	2	2.99E-02	Negative regulation of programmed cell death	3	2.95E-02
Regulation of response to stress	4	2.99E-02	Cell proliferation	2	4.98E-02

To further pinpoint genes associated with relevance biological functions to the HCC, these 45 genes were re-analyzed through IPA. Within the different functions provided, two functions standout from the rest which were incidence of malignant tumor and angiogenesis of tumor, both of which were decreased with *p* value of 7.49E-06 and 1.10E-05, respectively. Total of 8 genes involved even though one of these genes, *KDR* showed inconsistency while three genes *IQGAP1*, *KRAS* and *BRAF* showed unpredicted effect (Fig. 8).

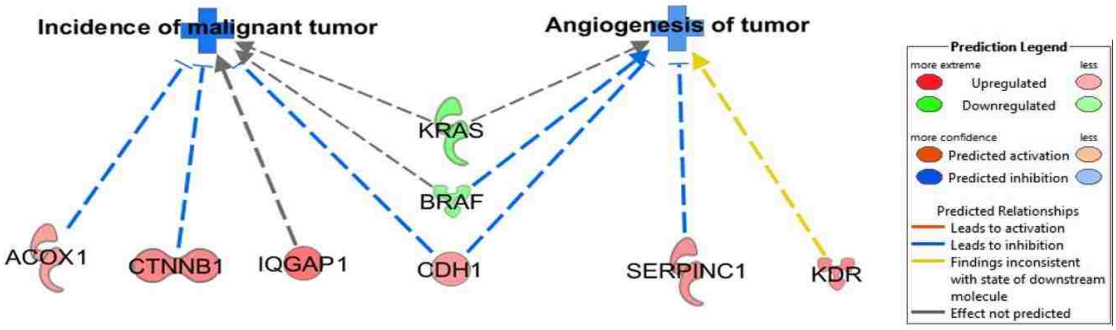

Figure 8: Incidence of malignant tumor and angiogenesis decline in G6 (H SF)

3.6 String network analysis

String network analysis provides an insight over the diverse interactions among the expressed proteins of interest. Theses interactions included the following: databases, experiments, gene neighborhood, gene fusion, gene co-occurrence, co-expression, text-mining and protein homology. The higher the number of association dedicated to a certain node or hub, the higher the importance it projects. Hence, the 45 genes involved in HCC inhibition and the 8 genes involved in incidence of malignant tumor and angiogenesis of tumor were examined for string network analysis (Fig. 9) and (Fig. 10) respectively.

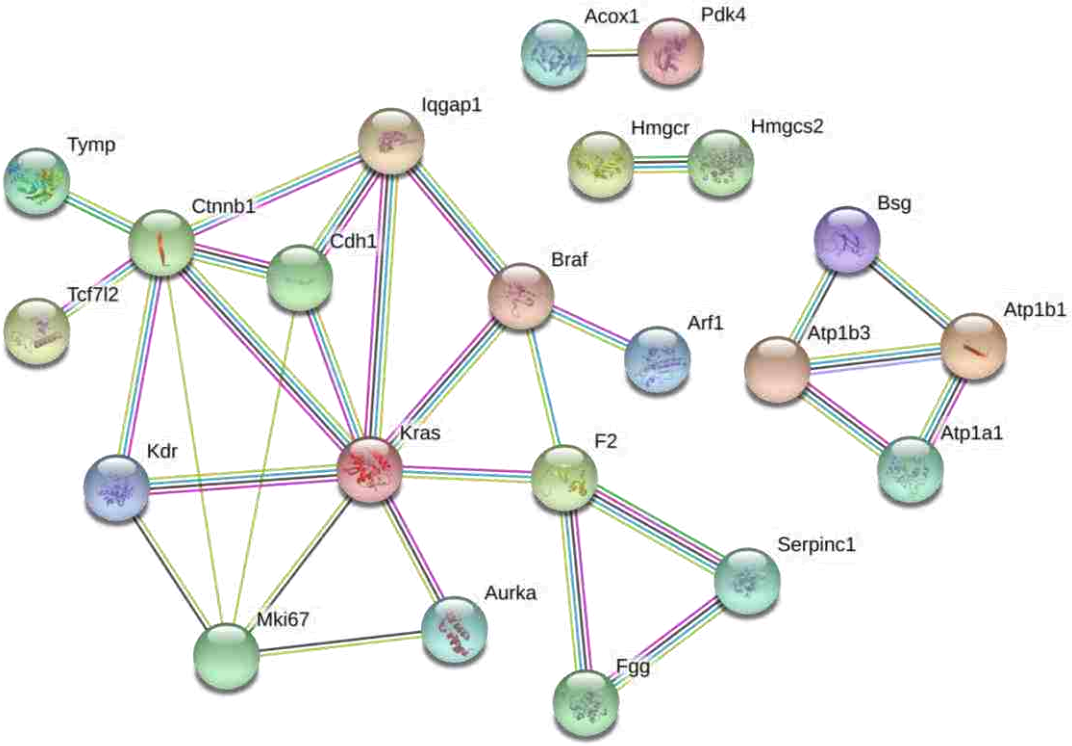

Figure 9: Network analysis of 45 proteins involved in HCC inhibition. The edges represents different protein-protein interaction colour coded; blue- curated databases, pink-experimentally determined, yellow- textmining, black- coexpression. Disconnected node were hidden.

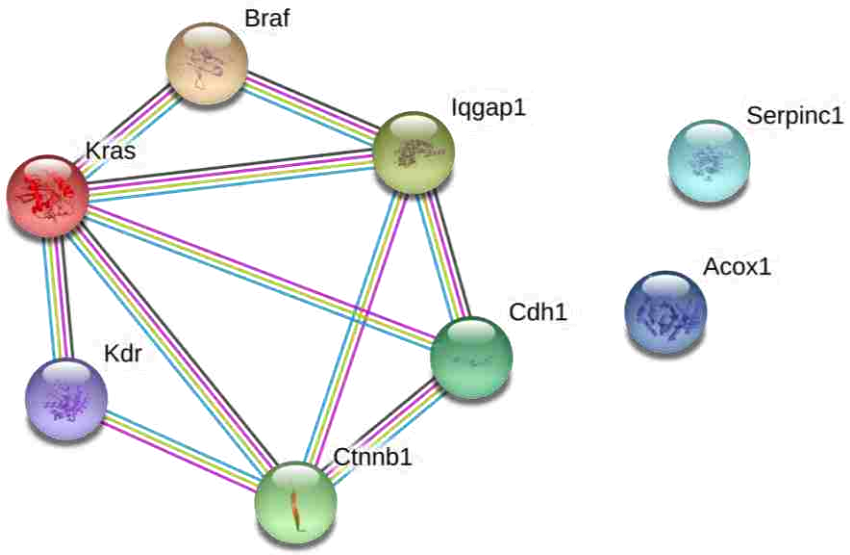

Figure 10: Network analysis of 8 proteins of interest. The edges represents different protein-protein interaction colour coded; blue- curated databases, pink-experimentally determined, yellow- textmining, black- coexpression.

3.7 qPCR quantification and validation

Finally, a validation through qPCR is needed for the accuracy and reproducibility of gene expression results produced by RNA sequencing data. Five random DEGs were chosen from G6 (H SF) which were *BTG1*, *PAWR*, *BMP4*, *DNAJB9* and *CIDEA*. The transcript level of these 5 genes were differently expressed and showed the same overall expression pattern (Fig. 11).

At first both *GAPDH* and *ACTB* were chosen as reference genes however, both were up regulated, *GAPDH* was up regulated by 3.9 folds change while *ACTB* was up regulated by 7 fold change. Eventually, *18s rRNA* was tested and shown a consistent expression hence was chosen as reference gene for qPCR data normalization.

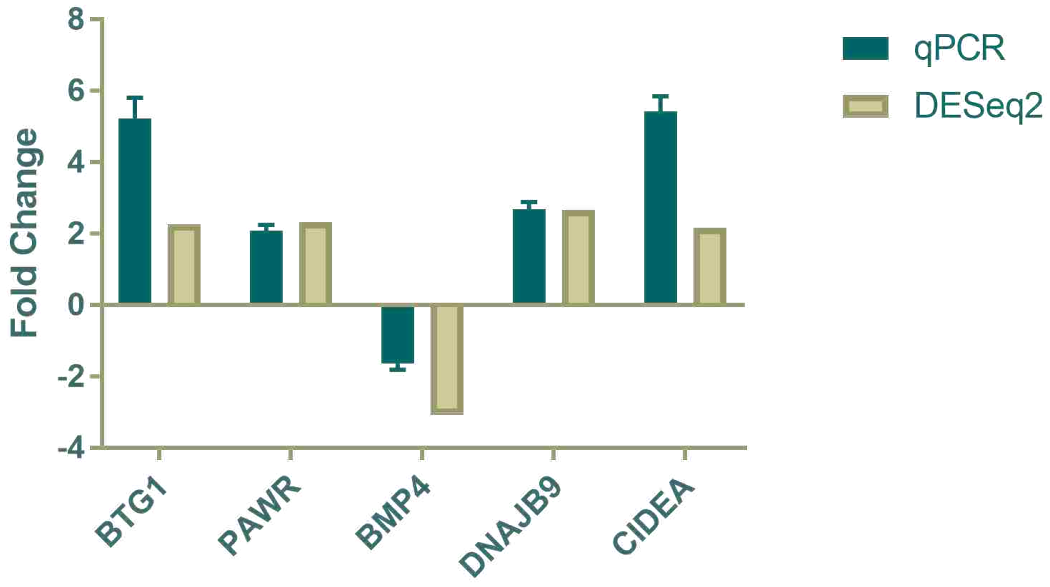

Figure 11: qPCR validation of five DEGs normalized against *18s rRNA*

Chapter 4: Discussion

With the ongoing search for an effective anti-cancer drug to tame down HCC aggressiveness and prolong its associated survival comes the need to characterize and define the underlying mechanisms of action. For once, the anti-cancer activity of saffron constituent, safranal, is widely known, yet, the precise mechanism still not fully understood. Identification of specific genes that mediate an inhibitory reaction toward HCC will improve the available conventional therapies and might introduce safranal as a novel natural-based chemotherapeutic agent.

In this study, a transcriptomic approach was used to assess safaranl's therapeutic effect in DEN-induced HCC *in vivo* model. Two methodologies of dimension reduction, hierarchical cluster analysis and PCA were used to obtain an overall view on RNA sequencing data. G5 (H) have shown the expected change in gene expression profile and major variance compared to the other groups. Excitingly, G6 (H SF) have shown similar gene expression profile to control group G1 (C W) resulting in similar clustering. Additionally, G6 (H SF) showed smaller variance when compared to other control groups (G3 (C SB) and G4 (C SF SB)).

Several studies invoke the cellular response of sorafenib that not only involve receptor tyrosine kinase (RTKs) signaling but extend it to mitochondrial quality control pathways that collectively lead to cancer suppression (Will et al., 2008; Coriat et al., 2012; Zhang et al., 2017). Sorafenib can induce the production of mitochondrial reactive oxygen species (mROS), although ROS are essential for cancer prosperity yet elevated level of extended ROS production can introduce cellular damage to the point where cell death becomes inevitable. Quite conveniently, cancer cells are extremely

sensitive to ROS induced by drugs (Chiou et al., 2009). In the present study, biological processes such as response to drug, response to stress, regulation of response to stress, response to oxidative stress, cellular respiration and electron transport chain have been consistently identified upon GO enrichment analysis on DEGs among the three experimental group G6 (H SF), G7 (H SB), G8 (H SF SB) and the common genes between them. This implies the fact that both safranal and sorafenib anti-cancer activities modulate mitochondrial quality control pathways.

As the focus of this study is G6 (H SF), pathway analysis was mapped to DEGs of G6 (H SF). Top three canonical pathways were interconnected metabolomic pathways including superpathway of cholesterol biosynthesis, superpathway of geranylgeranyl diphosphate biosynthesis I and mevalonate pathway I. Evidence to link between cancer and metabolomic pathways had emerged and now metabolomics alteration is considered among the hallmarks of cancer (Hanahan and Weinberg, 2011; Pavlova and Thompson, 2016). The constant need of cancer to grow and proliferate require an altered metabolic pathways that can meet its demands (Muñoz-Pinedo et al., 2012). Some even raise the question if cancer after all is another metablomic disease (Coller, 2014). Intriguingly, cancer metabolism can be viewed as the classic response of drug resistance (Rahman and Hasan, 2015).

Revisiting the top up regulated DEGs of G6 (H SF), *CYP2B1* and *CYP2B2* were found, both of which come from cytochromes P450 (P450s) family. P450s enzymes catalyze the metabolism of either endogenous substrates like steroids, fatty acids, and fat soluble vitamins or exogenous substrates like drugs and pesticides (Harleton, 2004). Enzymes from CYP2B family are involved in drug metabolism and xenobiotics (Schoedel and Tyndale, 2003). Hence, the action of metabolism of an anti-

cancer drug by P450s enzymes may deactivate the cytotoxic activities of that drug. On the other hand, several prodrugs can be activated by P450s enzymes where prodrugs are needed to be metabolized first before exhibiting the active cytotoxic form (McFadyen et al., 2004). The evident overexpression of *CYP2B1* and *CYP2B2* may increase the breakdown of safranal and hence its secretion. Therefore, the optimal effects of safranal might not be seen due to loss of maintaining the proper level of the drug within the body. This might serve as a mechanism for drug tolerance. It is worth mentioning that even popular therapeutic agents are subjected to such response. For instance, paclitaxel used for ovarian, breast and non-small cell lung cancer is metabolized by CYP2C8 and CYP3A into deactivated form. Doxorubicin used for sarcoma, breast and ovarian cancer is metabolized by CYP3A4 into deactivated form. Cyclophosphamide used for sarcoma, breast and ovarian cancer is metabolized by CYP2B6 and CYP3A4 into activated form reflecting on the fact that cyclophosphamide is prodrug. (Kivisto et al., 1995). And since the prediction behind P450s enzymes metabolic pathway either drug activation or deactivation is not clear as it might increase the intrinsic pharmacodynamics activity of the drug or just simply deactivate the drug and facilitate its clearance from the body (Furge and Guengerich, 2006). It is also conceivable that safranal's metabolite/s, rather than its intact version, is the major contributor to the drug's potent anti-cancer effect.

Sirtuin signaling pathway was the fourth canonical pathway displayed. Cellular redox changes occurring due to oxidative, metabolic or genotoxic stress are detected and an adequate response is directed by sirtuin proteins. For instance, SIRT1, stress sensor, in stress condition it implement cell survival and drive the cell in cytoprotective manner but in extreme stress condition it leads the cell to apoptosis (Raynes et al., 2013). Evidence have compiled over the dual functionality of sirtuin when it comes to

carcinogenesis (Bosch-Presegue and Vaquero, 2011; O'Callaghan and Vassilopoulos, 2017). Although none of the sirtuin proteins family have been significantly detected in sirtuin signaling pathway, 9 out of 19 genes found in sirtuin signaling pathway were involved in cell death of tumor cells based on IPA ingenuity knowledge base.

Mitochondrial dysfunction was the fifth canonical pathway observed. Mitochondrial dysfunction is greatly associated with cancer development and cancer metastatic potential (Chen, 2012; Hsu et al. 2016). However, contradicting data often resurface. To illustrate, various alterations in cellular redox state, respiratory chain complexes and mitochondrial functional parameters, induced oxidative stress and ROS production are all consequences of doxorubicin treatment that triggers mitochondria-mediated apoptosis (Kuznetsov et al., 2011). Similar scenario may describe safranal anti-cancer activity. In fact, safranal correlates impeccably with the previous GO enrichment analysis. 6 out of 13 genes found in mitochondrial dysfunction were involved in cell death of tumor cells based on IPA ingenuity knowledge base. Not to mention, oxidative phosphorylation was found within the canonical pathways indicating ROS production.

Unfolding protein response is another appealing canonical pathway existed in pathway analysis. With the up regulation of *DNAJB9, EDEM1* and *PDIA6* all of which indicate endoplasmic reticulum (ER) stress (Shen et al., 2002; Olivari and Molinari, 2007; Vekich et al., 2012). It has been reported that, if ER stress prolong and remain unresolved this might trigger autophagy and eventually leading to cell death (Luo and Lee, 2012).

Primarily, 45 genes were found to be involved in HCC inhibition however, most of their involvements were labelled as unpredicted effect. Therefore, in the

interest of more conclusive findings, theses 45 genes were re-analysed. In comparison to other experimental groups, gene expression pattern showed a clear difference in the expression of these 45 genes implying the specificity of these genes to G6 (H SF). In addition to that, GO term enrichment revealed important biological processes empathizing on the decline in cell development, regulation of wound healing, decrease in cell proliferation and the negative regulation of both apoptosis and programmed cell death was declined.

The re-analysis of these 45 genes reinforced the inhibition of two functions of interest; incidence of malignant tumor and angiogenesis. Genes involved in the decrease of incidence of malignant tumor were *ACOX1*, *CTNNB1* and *CDH1* whereas genes involved in the decrease of angiogenesis of tumor were *BRAF*, *CDH1* and *SERPINC1*. The rest of *IQGAP1*, *KARS* and *BRAF* shared unpredicted effect while *KDR* showed inconsistent relationship.

Acyl-CoA oxidase 1 (ACOX1), is a rate-limiting enzyme of the peroxisomal fatty acid β-oxidation pathway of very long chain fatty acids (VLCFAs) (Vluggens et al., 2010). It has been established that deficient ACOX1 resulted in accumulation of VLCFAs and led to growth retardation, microvesicular steatohepatitis, apoptosis, liver regeneration, oxidative stress and ultimately HCC development (Huang et al., 2011).

Catenin beta 1 (CTNNB1) is a cell surface cadherin protein complex subunit utilized as intracellular signal transducer in WNT signaling pathway; dysregulation in its activity has been strongly related to HCC progression (Monga, 2015). Unexpectedly, CTNNB1 has recently been introduced as tumor suppressive where KO mice experienced an intense HCC development after DEN treatment (Zhang et al., 2010) while another study documented the same results after DEN and phenobarbital

treatment (Rignall et al., 2010). E-cadherin (CDH1) is transmembrane glycoprotein within the cadherins family essential for cell-cell adhesion in calcium dependent manner. Vital E-cadherin is a calcium-dependent cell-cell adhesion molecule playing a crucial in epithelial architecture establishment and cell polarity and differentiation maintenance (Liu and Chu, 2014). CDH1 has been recognized as tumor suppressor in number of cancers including lobular breast cancer, diffuse gastric carcinoma, ovarian cancer and colorectal carcinoma (Schrader et al., 2007; Kim et al., 2016; Wang et al., 2016). CDH1 role in HCC was inconclusive and was thought early on to promote HCC growth (Wei, 2002). However, more recent accumulated evidence confirmed CDH1 role in suppressing liver carcinogenesis (Nakagawa et al., 2014; Schneider et al., 2014; Zhu et al., 2017). CDH1 is also interestingly involved in binding and regulating CTNNB1 thus mediating growth suppression (Gottardi et al., 2001).

Serpin family C member 1 (SERPINC1) is type of serine proteinase inhibitors also known as antithrombin, it prevents coagulation factors IX and X (Heit et al., 2013). Its deficiency is associated with several diseases including liver cirrhosis, liver cancer, nephropathy and disseminated intravascular coagulation (DIC) (Lu et al., 2017). SERPINC1 was described to effectively inhibit tumor angiogenesis in a mouse fibrosarcoma model (Larsson et al., 2000). However, its exact role in HCC is not well explored.

Mitogen-activated protein kinase (MAPK) pathway consist of series of serine/threonine kinases which convey intracellular signals from extracellular molecules such as growth factors, differentiation factors, hormones and tumor-promoting substance for cell proliferation, differentiation and survival purposes. Mainly, RAS, RAF, MEK and ERK are the key protein kinases in this pathway (Yang

and Liu, 2017). It has been found that 30% of cancers bar an activated RAS/RAF/MEK signaling cascade (Zhang et al., 2011). Thereby, a collective preclinical and clinical evidence compiled on irregular activation of Ras/Raf/MEK/ERK signaling pathway in HCC (Yang and Liu, 2017). Notably, blocking or inhibiting Ras/Raf/MEK/ERK signaling pathway has exhibited several anti-cancer activities (Li et al., 2016). In the present study, *KRAS*, a member of RAS family and *BRAF*, a member of RAF family, were significantly down regulated upon safranal treatment and were associated with decrease of angiogenesis and tumor incidence.

Finally and through string network analysis, regulatory hubs were identified based on the number of connections to other components in the same network. First network analysis of 45 genes involved in HCC inhibition, KRAS and CTNNB1 were recognized as regulatory hubs and similar outcome is drawn from the network analysis of 8 genes of interest. In addition to IQGAP1 which is strongly associated to HCC progression and can be used as HCC biomarker (Xia et al., 2014).

Taken together, this study has assessed safranal's anti-cancer properties on DEN induced HCC model through a transcriptomic analysis. Such analysis demonstrated a unique gene expression profile of safranal treated group and investigated associated biological processes and correlated canonical pathways in such a therapeutically treated animals. Current study, revealed key genes associated with HCC inhibition and explored further to attain their value as therapeutic candidates upon safranal treatment.

Chapter 5: Conclusion

In conclusion, transcriptomic analysis herein clearly indicated the therapeutic effects of safranal against HCC *in vivo* model. Although signs of drug tolerance was shown still that did not devalue its substantial anti-cancer effects. This study demonstrated safranal's involvement in mitochondrial dysfunction, induced oxidative stress and increased ROS production and ultimately leading to cell death. Moreover, safranal caused disruption of the HCC activated MAPK pathway or Ras/Raf/MEK/ERK signaling pathway which in turns effects cell proliferation, differentiation and survival.

Transcriptomic analysis using RNA sequence provides huge data to process yet it only offers one side of the story. The assumption that RNA transcripts corresponds perfectly to the expression of proteins generated is not always true. That is largely due to post-translational modifications. Hence, a proteomic study should follow up to deliver further insights into protein expression and interaction.

Given the growing realization on the fact that single anti-cancer agent may not be as successful combating cancer, combination therapy seems to hold captivating qualities evading drug resistance and expansion of drug synergy effects. Further studies are currently underway to investigate the synergistic properties of safranal and sorafenib.

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