# CURCUMIN AND EGCG: POTENTIAL CANCER THERAPEUTICS

By

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A Thesis Submitted in Partial Fulfillment

of the Requirements for the Degree

Master of Science

Major: Biology

West Texas A&M University

Canyon, Texas

May 2018

*Dedicated in the loving memory of my Father, my Hero*

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## ABSTRACT

The incidence rates of colon and liver cancer differ dramatically between Northern and Southern India. It has been suggested that diet plays an important role in their occurrence. The consumption rates of curcumin and EGCG in these regions are diverse as well. The compounds chosen for this study are curcumin, an active agent of turmeric and epigallocatechin gallate (EGCG), an active compound of green tea. Numerous studies have demonstrated the multi targeted and beneficial effects of curcumin and EGCG as chemo preventive agents. The problem of using high concentrations of natural compounds to be effects can be overcome by using a combination of these compounds. This study seeks to demonstrate the individual and combined effects of two natural compounds on liver and colon cancer cell lines and their role when used in combination with regorafenib, a chemotherapy drug used currently in the treatment for both colon and liver cancer. A probe of 84 cancer pathway genes determined that the curcumin and EGCG combination targets 28 genes in hepatocarcinoma (HCC) and 14 genes in colorectal cancer (CRC). Our study confirmed the beneficial effects of ARNT, FGF2, IGFBP7 and SERPINF1 expression in HCC and ANGPT2 and IGFBP7 expressions in CRC when treated with low concentrations of curcumin and EGCG. HepG2 cells treated with regorafenib showed regulatory effects on FGF2 expression very similar to curcumin and EGCG combination treatment. IGFBP7 expression was significantly affected in both HCC and CRC when treated with regorafenib, plus curcumin and EGCG combination. In CRC, ANGPT2 expression was significantly affected by regorafenib.

## ACKNOWLEDGEMENTS

First of all, I would like to express my heartfelt gratitude to my adviser Dr. Donna Byers. Her constant guidance, motivation and care helped me greatly to complete my thesis on time. Her affable nature, attention to details, and passion for science made these two years of research an exciting and memorable experience for me. I consider myself very lucky to have had her as my thesis adviser. I am also thankful to her for setting an excellent example as a successful researcher and mentor.

I would like to thank Dr. David Khan for giving great insights about my thesis and accommodating me in his lab. My sincere thanks to my committee members Dr. Rex Lee and Dr. Rocky Ward for their valuable time reviewing my thesis. I am especially grateful for the Killgore Research Committee members for offering me student grant for two consecutive years.

Most importantly I would like to thank my family, especially my mother for making all my dreams come true and being a strong wall. It is her love for science that inspired me to follow a career in biology. Her courage, love and strength are what crafted me the person I am today, and I owe my every success to her. I would like to thank my brother for being supportive and loving. I also express my heartfelt thanks to my best friend Asvin Vaidyanath for helping me throughout. Last but not the least I would like to thank my husband for his constant support and motivation, without whom none of this would have been possible.

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# CHAPTER 1

# INTRODUCTION

According to the World Health Organization (WHO) report of 2012, 14 million cases of cancer are reported globally each year. The US alone reported 1.6 million new cases in 2014. In addition, the incidence of cancer is expected to rise by 70% in the next 20 years worldwide. One of the promising treatments for cancer includes chemoprevention using natural compounds. It is estimated that approximately one-third of cancer deaths could be prevented by appropriate dietary modifications (Khan et al., 2008). There are several naturally occurring chemo preventive compounds that can be used in the treatment of cancer, among which curcumin and Epigallocatechin-3gallate (EGCG) are noteworthy. Colon cancer is the third most commonly diagnosed and second leading cause of cancer deaths among both men and women in the USA (American Cancer Society). Liver cancer is the tenth most common cancer and fifth major cause of cancer deaths in males, and eight major cause of cancer deaths in females in the US (American Society of Clinical Oncology 2016). Interestingly, the incidence of colon cancer is much lower in India compared to the USA, however the incidence of liver cancer is higher (Kumar et al., 2014). In fact, worldwide, hepatocarcinoma has the fifth highest mortality rate of all cancers reported (Forner et al., 2012).

### 1.1 Cancer mechanism

Cancer arises when the suppression of cell-level functions goes unchecked (Maynard Smith et al., 1995). DNA replication and cell division are a series of events that collectively form the cell cycle. There are two major types of cell cycle control mechanisms that are recognized. The first type of control involves the cyclin-dependent kinase (CDK) family which is highly regulated. Kinase activation requires an association with a second subunit, a cyclin, to create an active complex with unique substrate specificity. The second type of control is the establishment of checkpoints. Cell cycle checkpoints detect flaws during DNA replication and chromosome segregation (Elledge and Stephan., 1996). The connection between the cell cycle and cancer is inevitable. CDKs solely control the specific transitions in the cell cycle. In a non-cancerous cell, the beginning of replication is controlled by cyclin D-CDK4/6 at the restriction point (Musgrove et al., 2011). CDK4/6 initiates the phosphorylation of the retinoblastoma protein family, thereby activating E2F transcription factor which in turn initiates the S phase gene expression program (Malumbres and Barbacid., 2009). Many oncogenes overcome the restriction point by promoting CDK4/6 activity (Huillard et al., 2012) by amplification of CCND1 or homogenous deletion of CDKN2A (Pinyol et al., 1997).

Chk 1 protein kinase is one of the main components of the DNA damage and check point pathways. (Zhou and Elledge., 2000). Chk1 regulates the check points by targeting Cdc25A at the G1/S and S check points and Cdc25A and Cdc25C at G2/M check point (Peng et al., 1997). Chk 1 is activated when there is a DNA damage which in turn activates ATR/ATRIP complexes. Chk1 activation requires mediator genes such as, claspin, BRCA1, and TOBP1. Loss of the G1 check point is a leading cause of cancer due to

mutation of p53. This makes the DNA reliant on S and G2 check points for triggering cell death. Studies showed that when S and G2 check points are inhibited by the inactivation of ATR or Chk1, the DNA mutation is passed on to the next phase. (Carrassa et al., 2004). The Wee1 kinase family, along with Chk1 is considered a very important check point controlling protein that controls the G2 phase by regulating its timing. Wee1 is also important for maintaining the integrity of the genome during DNA replication (Sorensen and Syljuasen., 2012).

### 1.2 Oncogenes and Tumor Suppressors

Currently, the known list of verified oncogenes is 70 associated with germline mutations, and 342 associated with somatic mutations (American Cancer Society). Mutations that lead to cancer occur in two types of genes, namely the proto-oncogenes and tumor suppressor genes, which are very important for the normal functioning of the body. Proto oncogenes encode proteins that are important for regulation of cell division, differentiation, and apoptosis. When mutated, these proto oncogenes become oncogenes. In other words, because their normal function involves accurately directing the events of cell proliferation, their mutation results in unregulated cell replication, abnormal cell growth and aberrant differentiation. Oncogenic activation can be caused by point mutations, localized reduplication, or chromosomal translocation. Common oncogenic mutations are Her-2/neu, seen in breast cancer, K-ras mutation in pancreatic cancer, and MITF mutation in melanoma (Weinstein et al., 2006).

Mutations in tumor suppressor genes, the 'break mechanisms' of cell replication, lead to "loss of function" leading to cancer. The studies done on sequencing and characterization

of these genes involved in cancer show that they are normal genes gone awry and are not parts of foreign DNA. Tumor suppressor genes' natural function is to protect against neoplastic transformation. They have a long evolutionary history with the ability to regulate diverse and fundamental cellular processes including genome maintenance growth, division, differentiation, metabolism and death (Sherr., 2004; Pearson and Sanchez., 2008). The evolutionary pressure on tumor suppressor pathways relates to environmental influences and differences in metabolism and immune systems. This in fact leads to their activation and expression in a context dependent manner. This also accounts for why tumor suppressor pathways have different components in different species (Belyi, et al., 2010). In addition, the tumor suppressor gene function varies within a species with respect to developmental stage and age (Kim and Sharplers., 2006). Tumor suppressor genes could also play a very important role in tissue regeneration. However, in a context specific manner, some tumor suppressor genes also have regeneration suppressor activities.

### 1.3 Co-operation and Multicellularity

The breakdown in co-operation at the multicellular level is another main basis for cancer (Strassmann et al., 2010). Multicellularity refers to the combination and co-operation of cells, tissue, organs and the organism, leading to the development, maintenance and reproduction of the organism (Michod., 2007). Multicellularity has five major components which include apoptosis, inhibition of cellular proliferation (Alberts et al., 2002), division of labor (Maynard Smith et al., 1995), transportation of resources (Knoll., 2011), and maintenance of the extracellular environment. Malfunctioning in any of these domains can lead to tumorigenesis (Michod., 2007). Tumorigenesis is activated by carcinogens, tumor promoters, inflammatory agents and other factors. Carcinogens have the ability to

upregulate anti-apoptotic genes, thus inhibiting the process of programmed cell death. They also commonly interfere with the regulation of growth factors, cell cycle proteins, cell-adhesion molecules and transcription factors (Aggarwal and Shishodia., 2006). Apoptosis is a natural mechanism used by multicellular organisms to control proliferation of cells (Evan et al., 2001). Failure in the apoptosis mechanism leads to uncontrolled and unchecked cell replication leading to continuous cell proliferation (Hanahan et al., 2011). Division of labor is another significant feature of multicellularity (Ispolatov et al., 2012). The differentiated cells are assigned specific functions which are important for the efficient maintenance of tissue (Bell et al., 1997). In the case of cancer cells, inappropriate cell differentiation leads to increased severity of tumors (Tenen et al., 2003). Transportation of resources to cells via less wasteful mechanism are important, which requires a multi-step, complex co-operation of tissue and cell functions (Knoll et al., 2011). However, the disruption of this complex system enhances the severity of cancer by means of angiogenesis (Hanahan et al., 2011). Creation and maintenance of an extracellular environment, which consists of networks of proteins, is an essential factor for multicellularity. (Hynes., 2012). Cancer cells use factors such as matrix metalloproteinases (MMPs) to destroy the protein network leading to cancer cell invasion (Hanahan et al., 2000). The extracellular matrix is also invaded and destroyed by this process (Gatenby et al., 2003). The immune response, which is responsible for keeping the invading factors away is compromised, resulting in tumor growth via inflammation (Bissell et al., 2001).

### 1.4 Metastasis

Despite new advances in surgical techniques, radio therapies and molecular targeted therapies, cancer continues to prevail through the progressive growth of metastatic tumors

that are resistant to the current therapies. The real problem in cancer therapy is the development of metastases which undergo spontaneous mutations (Fidler., 2003).

One particular example of metastastatic cancer that does not benefit from therapy, compared to other metastasized cancers, is bone cancer. Bone metastasis pathophysiology is very complex and is primarily metastasized from breast cancer and prostate cancers (Guise., 2010). CXCL12, which plays a role in immune surveillance, embryogenesis, tissue maintenance, and tumor growth and metastasis, is expressed by the stromal cells in target organs of metastastatic breast cancer in bone, brain, liver, lung, lymph node but not in other tissues. An interesting study was done in 1998, where women with breast cancer were treated with adjuvant clodronate combined with surgery and other hormonal therapy. It demonstrated that new metastasis was significantly reduced but metastasis to bone was not reduced and after a follow-up of 36 months, invasion of bone marrow was observed. The presence of tumor cells in bone marrow indicates hematogenic metastasis. Three quarters of women in the study died of breast cancer that had metastasized to their bones (Diel et al., 1998).

Pathological bone remodeling in breast cancer is a result of paracrine signaling between breast cancer cells and stromal cells. Bone resorption releases TGF-β from bone matrix which activates a feedback mechanism for increased production of parathyroid hormone (Roodman., 2004) One possible option for targeting bone metastasis could be to target PTHrp (Para Thyroid hormone related protein). However this strategy is not effective for metastases to other organs, as only 17% of non-bone metastases express PTHrp. In addition, PTHrp targeting by neutralizing antibody therapy cannot be a reliable therapy for even bone metastasis because recent studies showed several PTHrp-independent osteolytic

pathways (Kang et al., 2005). Liver metastasis from breast cancer is primarily due to expression of VCAM-1 (Langley et al., 2001). Some reports show that, UEGFR-1 is activated in distal lung endothelial cells in order to increase their expression of MMP-9. Stromal products produced in the lung microenvironment can enhance resistance of pulmonary metastasis to chemotherapeutic drugs (Willmanns et al., 1992).

Liver is another frequent site of metastasis for breast cancer. Forty to fifty percent of women with metastatic breast cancer are diagnosed with liver metastasis (Diamond et al., 2009) TGFα/EGFR paracrine signaling networks create a microenvironment for liver metastasis. One dilemma with targeting metastatic cancer is that the sequence of mutational events leading to the tumor progression is most often not understood clearly. Another is that there are several alternative pathways involved, hence therapies for metastasis to one particular organ has to be very specific which will not work or have minimal effects on other metastasized cancers.

Tumor microenvironment targeting therapy for advanced cancers focuses on angiogenic blood supply that supports tumor growth and is gaining much interest in the current research. However, successful preclinical experiments fail to produce significant results when tested on patients (Fersara et al., 2004). Some examples such as bevacizumab, carboplatin and paclitaxel combination showed significant success in preclinical studies, but added only an increase of 2 months overall survival compared to non-targeted therapies in clinical studies (Sandler et al., 2006). This suggests that the tumor cells and host cells may both contribute to the treatment response (Bergers, et al., 2008). Therefore, one treatment which targets and is successful in one tumor microenvironment can potentially have no effect on another.

## 1.5 Colorectal Cancer Pathways

The incidence rate of colorectal cancer (CRC) is highly variable worldwide (Jemal et al., 2011). Colorectal carcinoma has a very complex pathogenesis and is influenced by a variety of factors including diet, lifestyle and genetic predisposition (Le Marchand et al., 1997). There are numerous pathways involved in the initiation of neoplastic transformation of CRC leading to metastasis and progression. One important pathway that most CRC follows is the Chromosomal Instability Pathway (CIN) which leads to chromosomal abnormalities (Leary et al., 2008). Approximately 15% of CRC is due to abnormalities in the DNA Mismatch Repair (MMR) system, leading to microsatellite instability (Lin et al., 2003). Abnormal DNA methylation and inflammation are other systems and pathways that contribute to the carcinogenesis of CRC.

In the CIN pathway, mutation in adenomatous polyposis coli (APC) tumor suppressor gene is the key for tumorigenic process initiation (Bardi et al., 1993). Mutations in APC are involved in both sporadic and familial polyposis (Shih et al., 2001; Sieber et al., 2002). This APC gene is very important for the β catenin pathway. Inactivation of the APC gene results in WNT pathway signaling by failing to degrade β catenin. The WNT/ β catenin pathway is altered by regulatory genes interacting with the APC gene (Chan et al., 1999). This results in increased proliferation and migration of colorectal cells (Sparks et al., 1998). The other genes such as CDK 8 and Cyclin D1 also are responsible for tumor progression. CDK8 gene overexpression stimulates Notch 1 and β catenin, resulting in the increase in oncogenic activity (Firestein et al., 2008). On the other hand, Cyclin D1, inhibits p27 and p21 leading to the development of neoplasia, by restricting apoptosis (Alao et al., 2007).

Following mutation of the CIN pathway, subsequent events in the RAS pathway lead to tumor progression (Malumbres and Barbacid 2003). Mutations in K-ras gene, which is a proto-oncogene, develops a permanent state that allows cell proliferation and restricts apoptosis. It is also known that dysregulated AMP-activated protein kinase (AMPK) results in the deactivation of p53 (Baba et al., 2010).

Dysregulated DNA MMR leads to a 100-fold increase in the mutation rate of colorectal cells (Thomas et al., 1996). Moreover, hMLH1 and hMLH2 mutations increase the risk of CRC (Aaltonen et al., 1994). TGF β receptor II mutations are found in about 80% of Microsatellite Instability system (MSI)-CRC (Riggins et al., 1997) which is further facilitated by mutations in smad4 (Zhang et al., 2010). In addition, the Activin receptor type-2B dysfunction is regulated by smad2 and smad3 proteins (Jung et al., 2004). Finally, a major cause for MSI-CRC is mutations in Bax, a critical pro-apoptotic protein. (Rampino et al., 1997).

The initiation and progression of colorectal cancer is highly influenced by inflammation. This is facilitated by increased DNA damage via activation of mutagenic reactive oxygen (ROS) and nitrogen species (NOS) and promoting anti-apoptotic systems (Coussens and Werb, 2002). TNFα levels, when high, can promote tumor growth. Interleukin-6 (IL-6) stimulates the STAT 3 pathway activation (Terzic et al., 2010). This in turn leads to the induction of expression of Bcl-2, cyclin D1, MMP 2-9 and Cox-2 (Ma et al., 2004) (Corvinus et al., 2005).

### 1.6 Hepatocellular Carcinoma Pathways

Hepatocellular carcinoma (HCC) is the result of a complex combination of genetic and epigenetic alterations. Mutation studies suggest that the most frequently mutated genes responsible for hepatocellular carcinoma are TP53 and CTNNB1 (Hoshida et al., 2010).

Pathways involved in cell proliferation, migration and angiogenesis are deregulated in HCC (Farazzi and Dephinho 2006). The most important pathways deregulated in HCC include growth factors such as Insulin like growth factor (IGF), Epidermal growth factor (EGF) and Vascular endothelial growth factor (VEGF) (Villanueva et al., 2007). IGF is important for the regulation of cell growth and development. The allelic loss of IGFR2 receptor and the overexpression of IGF2 ligand results in carcinogenesis in HCC (Villanueva et al., 2007). Studies show that the blocking of IGF signaling promoted anti-tumor effects in HCC models (Tovar et al., 2010). Another important pathway that contribute to HCC progression is the Human Growth Factor/ Mesenchymal-Epithelial Transition (HGF/MET) pathway and about 40% of HCC patients show MET activation (Kaposi-Novak et al., 2006). EGFR dysregulation is also an important factor for HCC development (Hoshida et al., 2004). A single nucleotide polymorphism in the EGF gene correlated with HCC prognosis by increasing the ligand half-life (Tanabe et al., 2008). Levels of VEGF are also significantly higher in HCC, leading to aggressive cancer behavior and angiogenesis (Poon et al., 2004).

Ras mutations, typically high in many other cancers, are rare in HCC (Villanueva and Llovet 2011), but AKT and MTORC1 mutations play an important role in HCC progression (Villanueva et al., 2008). Along with the growth factor-related pathways, WNT

signaling, Notch 1 and Hedgehog pathways also play a vital role in HCC prognosis (Villanueva et al., 2007).

### 1.7 Polyphenols

Nutraceuticals are polyphenols derived from natural products such as leaves, seeds and fruits, etc. (Bucar et al., 2013). It is known that these polyphenols play a vital role in the plant immune system (Tsao., 2010) which makes them potential therapeutics for several ailments including cancer. Many of the chemotherapeuticals used currently for cancer treatment are derived from plant sources (Newman and Cragg., 2016). The polyphenols are classified into three major groups, specifically, flavonoids, stilbenoids and phenolic acids (Tsao., 2010). Chemoprevention refers to using naturally occurring or synthetic compounds to completely prevent or control cancer (Ames et al., 1995). Polyphenols are not only potential cancer therapeutics, but also excellent agents for chemoprevention (Nihal Ahmed et al., 1997). Initially polyphenols gained interest in cancer therapy due to their antioxidant properties. However, recent research shows that polyphenols can interact with proteins, enzymes, receptors and transcription factors, thereby directly leading to physiological changes at the molecular level (Quideau et al., 2011). Tumorigenesis can be activated by environmental factors such as carcinogens, stress and inflammatory agents which activate the signal transduction pathway, providing an advantage to tumorigenesis (Aggarwal., 2006; Allavena et al., 2008). Many polyphenols have the ability to alter the regulation of the cell cycle, thereby inducing apoptosis (Fesus., 1995). Clinical and experimental studies have shown that curcumin and EGCG have the ability to inhibit proliferation and thereby induce apoptosis, making it a candidate for chemoprevention and therapy (Shimizu et al., 2011).

### 1.8 Mechanism of polyphenols

Carcinogenesis is a process by which a normal cell becomes a cancerous cell via progressive transformation (Bertram J S., 2000). The signal transduction pathways involved in carcinogenesis enhance oncogenic signals by interacting with each other, resulting in cell malignancy (Hanahan and Weinberg., 2000). Polyphenols can interact and interfere with these pathways, and thereby inhibit cancer by enhancing or inhibiting the genes involved in these pathways (Benvenuto et al., 2013). For example, ErbB receptors bind with specific ligands leading to trans-phosphorylation of ErbB receptors, which activate the mitogen-activated protein kinase (MAPK) pathway and lead to uncontrolled cell proliferation (McKay and Morrison., 2007). Polyphenols such as curcumin, EGCG and Resveratrol affect ErbB receptor downstream signaling in several cancer types, including breast, prostate, lung and pancreatic cancers (McKay and Morrison., 2007) (Cai X Z et al., 2009).

Curcumin has been shown to induce apoptosis, inhibit cell proliferation and decrease epidermal growth factor receptor (EGFR) in several cancer types (Sun., 2012). Curcumin can also induce apoptosis in a p53-independent fashion by activating p38 kinase (Watson et al., 2010). In addition, Curcumin can interfere with cell cycle progression and arrest at G2/M passage, thereby inducing apoptosis in medulloblastoma cells (Tang et al., 2010) EGCG, an anthocyanin, has the ability to inhibit cell proliferation by reducing nuclear translocation of NF-kB/ p65 in epidermal carcinoma (Gupta et al., 2008). Anthocyanin is capable of reducing the expression of NF-kB and COX-2 in esophageal tumors (Wang et al., 2009) Delphinidin, an anthocyanin responsible for the blue color to viola and delphinium, has been shown to reduce proliferation and promote apoptosis (Yun et al.,

2009). It can also induce cell growth arrest and caspase-dependent apoptosis by inhibiting NF-kB- DNA binding activity (Hafeez et al., 2008). Resveratrol, a potent anti-inflammatory found in red grapes, peanuts and legumes, down regulates Bcl-2 expression in MCF-7 breast cancer cell lines (Pozo-Guisado et al., 2005). The HH/GL1 cascade is a complex multi-step pathway for controlling cell proliferation and differentiation during embryogenesis (Mimeault et al., 2010). Curcumin and EGCG both have modulatory activity on the HH/GL1 pathway (Tang et al., 2010). Polyphenols have also been shown to interact with genes regulating angiogenesis in cancer (Platiet al., 2011)

### 1.9 Curcumin

Curcumin is a hydrophobic polyphenol isolated from *Curcuma Longa* (Aggarwal et al., 2009). Curcumin is used in traditional Ayurveda medicine for the treatment of several ailments such as ulcers, arthritis, psoriasis, jaundice and cancer (Singh, 2007). Curcumin has the capacity to interact and regulate multiple molecular targets such as growth factors, transcription factors, kinases, cytokines and apoptosis related proteins (Aggarwal and Mishra, 2009). Curcumin binds directly with more than 30 different kinds of proteins including DNA polymerases, Focal Adhesion kinases (FAK) and Protein Kinase C (Takuchi et al., 2006; Leu et al., 2003; Reddy and Aggarwal, 1994). Curcumin can also affect the expression of transcription factors by activating or inhibiting them, depending on their targets (Shin et al., 2009).

Studies have shown that curcumin inhibits the expression of activated protein 1(AP1) and NF-kB, both of which are important transcription factors that influence the expression of genes involved in inflammation, apoptosis, oncogenesis and lymphoid differentiation (Li Q

and Verma, 2002). Curcumin inhibits the expression of signal transducer and activator of transcription (STAT) proteins, increased levels of which result in angiogenesis (Bhattacharya et al., 2007). It also inhibits β catenin, which plays a crucial role in cell-cell adhesion, the malfunctioning of which leads to tumorigenesis (Prasad et al., 2009). Curcumin activates several transcription factors such as, aryl hydrocarbon receptor (ArH), important for cell proliferation, gene regulation and inflammation (Rinalde et al., 2002), early growth response-1, a tumor suppressor gene (Panagiotis et al., 2015) and activating transcription factors (ATF3) (Tanaka et al., 2011).

Curcumin plays an important role in inhibiting angiogenesis by controlling the regulation of growth factors such as VEGF, FGF and EGF (Strimpakos and Sharma., 2008). Studies have shown that curcumin exhibits anti-apoptotic and anti-proliferative effects by regulating the growth factors which play an important role in the normal processes of the cell cycle (Gupta et al., 2012). When these molecules are unregulated they lead to malignancy by inducing constitutive cell proliferation (de jong et al., 2001). Curcumin exhibits chemo-preventive effects in an estrogen receptor expression-independent pattern (Verma et al., 1998). CXCR4, a chemokine receptor which is widely expressed in immune and central nervous system (Zou et al., 1998), plays an important role in cell migration, proliferation and survival. Dysregulation of this chemokine receptor leads to metastasis in tumor cells (Xu et al., 2015). In one study, curcumin showed an inhibitory effect on CXCR4 expression in follicular lymphoma cells, suggesting strong anti-metastatic activity (Skommer et al., 2007). Curcumin has also been shown to induce apoptosis by several pathways. In one study using a microarray assay, it was demonstrated that curcumin affected the regulation of 104 out of 218 apoptotic genes (Ramachandran et al., 2005).

Curcumin may also functionally activate p53 gene expression. Studies have shown that curcumin induced apoptosis via overexpression of the p53 protein in glioma, breast cancer and prostate cancer cell lines (Choudhuri et al., 2005; Liu et al., 2007; Shanker and Srivastava, 2007). Curcumin has also been shown to regulate other important molecules such as HSP70, a potent buffering system for extrinsic and intrinsic cellular stress (Shen et al., 2007), multidrug resistance protein (Wortelboer et al., 2003) and cyclin D1 (Kannumakkara et al., 2009).

### 1.10 EGCG

(-) – Epigallocatechin-3-gallate (EGCG) is one of the major components of green tea. It constitutes about 50-80% of the total plant components. (Khan et al., 2006). EGCG has been shown to have beneficial effects against cancer, diabetes, stroke and obesity (Higdon and Frei., 2003; Shankar et al., 2008). EGCG is an antioxidant and has been demonstrated to suppress the inflammatory process which is the leading cause for transformation due to carcinogenesis (Thawonsuan et al., 2010). Studies suggests that EGCG has the ability to block the progression of tumors by sealing the receptors of the affected cells. EGCG can also alter the expression of receptors themselves. (Sigler and Ruch., 1993). There is also evidence that EGCG can bind directly to carcinogens and inactivate them, hence it can be used in potential cancer prevention (Sigler and Ruch., 1993). EGCG can potentially induce apoptosis by promoting cell growth arrest, and it plays an important role in activating caspases and downregulating NF-kB (Gupta et al., 2004). EGCG affects the mitogen-activated protein kinase dependent pathway and ubiquitin/proteasome degradation pathways (Khan et al., 2006). EGCG's ability to down-regulate NF-kB, also results in inhibition of the expression of Bcl-2 in human lung cancer PC-9 cells (Fujiki et al., 2001),

and stabilization of p53 expression (Hastak et al., 2003). EGCG also inhibits the activity of important cell cycle promoters CDK2, CDK4, CDK6 (Masuda et al., 2001). At low concentration (20μM), EGCG induced G0/G1 phase cell cycle arrest in head and neck squamous cell carcinoma cells (Masuda et al., 2001).

### 1.11 Combinations of polyphenols

Studies have shown that the treatment of cancer with combinations of polyphenols are much more effective than using polyphenols individually. In one study, Pterostilbene and Quercetin were used to treat highly malignant B16 melanoma F10 cell line. The combined polyphenols inhibited metastasis at the G0/G1 phase. This was later confirmed by *in vivo* studies (Ferrer et al., 2005). In another study, the combination of Geinstein and Thearubigin inhibited prostate cancer in the PC-3 cell line, however Thearubigin alone did not have any beneficial effect (Sakamoto., 2000). Combinations of Quercetin and EGCG showed inhibition of proliferation in human prostate cancer *in vitro* and *in vivo* more effectively compared to the effects of either of the compounds separately (Wang et al., 2012). A study done by Zhou et al, showed the combination of curcumin and EGCG at low concentrations effectively blocked the G1 and S/G2 phase, thereby inducing cell cycle arrest of NSCLC cell lines (Zhou et al., 2013). *In vivo* studies showed that the combination of curcumin and EGCG, in a lung cancer xenograft mouse model, not only suppressed the tumor growth, but also showed no toxicity, suggesting the combination of these compounds is effective in the treatment of human lung cancer (Zhou et al., 2013).

In a study done by Somers-Edgar, female athymic nude mice implanted with MDA-MB-231 cells, were treated with a combination of curcumin (200mg/kg/day) and EGCG

(25mg/kg/day) for 10 weeks. The mice showed significant reduction of tumor size, however this was not achieved when treated with either of the compounds separately (Somer-Edgar et al., 2008). The combination of curcumin, EGCG and arctigenin, an anti-inflammatory lignin, improved the chemopreventive effect in LNCaP prostate cancer cell line and MCF-7 breast cancer cell line. Both arctigenin and EGCG enhanced the ability of curcumin to induce apoptosis in these cancer cell lines. This effect was not achieved when the compounds were used separately. The combination of these compounds reduced the expression of NF-kB, P13 K/Akt, and STATS (Wang et al., 2014). Combinations of curcumin with raspberry extract and neem leaf extract induced apoptosis in human pancreatic cells by enhancing the activity of caspase 3 and caspase 7 and inhibiting the NF-kB –DNA-binding activity induced by radiotherapy (Veeraragavan et al., 2011).

### 1.12 Combination of polyphenols with combination drugs

Polyphenols are potential cancer therapeutics, as they can be combined with chemotherapy drugs, thereby increasing their efficacy and reducing the risk of side effects and drug resistance. Curcumin increased the anti-proliferative and apoptotic effects of Celecoxib, when used in combination in pancreatic adenocarcinoma cells (Lev-Ari et al., 2005). Mice with orthopedic pancreatic tumors, when treated with curcumin and Gemcitabine showed a decrease in the tumor volume via down-regulation of NF-kB-regulated genes such as cyclin D1, Bcl-2, Bcl-xL, COX-2, VEGF and apoptosis protein-1. This effect was not observed in mice treated with Gemcitabine alone. (Kannumakkara et al., 2007). In a study by Khan et al, curcumin inhibited Paclitaxel-induced NF-kB activation, thereby enhancing the induction of apoptosis by Paclitaxel in breast cancer cells. A study on athymic mice showed that, *in vivo*, the combination of curcumin and Paclitaxel led to tumor suppression

and reduced tumor proliferation rate (Kang et al., 2009). The anti-cancer activity of Centchroman (CC) was enhanced when breast cancer cells were pre-treated with a combination of curcumin and resveratrol. The combined treatment led to the up-regulation of caspase-9 expression, phosphorylation of p53 and altered Bcl-2/Bax ratio (Singh et al., 2012).

EGCG enhanced the activity of Oftamoxifen and Sulindac, drugs used for inducing apoptosis in PC-9 cells when used in combination (Suganuma et al., 1999). Likewise, EGCG when combined with Celecoxib upregulated growth arrest and expression of GADD153, thereby enhancing the induction of apoptosis in human lung cancer cell lines (Suganuma et al., 2006). EGCG, in combination with the Cox-2 inhibitor NS398, enhanced apoptosis by altering the Bax/Bcl-2 ratio and up-regulated the expression of caspase-9 and caspase-6 in prostate cancer cells (Stearns et al., 2011). Similar effects were seen with EGCG-Doxorubicin combination in prostate cancer (Stearns et al., 2010). EGCG and Doxorubicin also proved effective in the treatment of liver cancer cells. The combination led to suppressed proliferation and inhibited autophagy (Chen et al., 2014). EGCG increased the sensitivity of breast cancer cells to Paclitaxel, when used in combination, via activation of c-Jun N terminal kinases mediated by Paclitaxel (Luo et al., 2010). Finally, EGCG was shown to sensitize ovarian cancer cells to Cisplatin via delivery of hydrogen peroxide, suggesting an increase in oxidative stress may improve the efficiency of chemotherapy drugs (Chan et al., 2006).

### 1.13 Experimental Design

This study sought to test the hypothesis that the combination of two natural compounds, curcumin and EGCG, are effective nutraceuticals in the treatment of hepatocarcinoma and colorectal cancer. In addition, it is proposed that when used in combination with Regorafenib, these compounds will further enhance the effect of this drug on the viability and gene expression in both of these tumor cell lines. Three cell lines, control, HCC and CRC cell lines will be treated with Curcumin and EGCG, separately and in combination, in a time and dose-dependent manner to determine optimal treatment effect on viability and cancer gene expression. Following this, Regorafenib alone and in combination with the optimal dose of EGCG + curcumin, will be used to treat HCC and CRC cell lines. The optimal doses determined by viability results will be used to generate probes to access the effect of these compounds on the expression of 84 cancer pathway genes. Key candidate genes will then be selected for validation by quantitative PCR.

# CHAPTER 2

# MATERIALS AND METHODS

## 2.1 Cell Culture and Treatment:

Three cell lines were used, 1) Adult retinal pigmented epithelium (ARPE-19), 2) Hepato carcinoma (HepG2) and colorectal cancer (SW1417) cells (ATCC) were maintained using standard cell culture technique. The media used was DMEM: F-12, 1:1, supplemented with 1% Pen-Strep, 1% L-Glutamine and 10% FBS (HyClone). Curcumin and EGCG (Sigma) were prepared in advance and suspended in sterile 1X PBS (HyClone) at 1mM stock concentrations, aliquoted and stored at -20 C.

All cell culture experiments to determine dose response were performed in 12-well flat-bottomed culture plates with a concentration of 100,000 cells/well. Experimental exposures were with either curcumin or EGCG alone at 10, 50 and 100uM, or a combination of both compounds at 5, 10, and 25uM combinations. Each single or combination treatment was performed for both 24h and 48h. At the time of plating the necessary volume of drug was added to each well first, followed by the appropriate volume of cell suspension to achieve a final volume of 1.0 ml. All conditions were plated at n=3 and all experiments were incubated at 5% $CO_2$ and 37˚C. Cell viability was measured immediately following each assay.

## 2.2 Drug Dosage and Time Exposure

All cell lines were treated with different dosage of the drug separately and in combination. The varying dosages of curcumin used separately were 5 µM, 10 µM, 25 µM, 50 µM and 100 µM. the doses of EGCG used were 5 µM, 10 µM, 25 µM, 50 µM, 100 µM and 200 µM. the combination of drugs used were in low doses such as, 5 µM curcumin+5 µM EGCG, 5 µM curcumin+10 µM EGCG, 5 µM curcumin+25 µM EGCG, 10 µM curcumin+5 µM EGCG, 10 µM curcumin+10 µM EGCG, 10 µM curcumin+25 µM EGCG, 25 µM curcumin+5 µM EGCG, 25 µM curcumin+10 µM EGCG, 25 µM curcumin+25 µM EGCG and high doses such as, 50 µM curcumin+50 µM EGCG, 100 µM curcumin+100 µM EGCG. The cells were treated with the drugs separately and in combination and incubated for 24 and 48-hour time exposure. Each dosage and time dependent exposure were done in triplicates. Both HepG2 and SW1417 were treated with 2.5 µM regorafenib separately and in combination with 10 µM curcumin+10 µM EGCG and incubated for 48-hour time exposure.

## 2.3 Cell Viability and Statistical Analysis:

Cell viability was determined by manual counts of all samples using a hemocytometer and trypan blue. One-way ANOVA was performed for all treatment comparisons, followed by multiple-comparison T-test, using Prism 6 (GraphPad®) software.

## 2.4 Cell Proliferation

Cell proliferation was assayed using the Cell Counting Kit-8 (Sigma-Aldrich). The cells were suspended in 100 µl/well in a 96-well flat-bottom assay plate. The cells were then treated with different dosages of drugs separately and in combination for a 24 and 48-hour

time exposure. After incubation, 10 µl of CCK-8 solution was added to each well and incubated for 4 hours. The absorbance was measured at 460nm using a microplate reader. Statistical analysis was by One-way ANOVA for all treatment comparisons, followed by multiple-comparison T-test, using Prism 6 (GraphPad®) software.

**2.5 Total RNA isolation and quantification:**

Total RNA was isolated from the samples using the RNaqueous-Micro Total RNA Isolation Kit (Invitrogen™). The drug/media was aspirated from each well of a 6 well plate. 300µl of Cell Lysis buffer was added to each well and gently mixed to lyse the cells. 150µl of 100% ethanol was added and mixed thoroughly. The cell lysate was transferred to sterile 1.5ml microfuge tubes. The entire cell lysate was loaded onto labelled Micro Filter Cartridges. The samples were centrifuged for 10 sec. at maximum speed till all lysate passed through. The filtrate was discarded. 180µl of wash solution 1 was added and centrifuged for 10 sec at maximum speed. 180µl of wash solution 2 was added and centrifuged for 10 sec at maximum speed twice. The samples were centrifuged at maximum speed for 1 min. The columns were transferred to fresh elution tubes. 10µl of preheated elution buffer ($75^0$C) was added to the column and incubated at room temperature for 1 min. The RNA was eluted into the elution tube by centrifuging the tube at maximum speed for 30 sec. A second 10µl of elution buffer was added and the process repeated. The eluted RNA was quantified using NanoDrop Spectrophotometer (Thermo-Scientific™). Absorption was measured at 260nm and 280nm, and the A260/A280 ratios were also calculated to determine purity of samples. All samples were within the ratio range of 2.0 ± 0.1.

**2.6 cDNA synthesis:**

cDNA was synthesized from the isolated total RNA using Verso cDNA Synthesis Kit by Invitrogen® (Thermo-Scientific™). A total volume of 8µl of the master mix containing 5X buffer (4µl), dNTPs (2µl), polyT (1µl) Verso Enzyme (1µl) was prepared for each sample. 500ng of RNA from each sample was added to RNase-free $H_2O$ to equal 12µl, for a total reaction volume of 20µl. The reaction tubes were incubated at 42°C for 30 min. The tubes were then placed at 95°C for 2 min. The samples were then kept in ice for 5 min. A 1:10 dilution of the cDNA was made by adding 180µl of DNase-free $H_2O$.

**2.7 Probe preparation:**

For each condition, 20µl of the samples from that condition were transferred and pooled in a sterile PCR tube and stored at -20 °C, to be used for gene array analysis.

**2.8 qPCR Aliquots:**

50 µl aliquots of each sample was transferred to a corresponding second set of PCR Tubes and stored at -20 °C, to be used for qPCR validation of gene expression.

**2.9 Standard Curve:**

5µl of each sample was aliquoted to a common PCR tube. 100µl of the pooled samples were transferred to a sterile PCR tube and a 5-fold dilution series was prepared by transferring 20µl of the standard to a sterile PCR tube containing 80µl DNase-free water. The standards were stored with the working cDNA aliquots at -20°C.

## 2.10 Microarray

Previously prepared probes of cDNA from all samples in each condition (102µl) were used to assay expression of 84 genes using RT2 Profiler - Cancer Pathway Arrays (Qiagen). A total volume of 2500µl of the master mix containing 1250µl SYBR green, 100µl cDNA template, 1150µl RNase-free water, was prepared. The RT-plate was removed from the sealed bag. 25µl of the of the master mix was added to each well of the array plate. The array plate was then sealed with the Optical Thin-walled 8-cap strips. The array plate was centrifuged at 1000 x g at room temperature for 2 min. The RT array was placed in a CFX-96 Touch Thermocycler (Bio-Rad) and run for 40 cycles, followed by a dissociation (melt) curve.

## 2.11 Primer test for verification of candidate genes by quantitative PCR

Gene selection for verification were based on degree of change observed in RT2 assays, determined by $\Delta C_t$, novelty of expression and relevance to the literature. Gene specific primers were designed for candidate genes of interest and synthesized by IDT Technologies™. All primers were tested for efficiency and amplification of a single product before using for gene validation assays.

## 2.12 qPCR validation of target genes from cancer arrays

Pooled aliquots of cDNA from all samples were used to make a 4 x 5-fold dilution series for the standard curve. In a 96-welled plate, 2µl of the pooled standard and cDNA samples were added in triplicates. 20µl of the master mix containing 10µl of 2X PCR mix, 1µl each of forward and reverse primers and 8µl of DNase-free water was added to each well. Primer efficiency was determined by running a standard curve with each primer pair per

gene. qPCR was run using the following protocol:  3 min at 95°C, 40 cycles of 95°C for 15sec and 60°C for 30sec, followed by a dissociation curve. Ribosomal 18S was quantified for all samples as the internal standard. ARNT, ANGPT2, FGF2, IGFBP7 and SERPINF1 were the candidate genes selected for validation. IGFBP7 was quantified for both untreated and treated HepG2 and SW1417 cells. ARNT, SERPIN and FGF 2 were quantified for untreated and treated HepG2 cells. ANGPT2 was quantified for untreated and treated SW1417 cells.

# CHAPTER 3

# RESULTS

## 3.1 Determination of cell viability in a time-dosage dependent manner after treatment with curcumin and EGCG Separately and in combination

Based on the preliminary data, curcumin did not have any effect on the viability of ARPE-19 cells after 24 hours exposure for varying doses such as 10 µM, 50 µM, 100 µM. However, reduced viability was seen at 10 µM and 100 µM after 48 hours exposure. A significant reduction in viability was observed in ARPE-19 cells when treated with 200 µM after both 24 and 48 hours exposures, and when treated with 100 µM at 48 hours exposure (Fig. 1). In the case of HepG2 cells, curcumin at varying doses had no effect on viability after 24 hours exposure, but, after 48 hours exposure, the cell viability was seen to have reduced when treated with 50 µM and 100 µM. EGCG reduced the cell viability at 100 µM and 200 µM after 24 and 48 hours exposure significantly. It should be noted that, the time exposure did not have any significant difference in reduction of cell viability after 24 and 48 hours exposure on HepG2 cells (Fig. 3). Treatment with varying doses of curcumin showed reduction in cell viability in SW1417 cells after 24 hours, but showed no significant effects after 48 hours. SW1417 cells showed varying effects when treated with EGCG at 24 hours, however, a linear decrease in viability was observed after 48 hours treatment with EGCG (Fig. 5).

Combination of curcumin and EGCG at varying doses had no effect on the viability of HepG2 cells after 24 hours exposure (Fig. 4). Varying effects were seen in SW1417 cells. 48-hour exposure with 5µM of curcumin + 5µM of EGCG and 10µM curcumin + 10µM EGCG reduced viability in HepG2 cells and in SW1417 Cells. Reduced viability was also seen in combinations of 5µM curcumin + 10µM EGCG, 10µM curcumin + 10µM EGCG and 25µM curcumin + 10µM EGCG, suggesting 10µM curcumin + 10µM EGCG had the greatest effect in both cell lines (Figs. 4 & 6) Interestingly, ARPE-19 and HepG2 when treated with high concentrations of EGCG, 100 µM and 200 µM showed cell detachment when exposed for 24 and 48 hours.

Interestingly, the normal ARPE-19 cells had small but significant reduced viability at nearly every dose combination after 24h exposure, however this effect was not observed after 48h (Fig. 2). This suggests that in healthy tissue there may be an initial effect, but normally functioning cells have the ability to recover quickly, avoiding cell death.

The preliminary results were confirmed on a time-dosage dependent pattern by monitoring the cell proliferation and cell death using Cell Counting Kit 8 (Sigma-Aldrich). Statistical analysis was performed by One-way ANOVA followed by multiple comparison T-test using Prism 6 (GaphPad™).

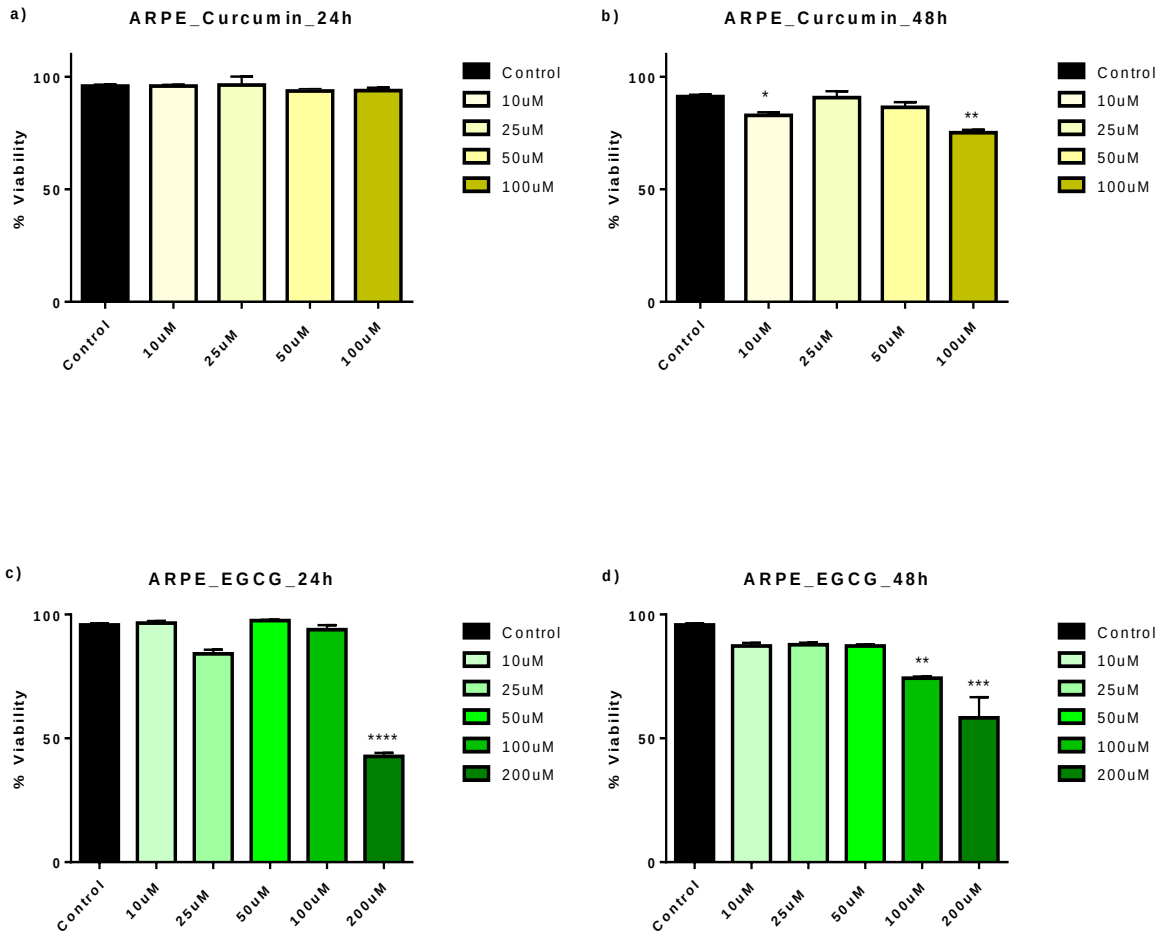


**Fig. 1.** Dose response of Curcumin and EGCG in ARPE-19 cells at 24h and 48h. ARPE-19 cells were treated with varying doses of curcumin (a, b) and EGCG (c, d) to observe the effect on viability of healthy cells. [Bars represent Mean ± S.E.M.; N = 3; * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.]

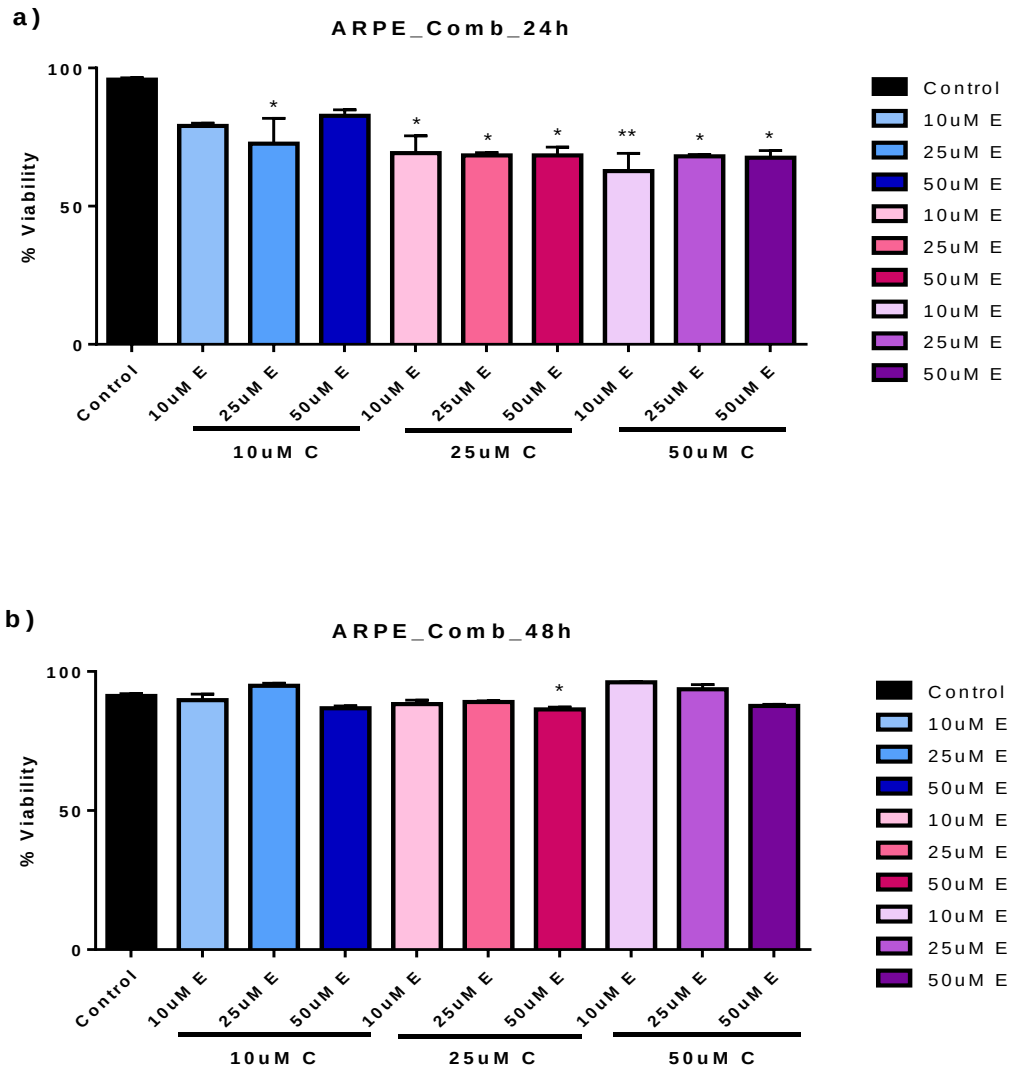


**Fig. 2.** Combination dose response of Curcumin and EGCG in ARPE-19 cells at 24h and 48h. ARPE-19 cells were treated with varying combined doses of curcumin and EGCG for 24h (a) or 48h (b) to observe the effect on viability of healthy cells. [Bars represent Mean ± S.E.M.; N = 3; * = $p<0.05$, ** = $p<0.01$.]

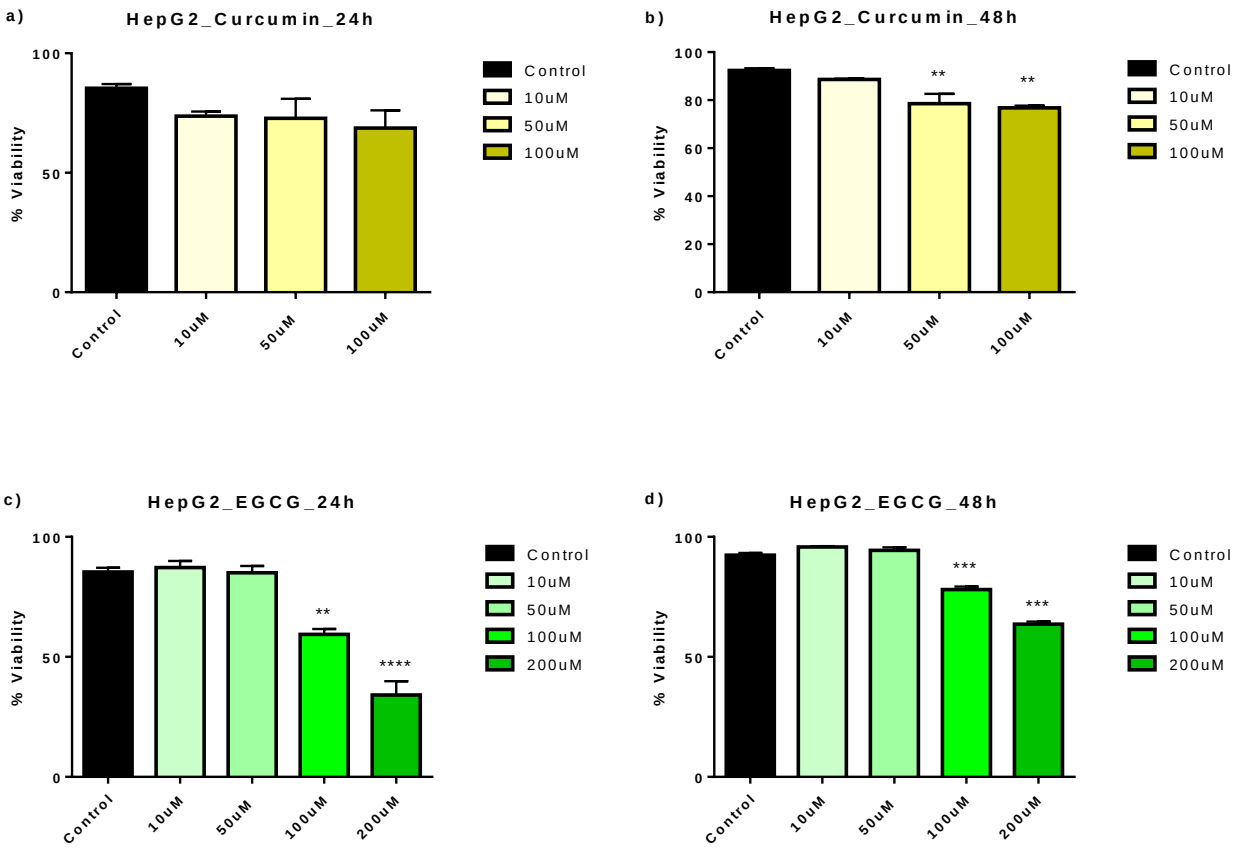


**Fig. 3.** Dose response of Curcumin and EGCG in HepG2 cells at 24h and 48h. HepG2 cells were treated with varying doses of curcumin (a, b) and EGCG (c, d) to observe the effect on viability of cancerous cells. [Bars represent Mean ± S.E.M.; N = 3; * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

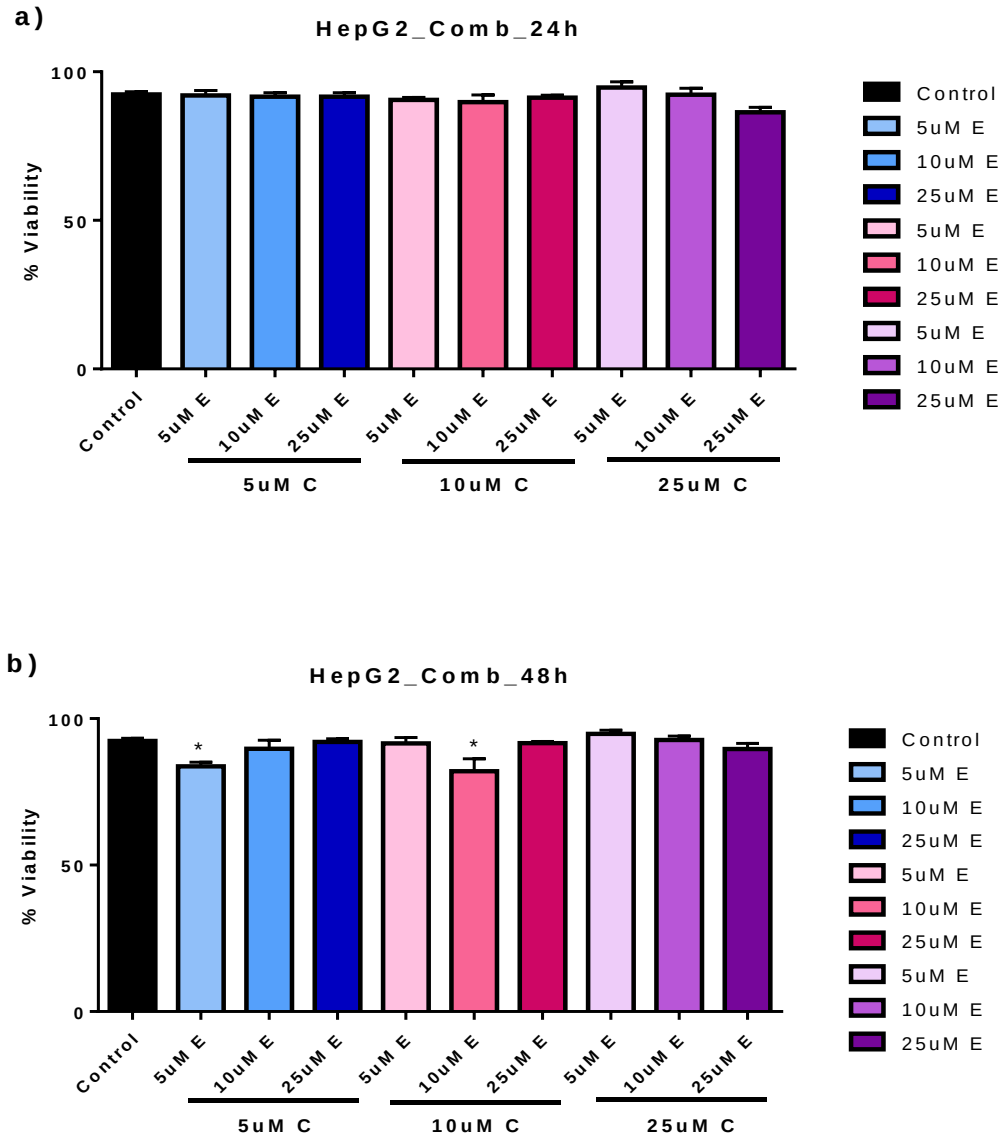


**Fig. 4.** Combination dose response of Curcumin and EGCG in HepG2 cells at 24h and 48h. HepG2 cells were treated with varying combined doses of curcumin and EGCG for 24h (a) or 48h (b) to observe the effect on viability of cancerous cells. [Bars represent Mean ± S.E.M.; N = 3; * = $p<0.05$.]

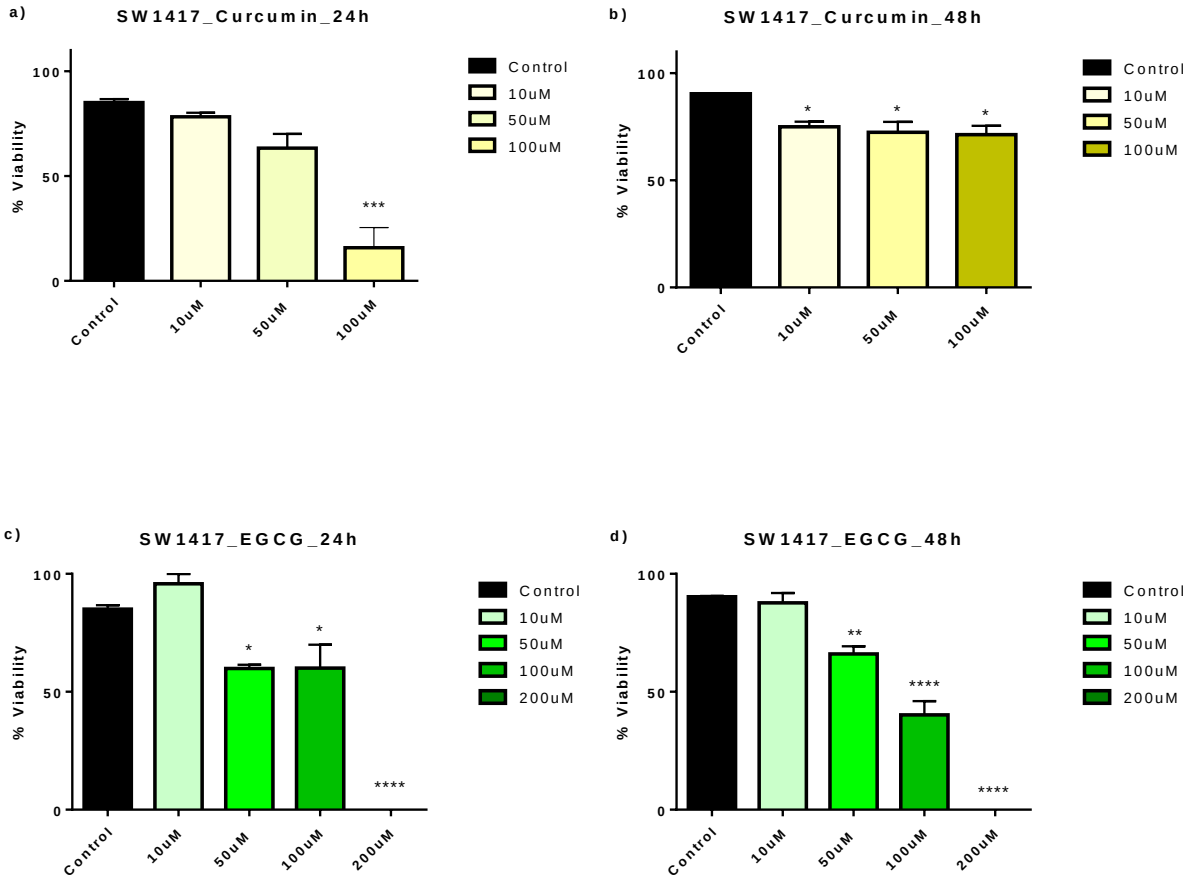


**Fig. 5.** Dose response of Curcumin and EGCG in SW1417 cells at 24h and 48h. SW1417 cells were treated with varying doses of curcumin (a, b) and EGCG (c, d) to observe the effect on viability of cancerous cells. [Bars represent Mean ± S.E.M.; N = 3; * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.]

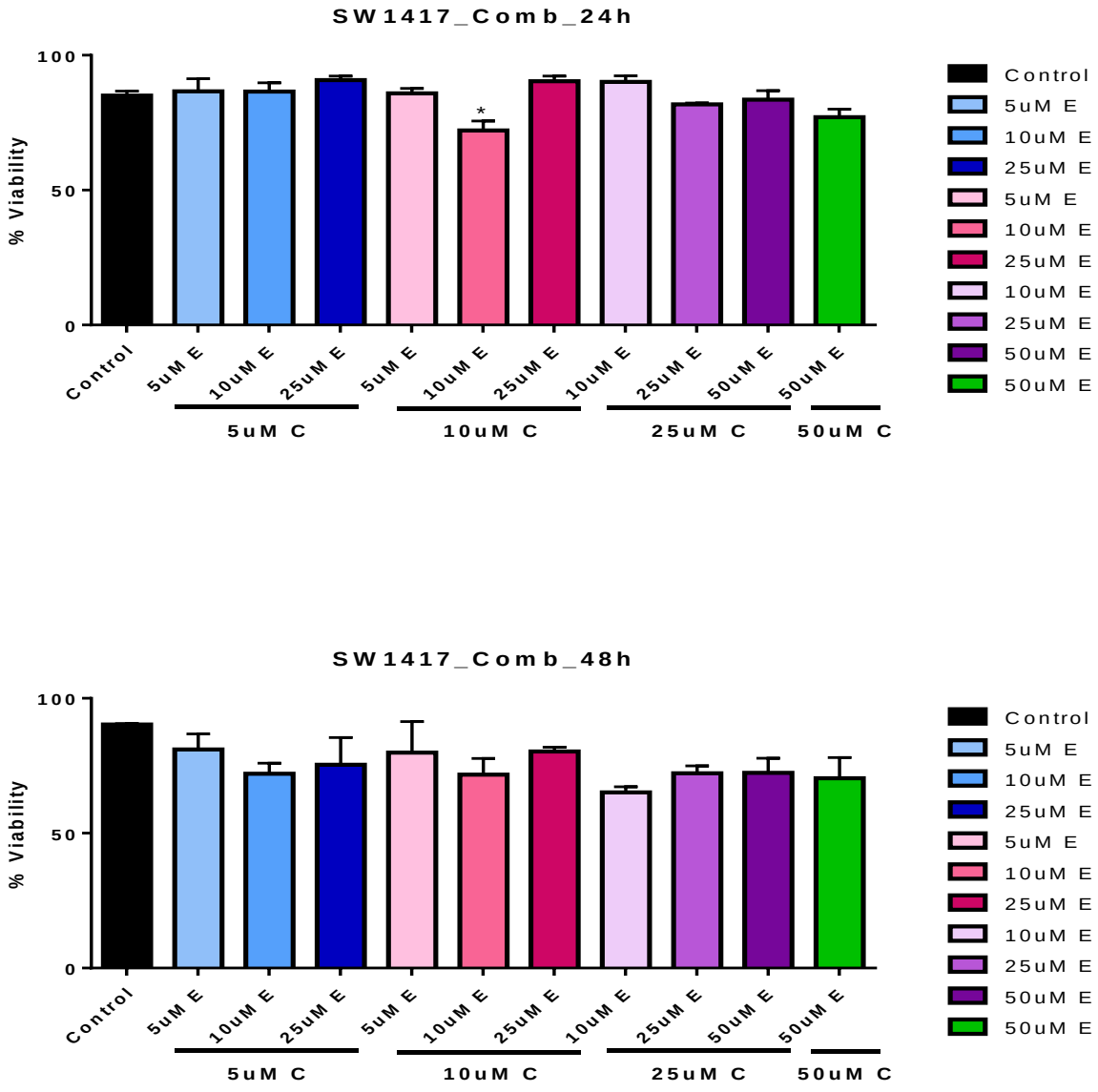


**Fig. 6.** Combination dose response of Curcumin and EGCG in SW1417 cells at 24h and 48h. HepG2 cells were treated with varying combined doses of curcumin and EGCG for 24h (a) or 48h (b) to observe the effect on viability of cancerous cells. [Bars represent Mean ± S.E.M.; N = 3; * = $p<0.05$.

### 3.2 Determination of proliferation of HepG2 and SW1417 cells treated with curcumin and EGCG separately and in combination after 48 hours

The cell proliferation was found to decrease with an increase in the dosage in both HepG2 (Fig. 8) and SW1417 (Fig. 9) cell lines after 48 hours. Significant decrease of cell proliferation in ARPE-19 was observed only in very high dosages when treated separately and in combination of curcumin and EGCG (Fig. 7). The maximum decrease in cell proliferation was found when treated with 100 µM curcumin and 100 µM EGCG in ARPE-19 and HepG2. However, in the case of SW1417 cells, there was not much difference with increasing dosage of curcumin (Fig. 9). This confirms the preliminary cell-viability results. Interestingly, ARPE-19 (Fig. 7) and HepG2 (Fig. 8) cells showed less proliferation when treated with 200 µM EGCG compared to 100 µM EGCG. When the cells were treated with a combination of 10 µM curcumin and 10 µM EGCG, ARPE-19 did not show any reduction in cell proliferation, indicating this to be a safe dose for normal cells. There was a reduction in cell proliferation at a high dose of 100 µM curcumin and 100 µM EGCG combination. In both SW1417 and HepG2, significant decrease in cell proliferation when treated with 10 µM curcumin and 10 µM EGCG combination (Figs. 8 & 9), suggesting this drug dosage to be a potential cancer therapeutic in both liver and colon cancer

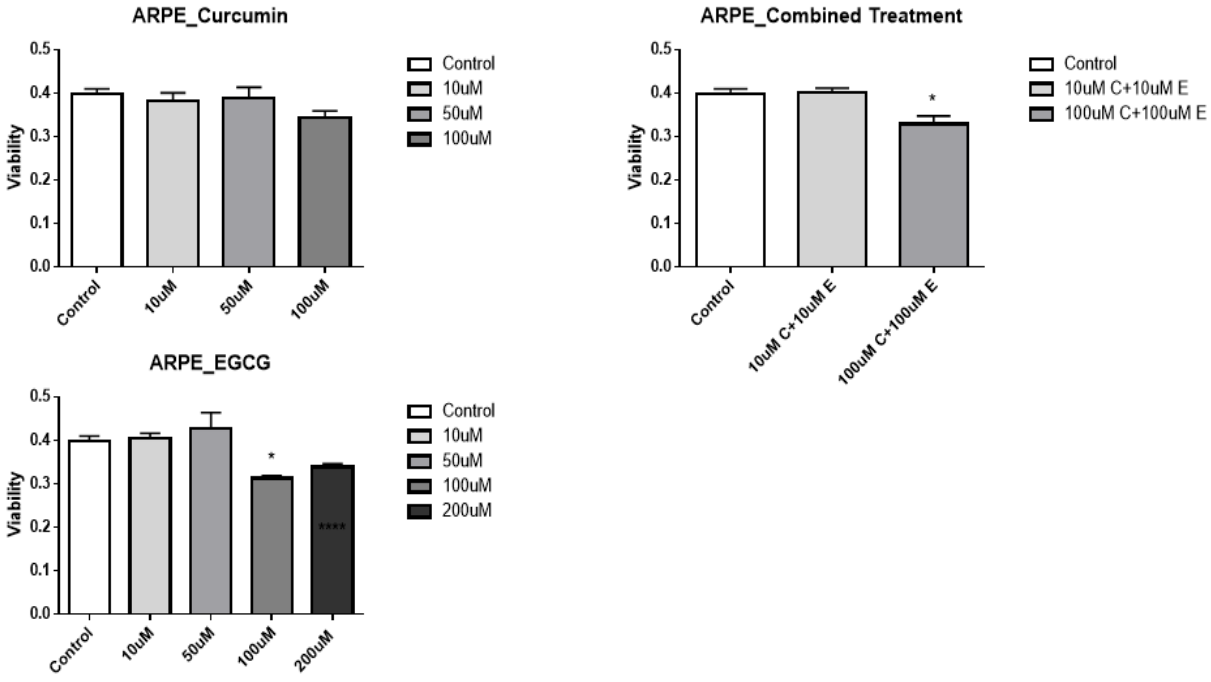


**Figure 7:** Cell proliferation in ARPE-19 cells when treated with different doses of curcumin and EGCG separately and in combination. [Bars represent Mean ± S.E.M.; N = 3; * = $p<0.05$.]

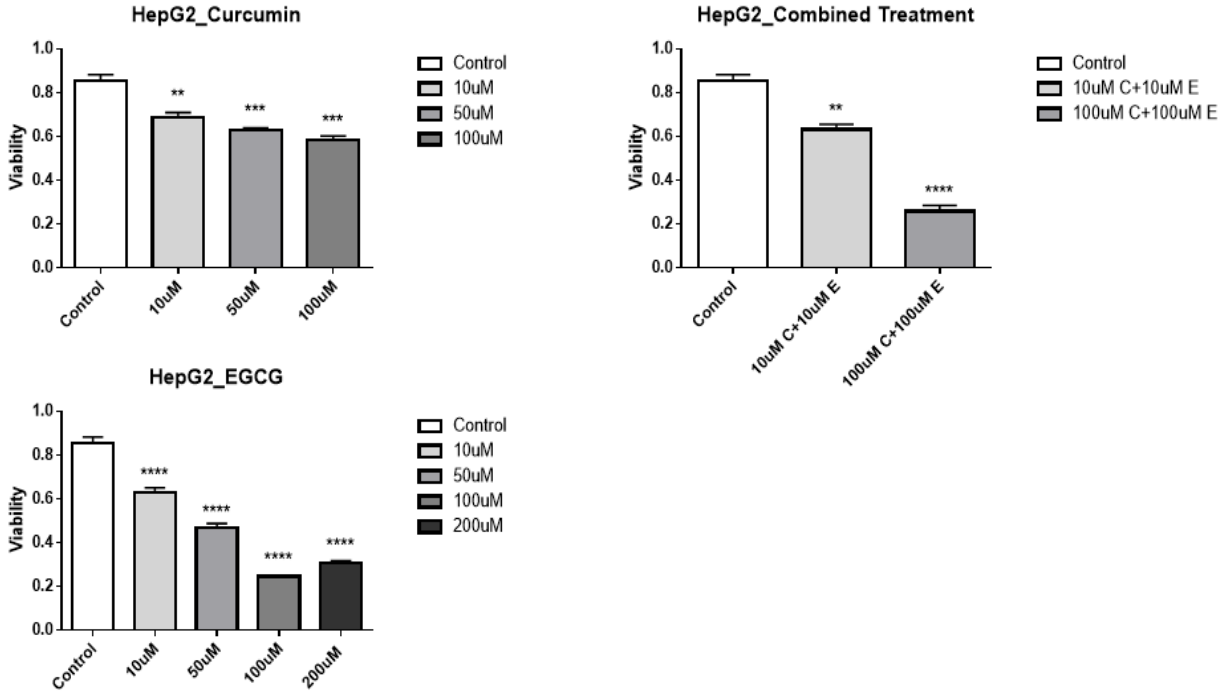


**Figure 8:** Cell proliferation in HepG2 cells when treated with different doses of curcumin and EGCG separately and in combination. [Bars represent Mean ± S.E.M.; N = 3; * = $p<0.05$.]

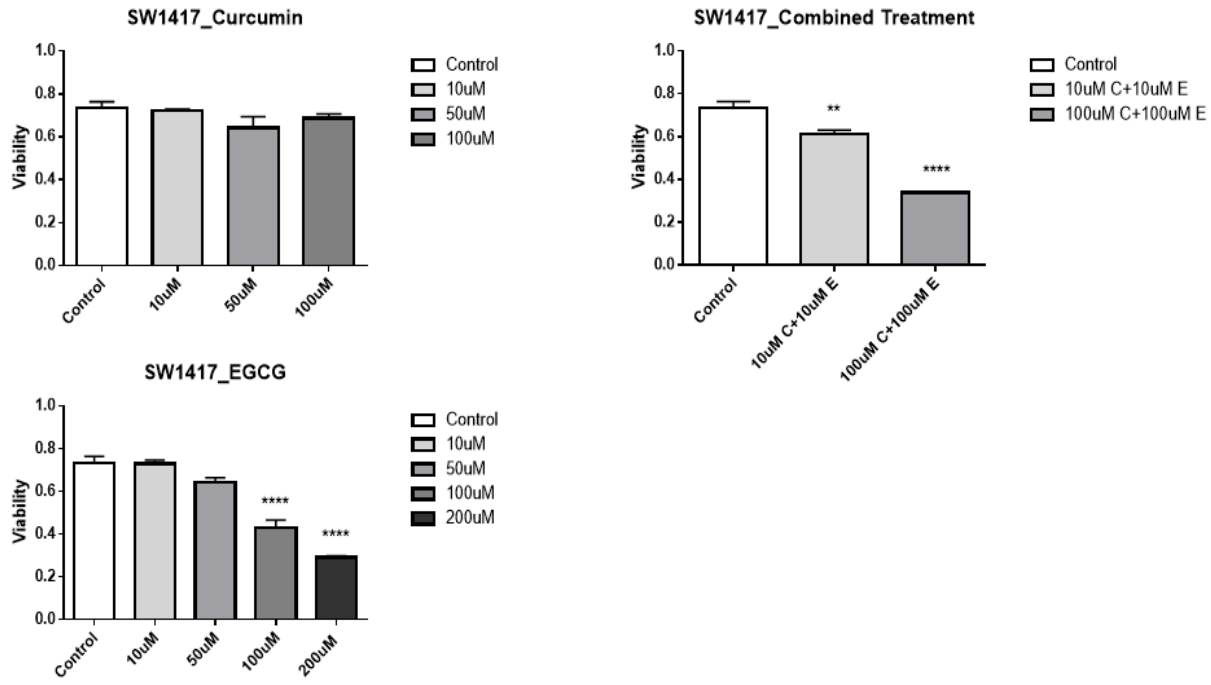


**Figure 9:** Cell proliferation in SW1417 cells when treated with different doses of curcumin and EGCG separately and in combination. [Bars represent Mean ± S.E.M.; N = 3; * = $p<0.05$.]

### 3.3 Gene regulation in HepG2 and SW1417 cells 48 hours after treatment with the curcumin and EGCG combination

The genes that demonstrated changes in regulation when HepG2 and SW1417 were exposed to 10µM curcumin+10µM EGCG were determined using cancer arrays. A minimum of at least a 1-fold change was the criteria for identifying an effect of treatment. In HepG2 cells, 28 cancer genes were observed to have at least the minimum change in their expression after treatment. APAF1, AURKA, BIRC3. CASP7, CCL2, CDH2, COX5A, ETS2, FGF2, IGFBP3, IGFBP7, KRT14, SLC2A1, VEGFC were the genes that were down-regulated and ACSL4, ARNT, BCL2L11, CASP7, CASP9, CCND3, DKC1,

ERCC3, FOXC2, POLB, SERPINF1, SKP2, SNAI1, TBX2 were up-regulated (Table 1). In SW1417, 14 genes were regulated. ACSL4, FOXC2, IGFBP3, IGFBP7, SERPINF1, SNAI3 were down-regulated and ANGPT2, CASP7, CCL2, COX5A, DKC1, E2F4A, ERCC3 were up-regulated (Table 2). The regulation of genes in both the cell lines were very different even though the drug dosage and exposure time are the same, showing that the drug targets different genes in different cancer types.

**Table 1:** Genes that were up- or down-regulated by at least a 1-fold difference in HepG2 cells after treatment with 10µM Curcumin+10 µM EGCG.

| **Genes** | **Control** | **reated** | **Δ (Ct)** |
|---|---|---|---|
| ACSL4 | 24.2 | 22.97 | 1.23 |
| APAF1 | 27.66 | 28.42 | -0.76 |
| ARNT | 30.26 | 27.67 | 2.59 |
| AURKA | 24.54 | 25.77 | -1.23 |
| BCL2L11 | 29.26 | 28.27 | 0.99 |
| BIRC3 | 28.82 | 31.34 | -2.52 |
| CA9 | 25.18 | 28.44 | -3.26 |
| CASP7 | 27.87 | 26.87 | 1 |
| CASP9 | 28.19 | 27.28 | 0.91 |
| CCL2 | 27.21 | 28.53 | -1.32 |
| CCND3 | 29.15 | 27.41 | 1.74 |
| CDH2 | 25.03 | 27.19 | -2.16 |

| Genes | Control | Treated | Δ (Ct) |
|---|---|---|---|
| COX5A | 24.58 | 25.81 | -1.23 |
| DKC1 | 26.22 | 25.15 | 1.07 |
| ERCC3 | 27.78 | 26.2 | 1.58 |
| ETS2 | 27.41 | 28.42 | -1.01 |
| FGF2 | 25.22 | 30.43 | -5.21 |
| FOXC2 | 31.4 | 30.08 | 1.32 |
| IGFBP3 | 26.36 | 27.34 | -0.98 |
| IGFBP7 | 22.18 | 26.49 | -4.31 |
| KRT14 | 28.08 | 29.05 | -0.97 |
| POLB | 27.32 | 26.4 | 0.92 |
| SERPINF1 | 23.06 | 21.05 | 2.01 |
| SKP2 | 27.46 | 26.57 | 0.89 |
| SLC2A1 | 24.61 | 25.86 | -1.25 |
| SNAI1 | 29.23 | 28.38 | 0.85 |
| TBX2 | 29 | 28.07 | 0.93 |
| VEGFC | 27.29 | 28.38 | -1.09 |

**Table 2:** Genes that were up- or down-regulated by at least a 1-fold difference in SW1417 cells after treatment with 10μM Curcumin+10 μM EGCG.

| Genes | Control | Treatment | Δ(Ct) |
|---|---|---|---|
| ACSL4 | 25.74 | 26.42 | -0.68 |
| ANGPT2 | 35.19 | 30.29 | 4.9 |
| CASP7 | 29.02 | 27.34 | 1.68 |
| CCL2 | 31.96 | 28.37 | 3.59 |
| COX5A | 24.02 | 23.08 | 0.94 |
| DKC1 | 26.51 | 24.35 | 2.16 |
| E2F4A | 28.51 | 27.16 | 1.35 |
| ERCC3 | 27.98 | 25.77 | 2.21 |
| FOXC2 | 30.11 | 31.13 | -1.02 |
| IGFBP3 | 25.44 | 26.41 | -0.97 |
| IGFBP7 | 26.24 | 27.77 | -1.53 |
| SERPINF1 | 25.62 | 26.59 | -0.97 |
| SNAI3 | 28.49 | 29.47 | -0.98 |
| TNKS | 27.52 | 28.42 | -0.9 |

### 3.4 Determination of candidate genes for validation in HepG2 AND SW1417 cells treated with curcumin and EGCG, regorafenib, regorafenib, curcumin and EGCG combinations.

The determination of candidate gene expression was done by selecting genes which showed significantly more than one-fold increase or decrease when treated with a combination of 10 µM curcumin and 10 µM EGCG compared to the cells that were untreated in the gene array. The candidate genes were selected based on their importance in tumorigenesis, proliferation, and apoptosis. ARNT, FGF2, IGFBP7, SERPINF1 were genes selected for the confirmation of expression in HepG2 cells; ANGPT2 and IGFBP7 were the genes selected for confirmation of expression in SW1417 cells. The candidate genes were used to study the effects of treatment with regorafenib and regorafenib combined with curcumin and EGCG.

**Table 3:** Four genes selected for validation by qPCR for HepG2.

| GenBank # | Abbreviation | Scientific Name | Fold Change* |
|---|---|---|---|
| NM_001197325.1 | ARNT | Aryl hydrocarbon receptor nuclear translocator | +2.59 |
| NM_002006.4 | FGF2 | Fibroblast growth factor 2 | -5.21 |
| NM_001253835.1 | IGFBP7 | Insulin like growth factor binding protein 7 | -4.31 |
| NM_002615 | SERPINF1 | Serpin family F member 1 | +2.01 |

***increase or decrease from Control Cq value to treatment Cq value**

**Table 4:** Two genes selected for validation by qPCR for SW1417.

| GenBank # | Abbreviation | Scientific Name | Fold Change* |
|---|---|---|---|
| NM_001147 | ANGPT2 | Angiopoietin 2 | +4.90 |
| NM_001253835.1 | IGFBP7 | Insulin like growth factor binding protein 7 | -1.53 |

***increase or decrease from Control Cq value to treatment Cq value.**

### 3.5 Validation of the expression of candidate genes in HepG2 and SW1417 cells treated with a combination of curcumin and EGCG regorafenib, regorafenib, curcumin and EGCG combinations

The expression of the candidate genes selected were validated by qPCR and the amplification of the target genes were confirmed by observing a single dissociation peak in all cases. The candidate genes were then analyzed for up- or down-regulation when the cells were treated with 2.5 µM regorafenib separately and in combination with 10 µM curcumin and 10 µM EGCG. The results were compared to the 10 µM curcumin and 10 µM EGCG combinations to determine if the chemotherapy drug affected the same target genes and how expression of the genes were affected.

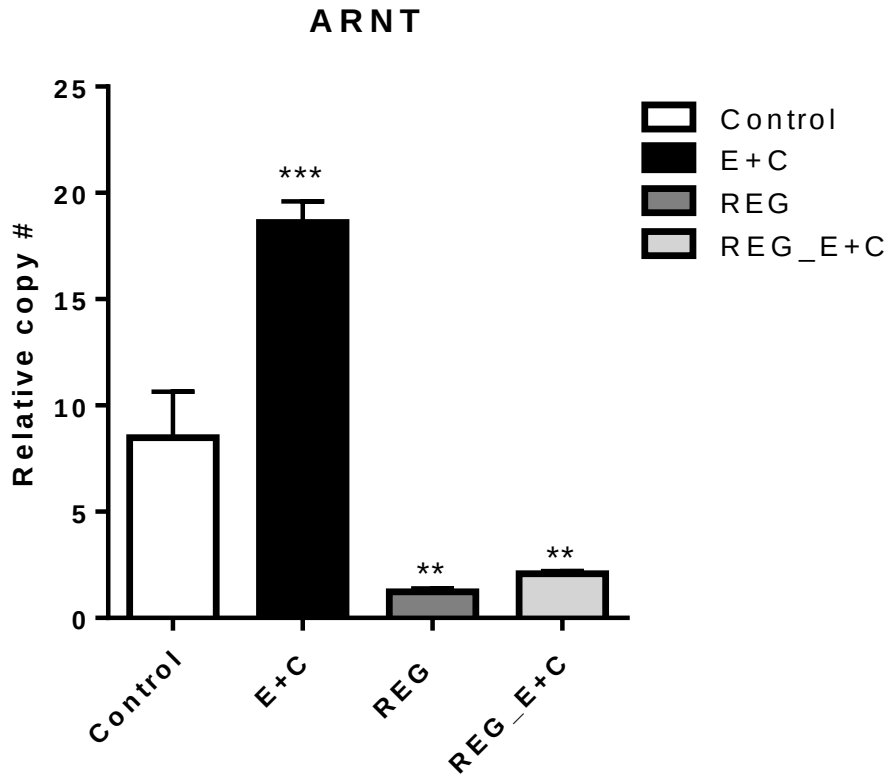


**Figure 10:** qPCR confirmed up-regulation of ARNT in hepatocarcinoma (HepG2) cells treated with 10uM EGCG + 10uM Curcumin and 2.5uM regorafenib +10uM EGCG + 10uM Curcumin [Bars represent Mean ± S.E.M.; ** = $p<0.01$; N = 6]

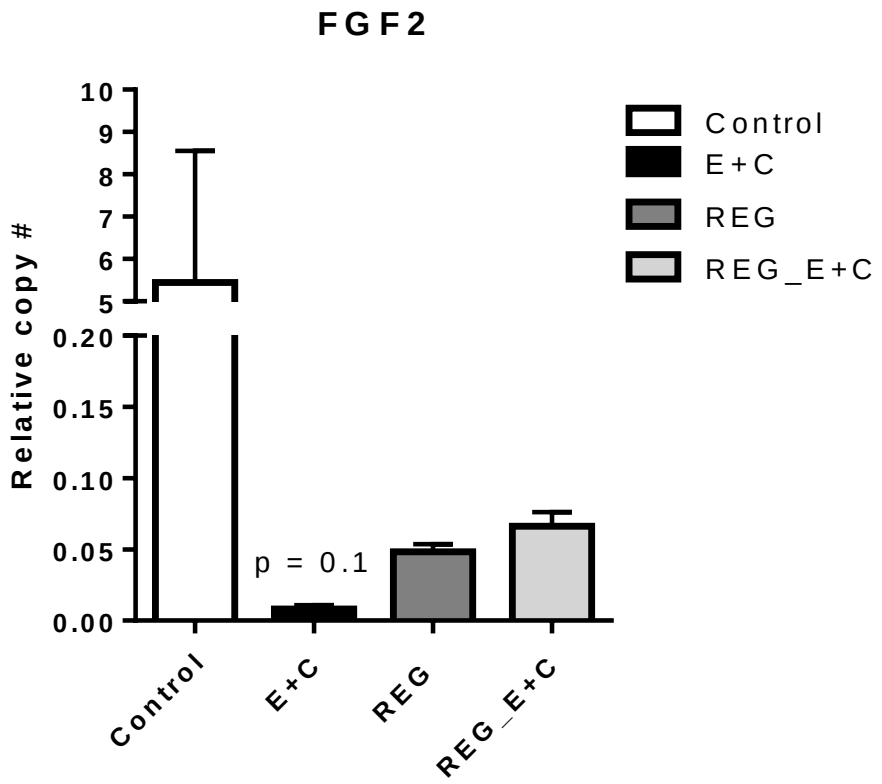


**Figure 11**: qPCR confirmed down-regulation of FGF2 in hepatocarcinoma (HepG2) cells treated with 10uM EGCG + 10uM Curcumin and 2.5uM regorafenib +10uM EGCG + 10uM Curcumin, however due to the high variance in the control data, this difference was not significant. [Bars represent Mean ± S.E.M.; N = 6]

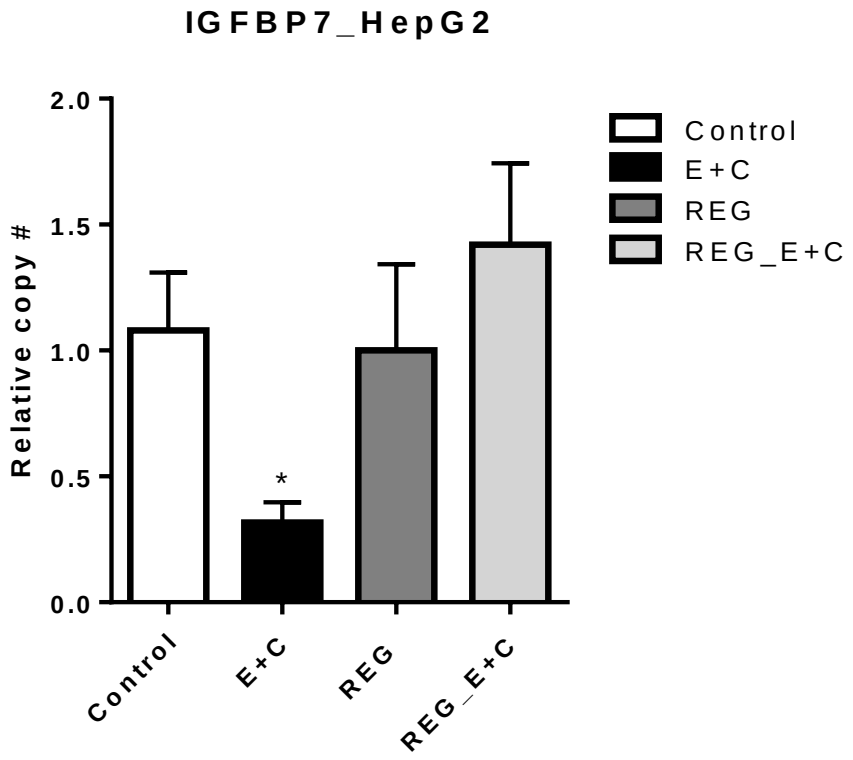


**Figure 12.** qPCR confirmed down-regulation of IGFPB7 in hepatocarcinoma (HepG2) and cells treated with 10uM EGCG + 10uM Curcumin and 2.5uM regorafenib +10uM EGCG + 10uM Curcumin. [Bars represent Mean ± S.E.M.; * = $p<0.05$; N = 6]

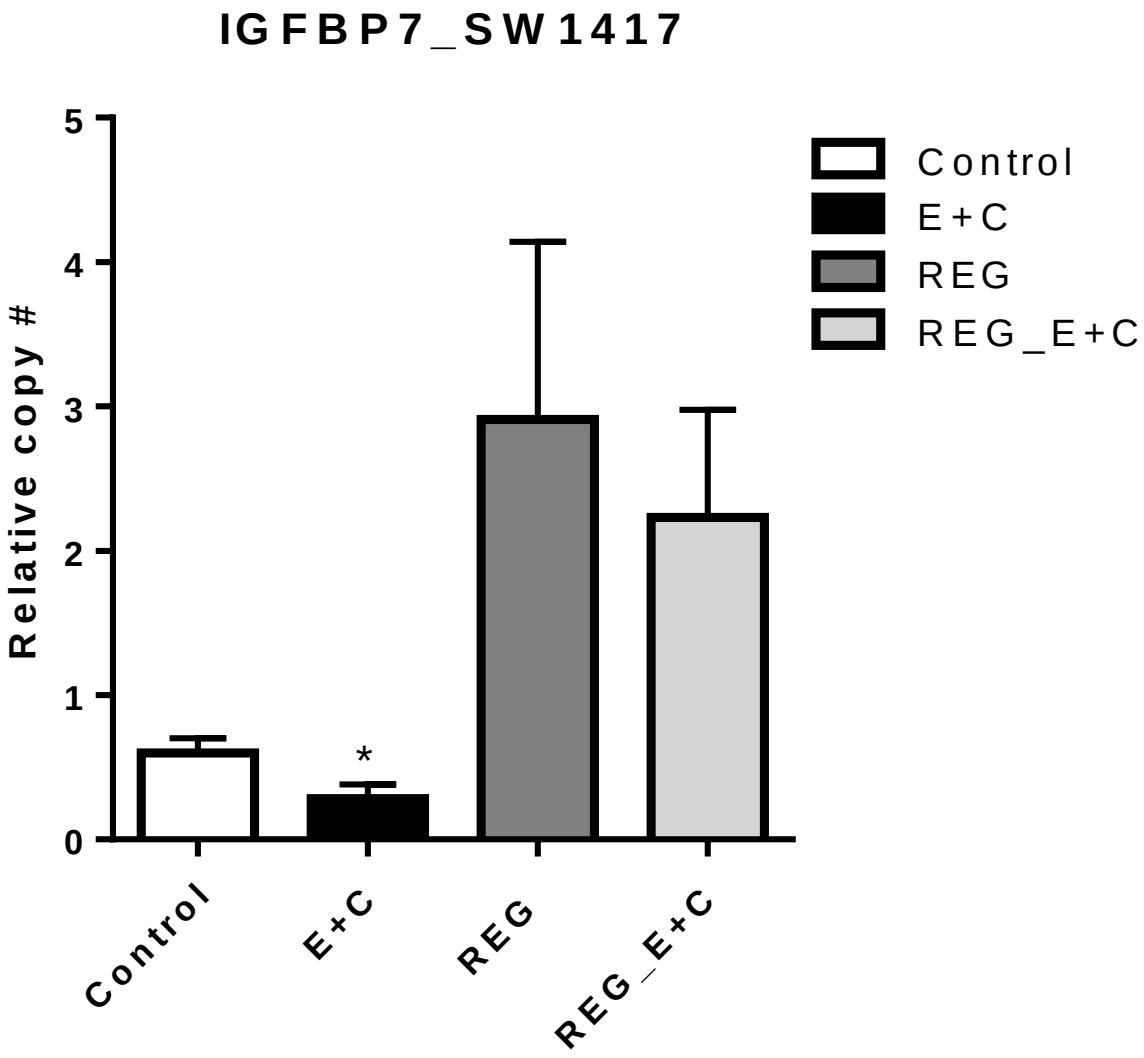


**Figure 13**. qPCR confirmed down-regulation of IGFPB7 in colorectal adenocarcinoma (SW1417) cells treated with 10uM EGCG + 10uM Curcumin and 2.5uM regorafenib +10uM EGCG + 10uM Curcumin. [Bars represent Mean ± S.E.M.; * = $p<0.05$; N = 6]

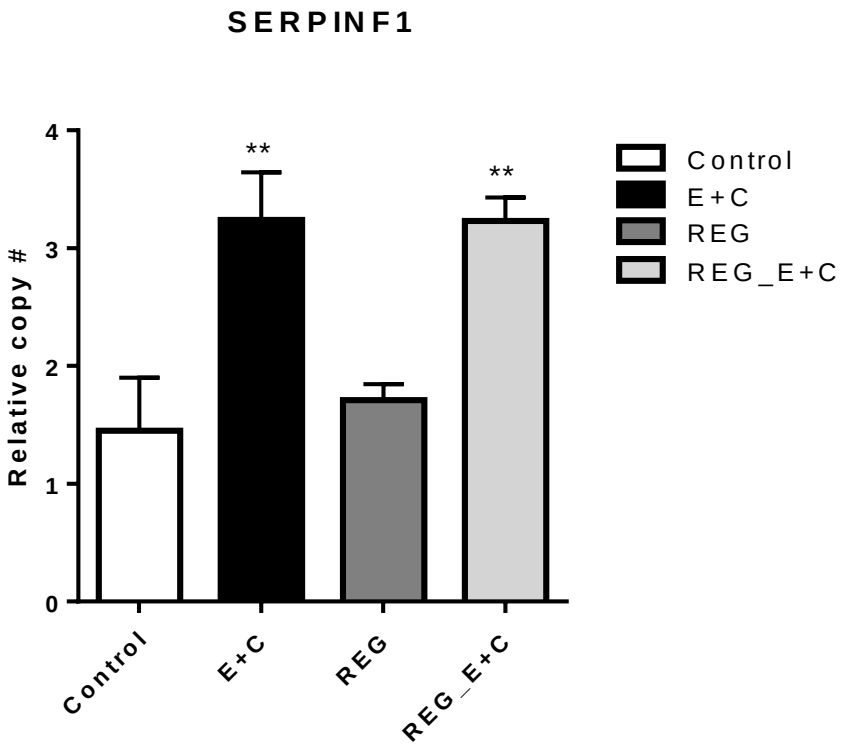


**Figure 14.** qPCR confirmed up-regulation of SERPINF1 in hepatocarcinoma (HepG2) cells treated with 10uM EGCG + 10uM Curcumin and 2.5uM regorafenib +10uM EGCG + 10uM Curcumin. [Bars represent Mean ± S.E.M.; ** = $p<0.01$; N = 6]

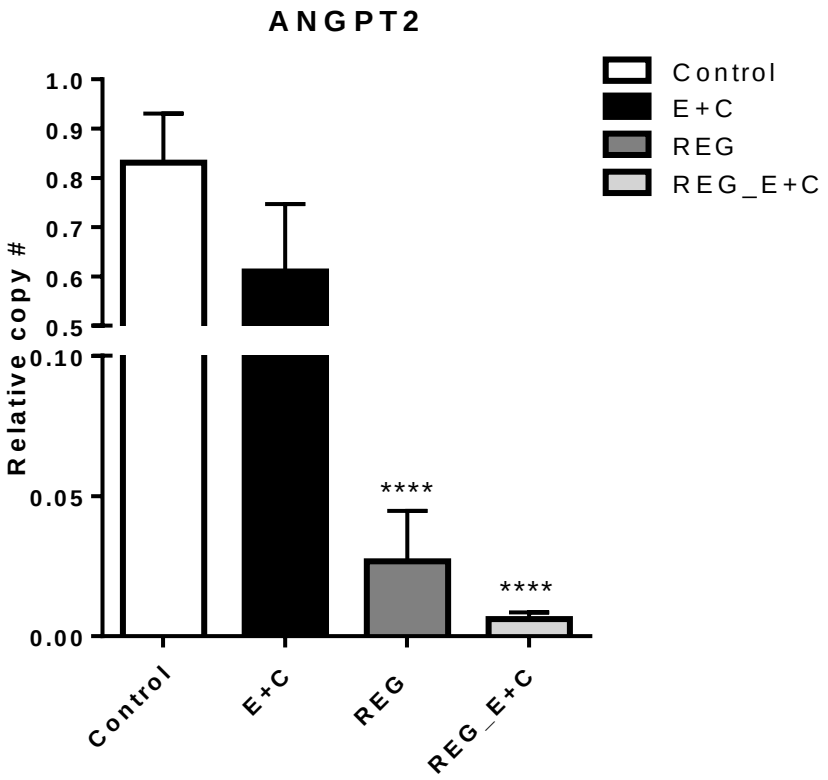


**Figure 15.** qPCR did not confirm upregulation of ANGPT2 in colorectal adenocarcinoma (SW1417) cells treated with 10uM EGCG + 10uM Curcumin and 2.5uM regorafenib +10uM EGCG + 10uM Curcumin. [Bars represent Mean ± S.E.M.; N = 6]

# CHAPTER 4

# DISCUSSION

## 4.1 Up regulation of ARNT gene in HepG2 treated with curcumin and EGCG combination

The aryl hydrocarbon receptor nuclear translocator is essential for normal growth and development of cells (Wenger and Glassmann., 1997). It is a member of basic helix-loop-helix family of transcriptional factors (Hasen et al., 2015) which is important for the regulation of aryl hydrocarbon receptor (ARH) and HIF-1α. ARH and HIF-1α play a vital role in cancer proliferation (Chandel and Simon., 2008). It is shown that, in normal cells, the depletion or down-regulation of ARNT can lead to abnormal angiogenesis, defective hematopoiesis and problems related to cardiac and cranial dysfunction (Kozak et al., 1997). ARNT also plays a vital role in adaptations to environmental stress (Gu et al., 2000).

Metastasis results in cancer lethality, leading to an increase in malignancy and resistance to anti-cancer drugs (Gottesman., 2002). Huang et al. demonstrated that the loss of ARNT led a series of events that led to a pro-metastatic phenotype in colorectal cancer (Huang et al., 2015). ARNT expression itself plays an important role in tumor growth (Liang et al., 2012). Studies have demonstrated that ARNT expression is reduced in advanced stages of colorectal cancer (Huang et al., 2015), suggesting that the up-regulation of ARNT might be significantly involved in reducing metastasis in cancer. It was also shown that the depletion of ARNT led to tumor invasion and metastasis via activation of β1/ FAK signaling.

The cells treated with the chemotherapy drug, regorafenib showed down-regulation of ARNT in HepG2 cells. The combination of curcumin and EGCG, when used with regorafenib, showed very little added effects. However, the HepG2 cells treated with the combination of curcumin and EGCG, showed significant over expression of the gene.

ARNT plays both positive and negative roles in tumor growth, however it is important to understand the stage of cancer for targeted therapy, as ARNT has positive effects and prevents metastasis only in later stages of cancer (Huang et al., 2015). In case of early stages of colorectal cancer, ARNT has been shown to regulate anti-oxidant and chemotherapy drug resistance promoting tumor growth (Shi et al., 2009). In this study, the HepG2 cells used were obtained from a 15-year-old patient in later stages of cancer. Hence, the combined effects of curcumin and EGCG in these cells showed potential therapeutic effect.

### 4.2 Up regulation of SERPINF1 gene in HepG2 treated with curcumin and EGCG combination

Serpin family 1 (SERPINF1), also known as pigment epithelium derived factor (PEDF) is a multifunctional protein responsible for inhibiting metastasis and angiogenesis (Rychli et al., 2009). SERPINF1 also plays a vital role in stimulating the maturation of the vascular environment (Wretcha et al., 2015). SERPINF1 also plays an important role in induction of apoptosis in cancer cells (Hoshina et al., 2010). SERPINF1 can also inhibit the proliferation and migration of cancer cells that are induced by VEGF (Becrra et al., 2013). Bing et al. showed that when the SERPINF1 is down-regulated, the levels of Thioredoxin Domain Containing 5 (TXNDC5) increase. TXNDC5 is very important for the migration

and proliferation of tumor cells, also for the formation of vascular network in endothelial cells (Bing et al., 2017).

Our study showed, significant increase in the levels of SERPINF1 in HepG2 cells when treated with the curcumin and EGCG combination, suggesting potential therapeutic effects. However, expression was not affected when the HepG2 cells were treated with regorafenib alone. Also, the levels of SERPINF1 did not increase when HepG2 cells were treated with curcumin, EGCG and regorafenib, suggesting 1) regorafenib alone does not affect the expression of SERPINF1, 2) there is no effect of combined EGCG and curcumin on the drug, and 3) SERPINF1 is up-regulated by the treatment of EGCG and curcumin combined.

### 4.3 Down regulation of FGF gene in HepG2 treated with curcumin and EGCG, and regorafenib, curcumin and EGCG combinations

FGF2 belongs to the Fibroblast growth factor family (Powers et al., 2000). FGFs play a vital role in hematopoiesis, tissue regeneration and embryonic development (Korc et al., 2009). Studies have shown that FGF2 is a prognostic marker for different types of cancers. In normal cells, FGF2 activity is highly regulated, and terminated via receptor internalization (Korc et al., 2009). However, in the case of cancer cells, FGF2 signaling is dysregulated and is one of the key promoting factors for carcinogenesis (Powers et al., 2009). The overexpression of FGFR2 is followed by C-terminal exon abrogation, leading to disrupted internalization (Ahmed et al., 2012; Cha et al., 2009). FGF2 is also identified as a strong pro-angiogenic growth factor (Gualandris et al., 1996). FGF2 also causes the dysregulation of FGFR1 and FGFR2, leading to increased cell proliferation, metastasis and angiogenesis (Tanghetti et al., 2002). The transient expression of FGF2 controls the

expression of genes important for cell cycle, adhesion and differentiation in endothelial cells (Dell Era et al., 2002).

FGF2 can contribute to cancer metastasis via alternative mechanisms (Karajannis et al., 2006). For example, the majority of pancreatic ductal carcinomas were positive for VEGF and FGF2, but in the case of parathyroid hyperplasia, correlation between FGF2 and MVD was not observed, compared to the correlation between VEGF and MVD (Kuwahara et al., 2003). Hence, FGF2 can function in both a paracrine and autocrine manner in cancer cells.

In this study, curcumin and EGCG combination showed significant downregulation of FGF2 in HepG2 cells compared to the treatment with regorafenib. However, the differences in downregulation of FGF2 between the treatment using regorafenib alone and regorafenib, curcumin and EGCG combination was minimal. This suggests the curcumin and EGCG combination treatment is equally effective as regorafenib in targeted therapy for FGF2 in liver cancer treatment.

### 4.4 Down regulation of ANGPT2 gene in SW1417 treated with curcumin and EGCG, and regorafenib, curcumin and EGCG combinations

Angiogenic factors are essential for tumor neovascularization leading to the shift of the angiogenic balance towards pro-angiogenic phenotype (Folkman., 1995). ANGPT2 has been shown to play a vital role in tumorigenesis by increasing the initiation of tumor vessel sprouts (Nasarre et al., 2009; Hashizume et al., 2010). It is also known that ANGPT2 leads to the dissociation of cell-cell interaction in endothelial cells by induction of permeability (Sharptnecker, 2005).

In normal cells, ANGPT2 is secreted by endothelial cells and is important for the initiation of vascularization (Zheng et al., 2016). However, the levels of ANGPT2 are highly increased in tumor cells making them more susceptible for angiogenesis (Zheng et al., 2016). ANGPT2 in normal cells is stored in Weibel-palade bodies and is released only when stimulated by an appropriate signal (Sfiligoi et al., 2003). ANGPT2 also acts as a regulator for endocrine functions in normal cells (Mitsuhashi et al., 2003).

In our study, the cancer array showed significant decrease in the expression of ANGPT2 levels in HepG2 cells treated with a combination of curcumin and EGCG. However, this could not be confirmed, as the qPCR showed no significant decrease in the levels of ANGPT2 between the control and treated cells. The reason for this is unclear. On the other hand, HepG2 cells treated with regorafenib showed a significant decrease in the ANGPT2 levels. When HepG2 cells were treated with regorafenib plus EGCG and curcumin the effect of regorafenib was enhanced, although there was no significant difference between the two treatments. The inconsistency of the gene expression data suggests that a follow-up assay needs to be performed to further observe the effect of EGCG and curcumin on ANGPT2 expression in HepG2 cells.

### 4.5 Regulation of IGFBP7 gene in HepG2 and SW1417 treated with curcumin and EGCG, and regorafenib, curcumin and EGCG combinations

Insulin like growth factor plays an important role in the carcinogenesis of HCC and CRC. Insulin like growth factor binding protein 7 (IGFBP7) binds to IGF and blocks its activation (Evdokimova et al., 2012). The expression of IGFBP7 and its correlation with various cancer types is inconsistent. For example, IGFBP7 was shown to promote tumor cell proliferation and angiogenesis of brain endothelial cells (Jiang et al., 2008), whereas it

showed direct induction of apoptosis in gastric cancer (Kim et al., 2018). Hence it is important to know the type of cancer and the behavior of IGFBP7 with that specific cancer type for targeted gene therapies.

In case of CRC, IGFBP7 can promote anchorage-independent growth in malignant cells, when it is expressed by the tumor cells themselves and can induce colony formation in colon cancer cells co-cultured with IGFBP7-expressing CAFs by a paracrine tumor-stroma interaction (Rupp et al., 2014). Alternatively, the loss of IGFBP7 resulted in the activation of IGF pathway leading to increased proliferation of HCC cells (Maaged et al., 2017)

In our study, regorafenib had no effect on the IGFBP7 in HCC, however, IGFBP7 was overexpressed when regorafenib was used in combination with curcumin and EGCG. In CRC, regorafenib showed overexpression of IGBP7 gene, but those levels were decreased when regorafenib was used in combination with curcumin and EGCG. This suggests the combination of regorafenib, curcumin and EGCG has beneficial effects in both HCC and CRC compared to the treatment of regorafenib or curcumin and EGCG separately.

# CHAPTER 5

# CONCLUSIONS

The combination of curcumin and EGCG at low doses efficiently regulated important genes responsible for cancer initiation and proliferation in both HCC and CRC. The comparative analysis of the molecular targets of curcumin and EGCG combination and regorafenib showed curcumin and EGCG combination to be significantly effective and targeted. Curcumin and EGCG induced an additive effect of regorafenib on only one of the candidate genes. Hence, curcumin and EGCG may have promising applications in cancer therapy when used in combination, but minimal additive effect when used in treatment with regorafenib.

# CHAPTER 6

## FUTURE DIRECTIONS

Regorafenib is associated with a number of adverse reactions which need to be identified and managed in the course of medication. Diarrhea, fatigue, hypertension, anemia and thrombocytopenia are some of the common symptoms associated with regorafenib. Stomatitis is a complication that can be of significant risk leading to sepsis among patients treated with regorafenib. It may cause stomatitis by causing direct DNA damage to mucosal surface. This repeated sepsis may also lead to the stimulation of secondary mediators of injury. On the other hand, low doses of curcumin and EGCG did not show any harmful or toxic effect in normal cells at short and long exposure durations. Curcumin and EGCG combination showed significant changes in expression of important cancer genes, as well as different target effects in HCC and CRC. The potential therapeutic benefits with little or no harmful effects certainly warrant additional studies to further investigate the potential usefulness of these two compounds in the co-treatment of other types of cancer.

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# APPENDICES

## Appendix A: Cell viability

**Curcumin -48hr**

| Sample/Well | Live | Dead | % viability |
|---|---|---|---|
| Control | 9 | 1 | 90 |
| Control | 10 | 1 | 90.9 |
| Control | 9 | 1 | 90 |
| 10µM curcumin | 10 | 3 | 76.9 |
| 10µM curcumin | 7 | 3 | 70 |
| 10µM curcumin | 7 | 2 | 77.7 |
| 50µM curcumin | 4 | 1 | 80 |
| 50µM curcumin | 7 | 4 | 63.6 |
| 50µM curcumin | 14 | 5 | 73.6 |
| 100µM curcumin | 7 | 4 | 63.6 |
| 100µM curcumin | 8 | 3 | 72.7 |
| 100µM curcumin | 7 | 2 | 77.7 |

**EGCG - 48h**

| Sample/Well | Live | Dead | % viability |
|---|---|---|---|
| 10µM EGCG | 17 | 1 | 94.4 |
| 10µM EGCG | 8 | 1 | 88.8 |
| 10µM EGCG | 8 | 2 | 80 |
| 50µM EGCG | 5 | 2 | 71.4 |
| 50µM EGCG | 6 | 3 | 66.6 |
| 50µM EGCG | 3 | 2 | 60 |
| 100µM EGCG | 6 | 7 | 46 |
| 100µM EGCG | 2 | 5 | 28.5 |
| 100µM EGCG | 6 | 7 | 46 |
| 200µM EGCG | 0 | 10 | 0 |
| 200µM EGCG | 0 | 7 | 0 |
| 200µM EGCG | 0 | 7 | 0 |

**EGCG + Curcumin - 48h**

| Sample/Well | Live | Dead | % viability |
|---|---|---|---|
| 5µM curcumin | 10 | 1 | 90.9 |
| 5µM curcumin | 14 | 5 | 73.6 |
| 5µM curcumin | 6 | 2 | 75 |
| 25µM curcumin | 10 | 3 | 76.9 |
| 25µM curcumin | 6 | 2 | 75 |
| 25µM curcumin | 11 | 3 | 84.6 |
| 5µM EGCG | 6 | 1 | 85.7 |
| 5µM EGCG | 9 | 2 | 81.8 |
| 5µM EGCG | 16 | 2 | 88.8 |
| 25µM EGCG | 13 | 3 | 81.2 |
| 25µM EGCG | 8 | 2 | 80 |
| 25µM EGCG | 10 | 1 | 90 |
| 5µM C+ 5µM E | 12 | 3 | 80 |
| 5µM C+ 5µM E | 11 | 1 | 91.6 |
| 5µM C+ 5µM E | 10 | 4 | 71.4 |
| 5µM C+ 10µM E | 9 | 5 | 64.2 |
| 5µM C+ 10µM E | 12 | 4 | 75 |
| 5µM C+ 10µM E | 10 | 3 | 76.9 |
| 5µM C+ 25µM E | 5 | 1 | 83.3 |
| 5µM C+ 25µM E | 7 | 1 | 87.5 |
| 5µM C+ 25µM E | 5 | 4 | 55.5 |

| Sample/Well | Live | Dead | % viability |
|---|---|---|---|
| 10µM C+ 5µM E | 4 | 3 | 57.1 |
| 10µM C+ 5µM E | 8 | 1 | 88.8 |
| 10µM C+ 5µM E | 15 | 1 | 93.75 |
| 10µM C+ 10µM E | 4 | 1 | 80 |
| 10µM C+ 10µM E | 3 | 2 | 60 |
| 10µM C+ 10µM E | 6 | 2 | 75 |
| 10µM C+ 25µM E | 5 | 1 | 83.3 |
| 10µM C+ 25µM E | 8 | 2 | 80 |
| 10µM C+ 25µM E | 14 | 4 | 77.7 |
| 25µM C+ 10µM E | 7 | 4 | 63.6 |
| 25µM C+ 10µM E | 9 | 4 | 69.2 |
| 25µM C+ 10µM E | 5 | 3 | 62.5 |
| 25µM C+ 25µM E | 6 | 3 | 66.6 |
| 25µM C+ 25µM E | 6 | 2 | 75 |
| 25µM C+ 25µM E | 3 | 1 | 75 |
| 25µM C+ 50µM E | 23 | 10 | 69.6 |
| 25µM C+ 50µM E | 11 | 7 | 64.7 |
| 25µM C+ 50µM E | 29 | 6 | 82.8 |
| 50µM C+ 50µM E | 17 | 12 | 58.6 |
| 50µM C+ 50µM E | 15 | 7 | 68.1 |
| 50µM C+ 50µM E | 11 | 3 | 84.6 |

**Appendix B: RNA quantification and purity validation**

**Total RNA: ARPE-19 control and treated with 10µM curcumin+ 10 µM EGCG**

| | ng/ul | 260/280 | ul/600ng |
|---|---|---|---|
| **C1** | 248.90 | 2.03 | 2.41 |
| **2** | 298.40 | 2.02 | 2.01 |
| **3** | 257.40 | 2.00 | 2.33 |
| **4** | 607.40 | 2.05 | 0.99 |
| **5** | 355.80 | 2.04 | 1.69 |
| **6** | 416.80 | 2.04 | 1.44 |
| **T1** | 189.50 | 2.04 | 3.17 |
| **2** | 211.40 | 2.08 | 2.84 |
| **3** | 240.5 | 2.03 | 2.49 |
| **4** | 295 | 2.00 | 2.03 |
| **5** | 239 | 2.02 | 2.51 |
| **6** | 206.6 | 2.01 | 2.90 |

**Total RNA: HepG2 control and treated with 10µM curcumin+ 10 µM EGCG**

| | ng/ul | 260/280 | ul/600ng |
|---|---|---|---|
| **C1** | 445.60 | 2.03 | 1.3 |
| **2** | 42.60 | 1.98 | 14.1 |
| **3** | 246.20 | 2.02 | 2.4 |
| **4** | 241.60 | 2.00 | 2.5 |
| **5** | 236.40 | 2.01 | 2.5 |
| **6** | 530.00 | 2.03 | 1.1 |
| **T1** | 313.60 | 2.03 | 1.9 |
| **2** | 284.40 | 1.86 | 2.1 |
| **3** | 252.7 | 2.00 | 2.4 |
| **4** | 328.1 | 2.03 | 1.8 |
| **5** | 171.4 | 1.98 | 3.5 |
| **6** | 269.4 | 2.01 | 2.2 |

**Total RNA: SW1417 control and treated with 10µM curcumin+ 10 µM EGCG**

| | ng/ul | 260/280 | ul/600ng |
|---|---|---|---|
| **C1** | 159.79 | 2.04 | 3.75 |
| **2** | 412.01 | 2.01 | 1.46 |
| **3** | 188.36 | 2.05 | 3.19 |
| **4** | 280.71 | 2.01 | 2.14 |
| **5** | 187.53 | 2.02 | 3.20 |
| **6** | 208.72 | 2.03 | 2.87 |
| **T1** | 329.50 | 1.99 | 1.82 |
| **2** | 240.10 | 2.00 | 2.50 |
| **3** | 362.5 | 2.00 | 1.66 |
| **4** | 333.1 | 2.00 | 1.80 |
| **5** | 271 | 2.02 | 2.21 |
| **6** | 338.8 | 2.00 | 1.77 |

**Total RNA: HepG2 treated with 2.5 µM Regorafenib and 2.5 µM Regorafenib+ 10µM curcumin+ 10 µM EGCG**

| | ng/ul | 260/280 | ul/ 500ng |
|---|---|---|---|
| **HR1** | 101.20 | 2.00 | 4.9 |
| **2** | 142.70 | 2.01 | 3.5 |
| **3** | 186.70 | 2.03 | 2.7 |
| **4** | 149.40 | 1.99 | 3.3 |
| **5** | 83.40 | 1.97 | 6.0 |
| **6** | 96.20 | 1.99 | 5.2 |
| **HC1** | 181.90 | 2.01 | 2.7 |
| **2** | 125.70 | 2.01 | 4.0 |
| **3** | 110.7 | 2.02 | 4.5 |
| **4** | 132.3 | 2.02 | 3.8 |
| **5** | 118 | 2.01 | 4.2 |
| **6** | 157.5 | 2.00 | 3.2 |

**Total RNA: SW1417 treated with 2.5µM Regorafenib and 2.5µM Regorafenib + 10µM curcumin + 10 µM EGCG**

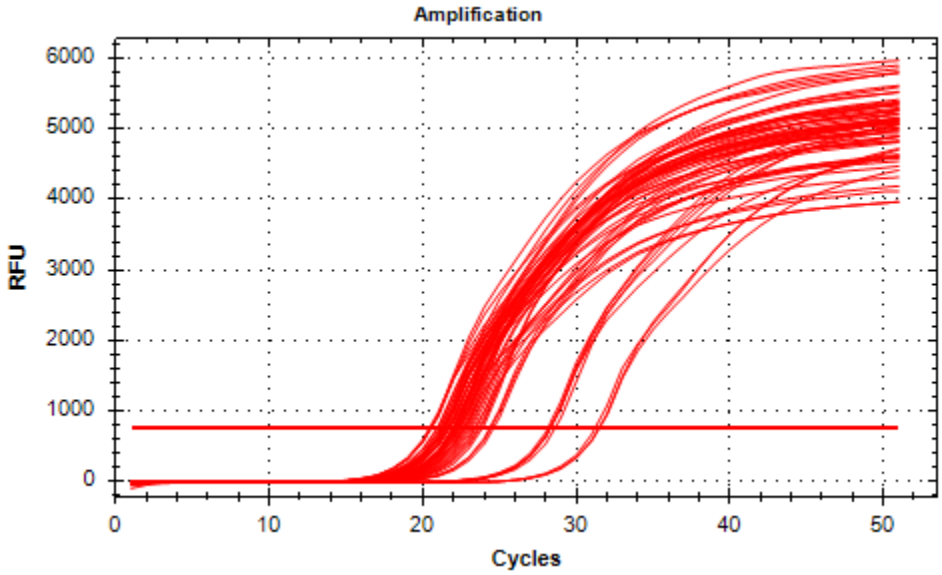
| | ng/ul | 260/280 | ul/ 500ng |
|---|---|---|---|
| **CR1** | 92.60 | 2.10 | 5.4 |
| **2** | 120.60 | 2.20 | 4.1 |
| **3** | 190.10 | 2.03 | 2.6 |
| **4** | 172.30 | 2.04 | 2.9 |
| **5** | 132.60 | 1.69 | 3.8 |
| **6** | 60.60 | 2.13 | 8.3 |
| **CC1** | 81.00 | 2.06 | 6.2 |
| **2** | 124.40 | 1.82 | 4.0 |
| **3** | 135.8 | 2.04 | 3.7 |
| **4** | 77.9 | 2.03 | 6.4 |
| **5** | 119.8 | 2.04 | 4.2 |
| **6** | 163.8 | 2.02 | 3.1 |

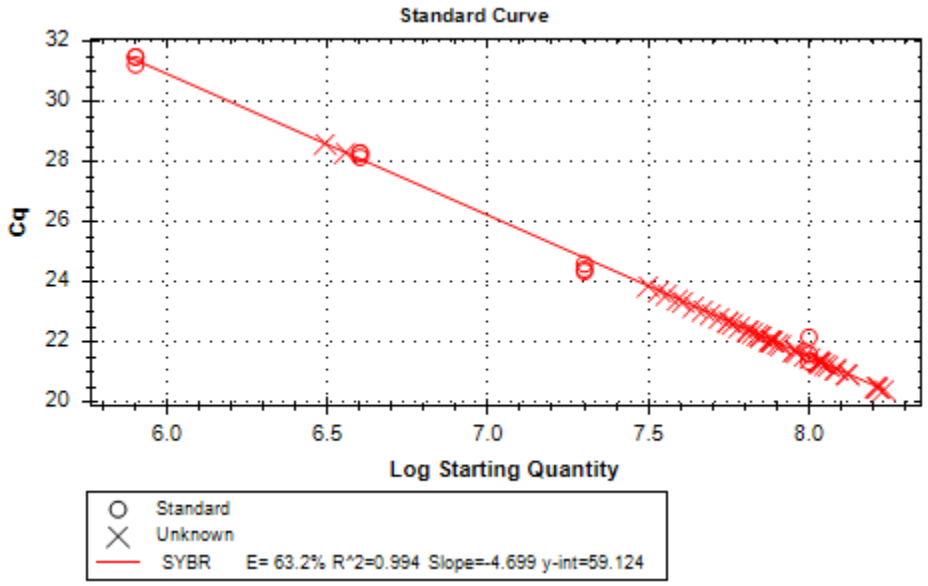
## Appendix C: Gene specific primers used for qPCR

| Gene | Primer Sequence |
|---|---|
| ARNT - F' | 5' - TCT CCC TCC CAG ATG ATG AC – 3' |
| ARNT – R' | 5' - CAA TGT TGT GTC GGG AGA TG – 3' |
| ANGPT2 – F' | 5' - ACT GTG TCC TCT TCC ACC AC – 3' |
| ANGPT2 – R' | 5' - GGA TGT TTA GGG TCT TGC TTT – 3' |
| FGF2 – F' | 5' - AAG AGC GAC CCT CAC ATC AA – 3' |
| FGF2 – R' | 5' - CAG TTC GTT TCA GTG CCA CA – 3' |
| IGFBP7 – F' | 5' - CTG CCC CTC TCC TCT TCC T – 3' |
| IGFBP7 – R' | 5' - GGG ATT CCG ATG ACC TCA CA – 3' |
| SERPINF1 – F' | 5' - TTC AAA GTC CCC GTG AAC AAG – 3' |
| SERPINF1 – R' | 5' - GAG AGC CCG GTG AAT GAT GG – 3' |

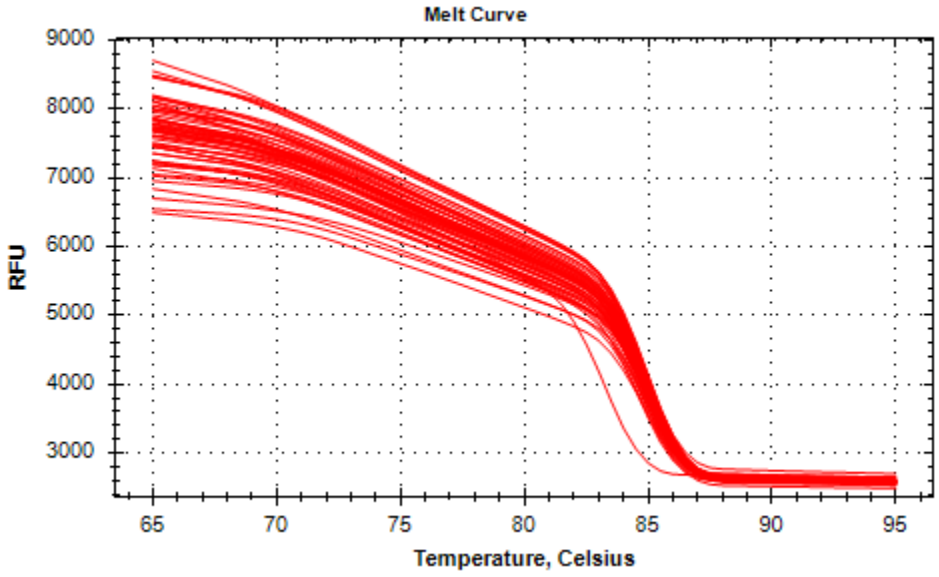
## Appendix D: Validation Data for Quantitative PCR-Amplification

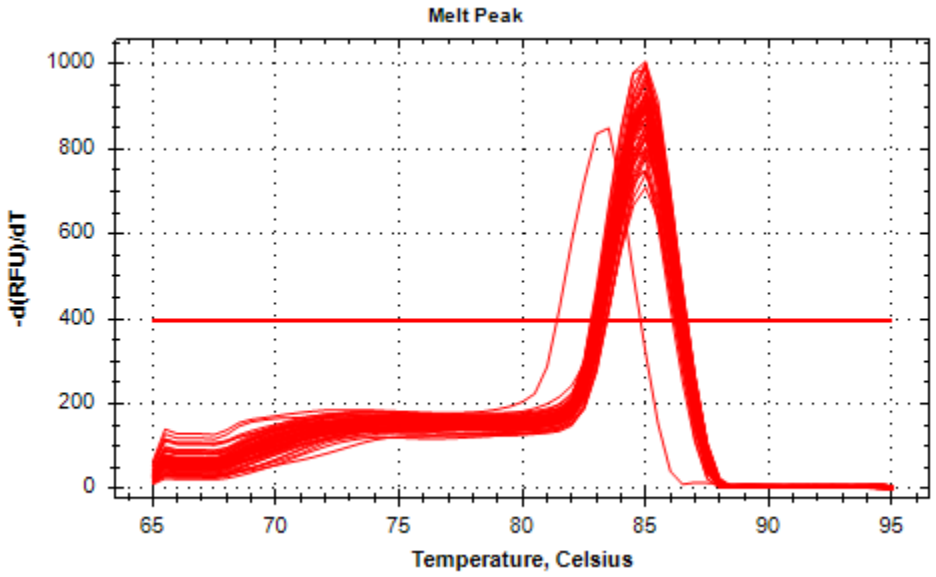
**IGFBP7**



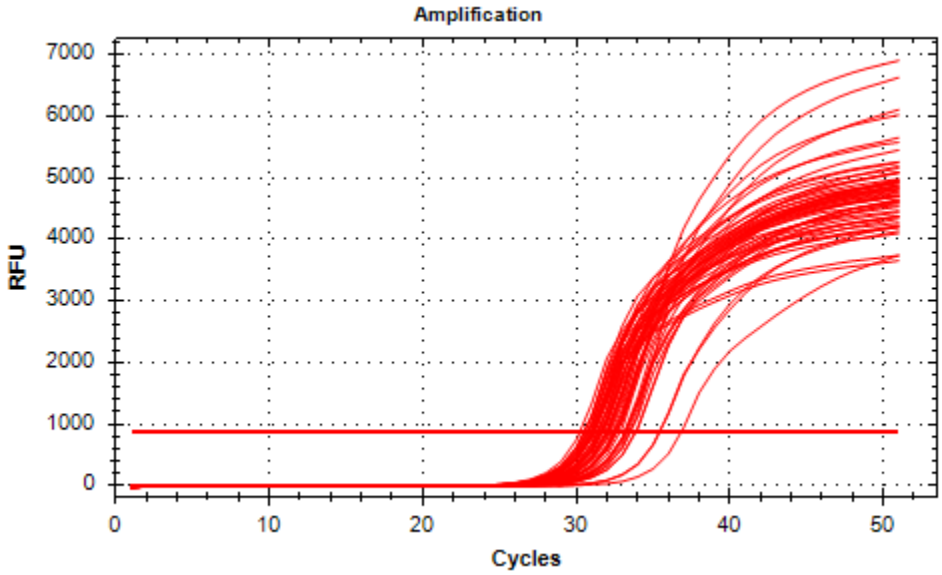



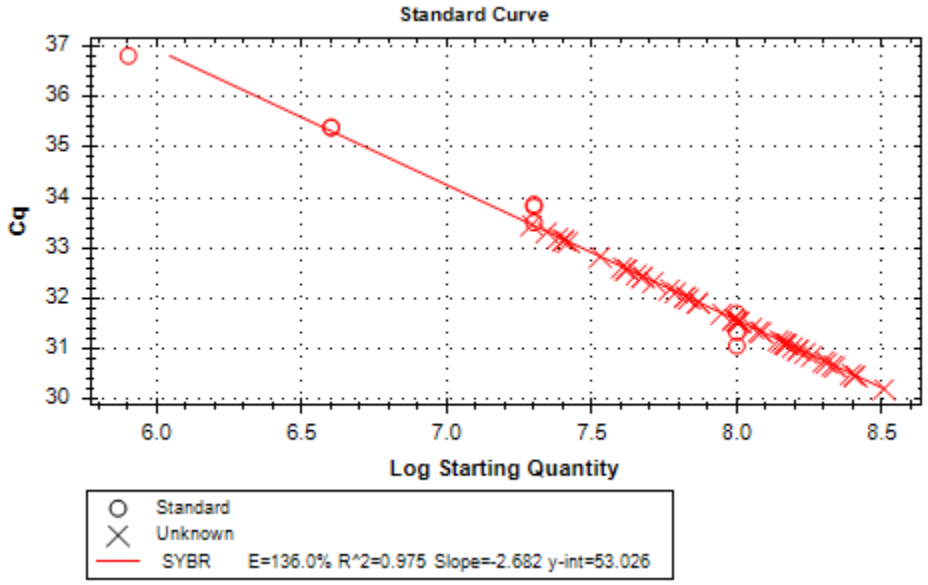
**IGFBP7**


Melt Curve
RFU
9000
8000
7000
6000
5000
4000
3000
65
70
75
80
85
90
95
Temperature, Celsius

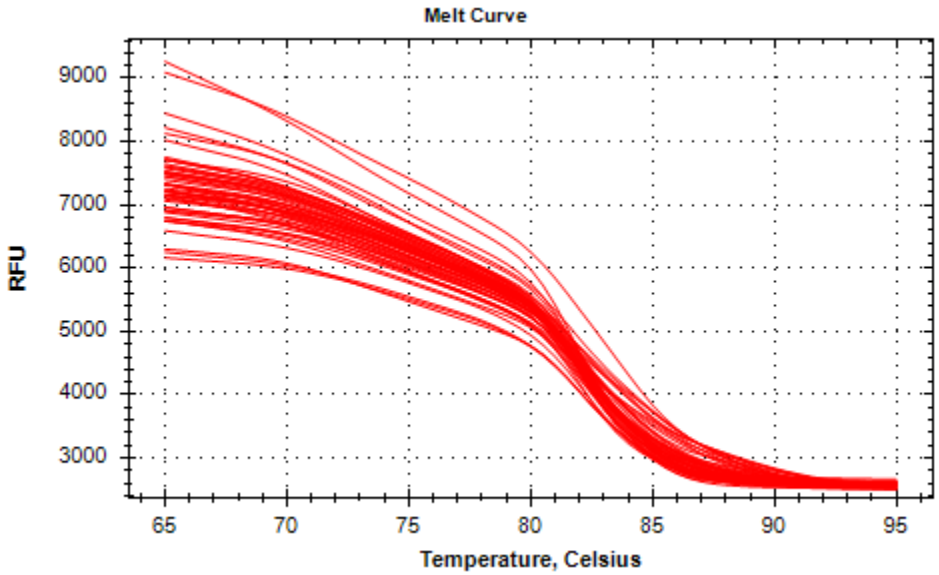

Melt Peak
-d(RFU)/dT
1000
800
600
400
200
0
65
70
75
80
85
90
95
Temperature, Celsius

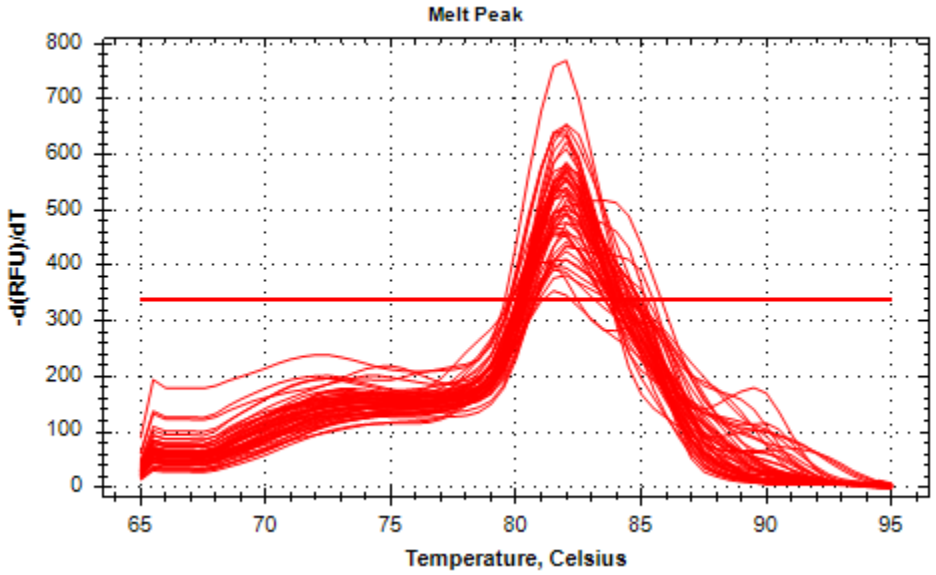
**SERPINF1**


Amplification
RFU
7000
6000
5000
4000
3000
2000
1000
0
0
10
20
30
40
50
Cycles

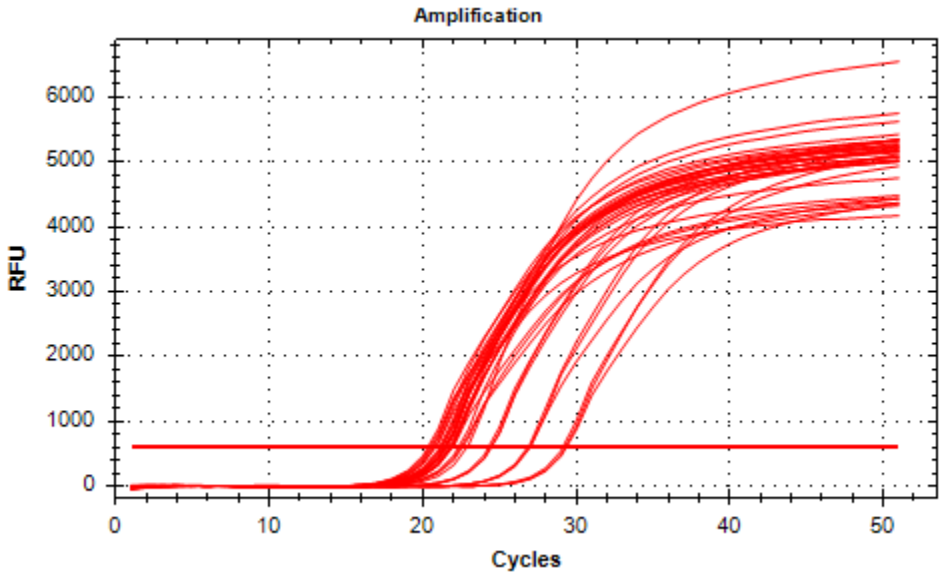

Standard Curve
Cq
37
36
35
34
33
32
31
30
6.0
6.5
7.0
7.5
8.0
8.5
Log Starting Quantity
Standard
Unknown
SYBR E=136.0% R^2=0.975 Slope=-2.682 y-int=53.026

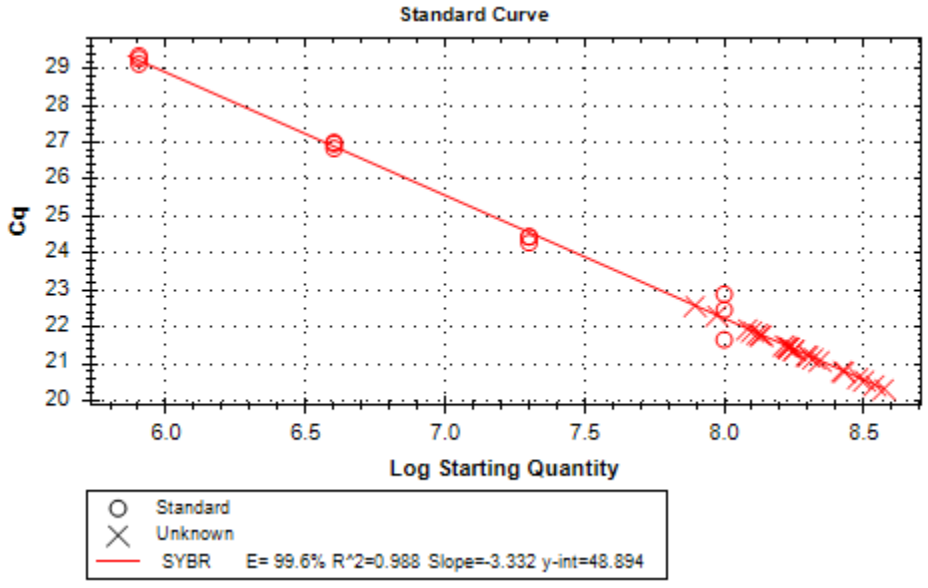
**SERPINF1**


Melt Curve
RFU
9000
8000
7000
6000
5000
4000
3000
65
70
75
80
85
90
95
Temperature, Celsius

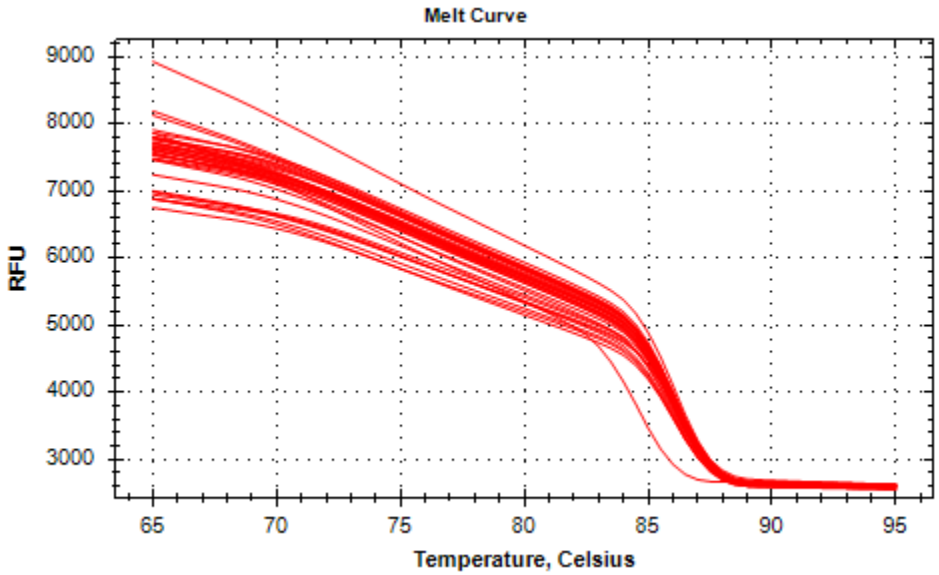

Melt Peak
-d(RFU)/dT
800
700
600
500
400
300
200
100
0
65
70
75
80
85
90
95
Temperature, Celsius

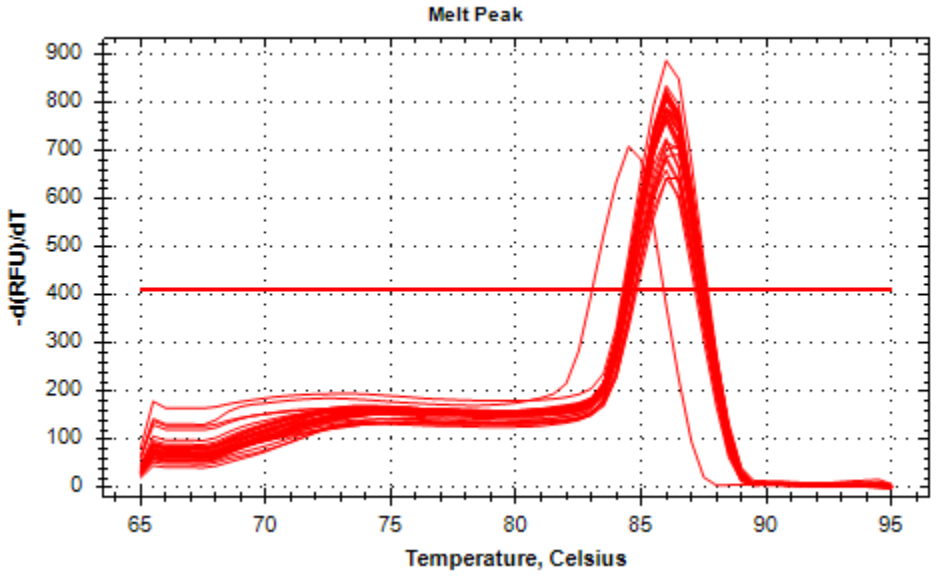
**ANGPT2**


Amplification
RFU
6000
5000
4000
3000
2000
1000
0
0
10
20
30
40
50
Cycles

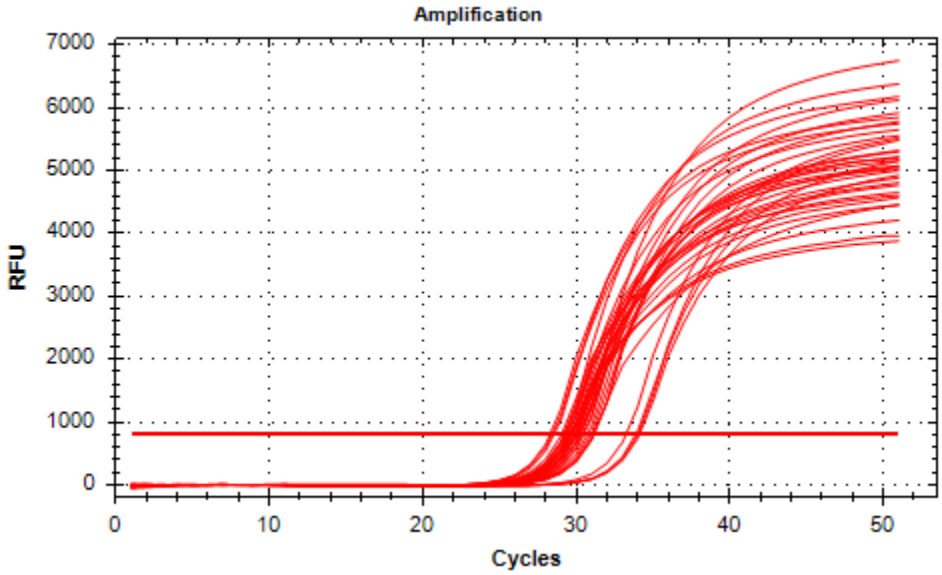

Standard Curve
Cq
29
28
27
26
25
24
23
22
21
20
6.0
6.5
7.0
7.5
8.0
8.5
Log Starting Quantity
Standard
Unknown
SYBR E= 99.6% R^2=0.988 Slope=-3.332 y-int=48.894

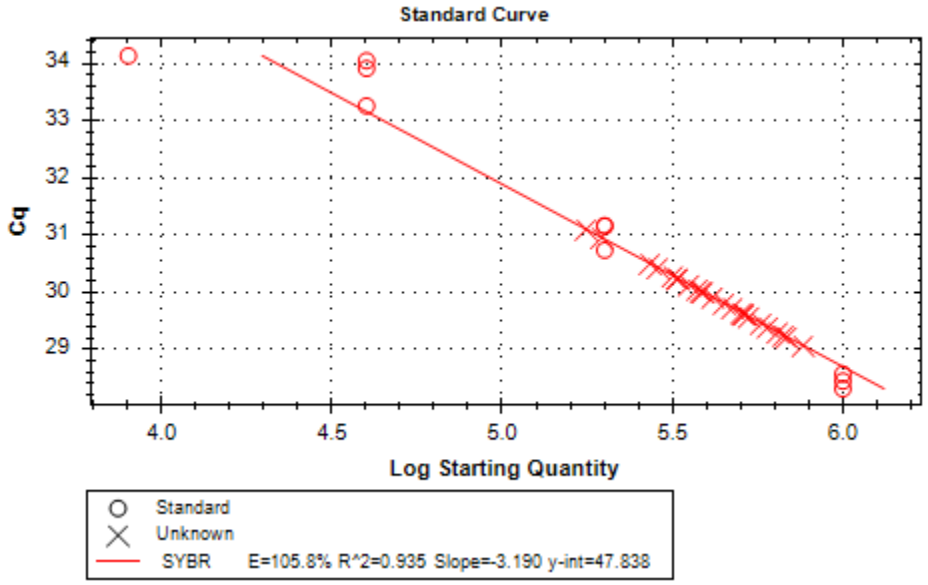
**ANGPT2**


Melt Curve
RFU
9000
8000
7000
6000
5000
4000
3000
65
70
75
80
85
90
95
Temperature, Celsius

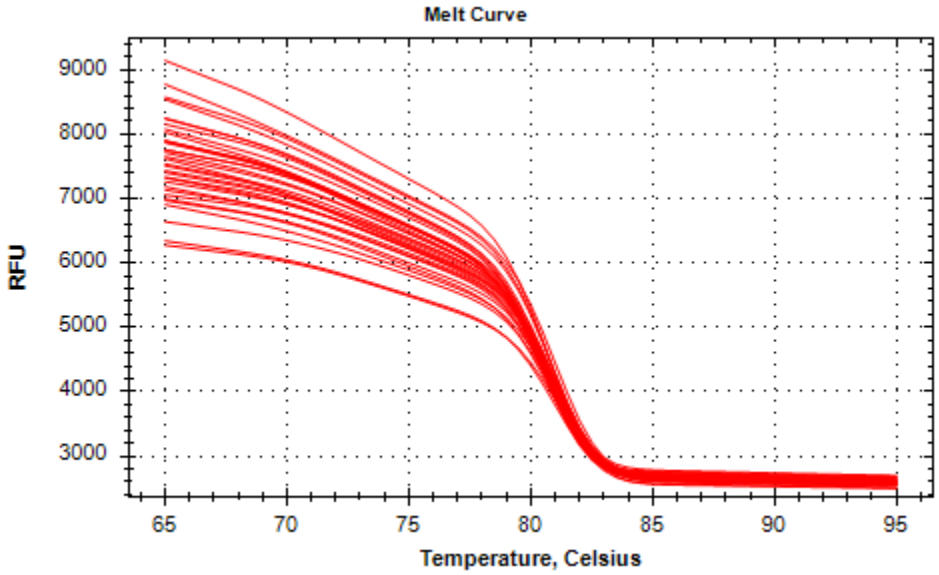

Melt Peak
-d(RFU)/dT
900
800
700
600
500
400
300
200
100
0
65
70
75
80
85
90
95
Temperature, Celsius

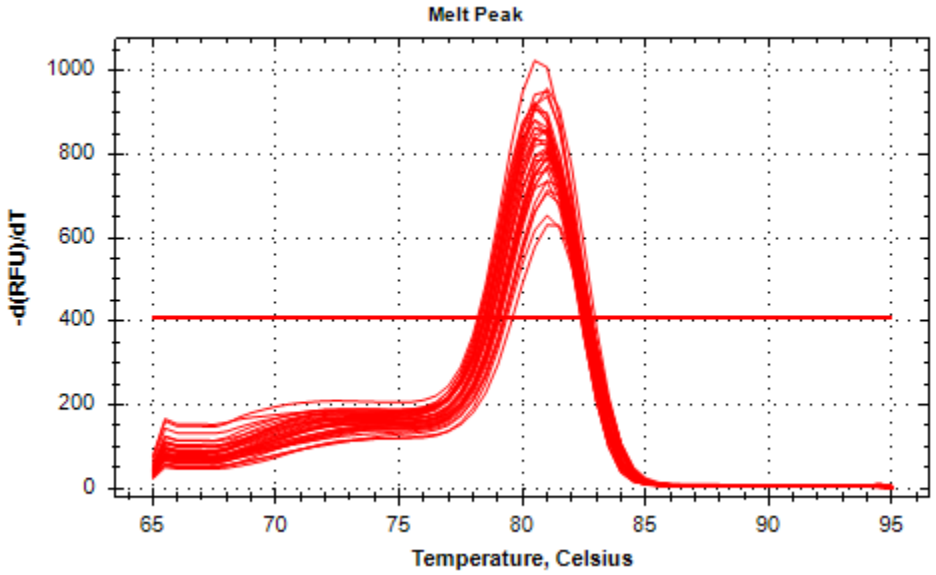
**ARNT**


Amplification
RFU
7000
6000
5000
4000
3000
2000
1000
0
0
10
20
30
40
50
Cycles

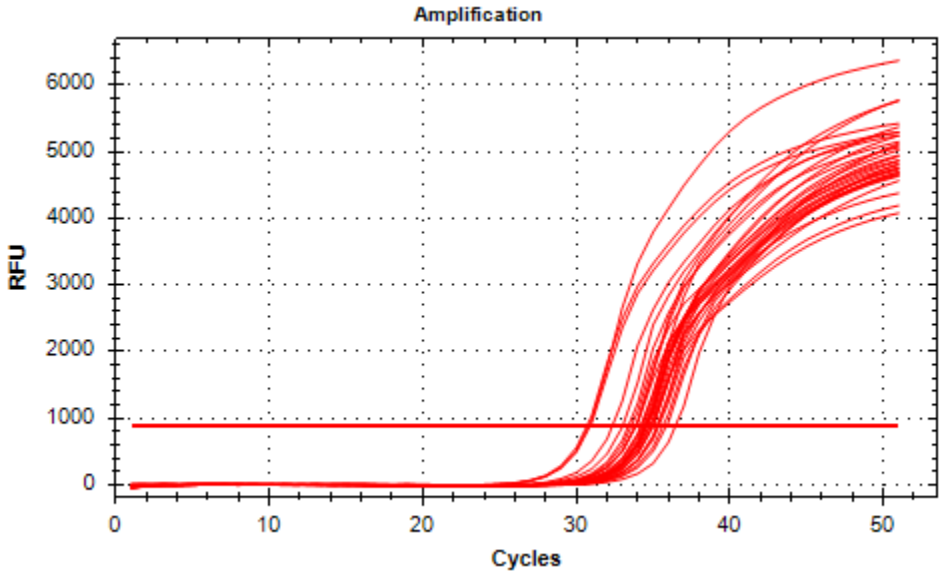

Standard Curve
Cq
34
33
32
31
30
29
4.0
4.5
5.0
5.5
6.0
Log Starting Quantity
Standard
Unknown
SYBR E=105.8% R^2=0.935 Slope=-3.190 y-int=47.838

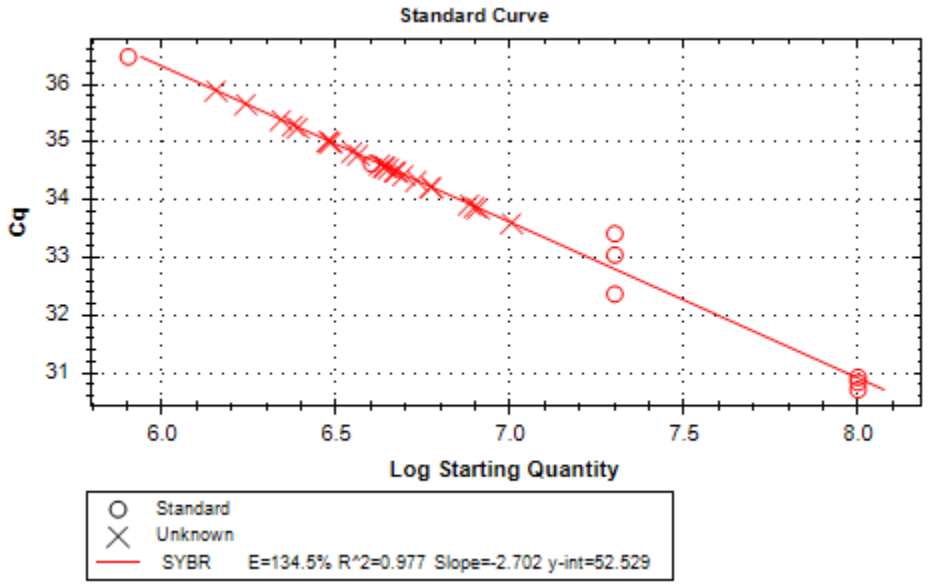
**ARNT**


Melt Curve
RFU
9000
8000
7000
6000
5000
4000
3000
65
70
75
80
85
90
95
Temperature, Celsius

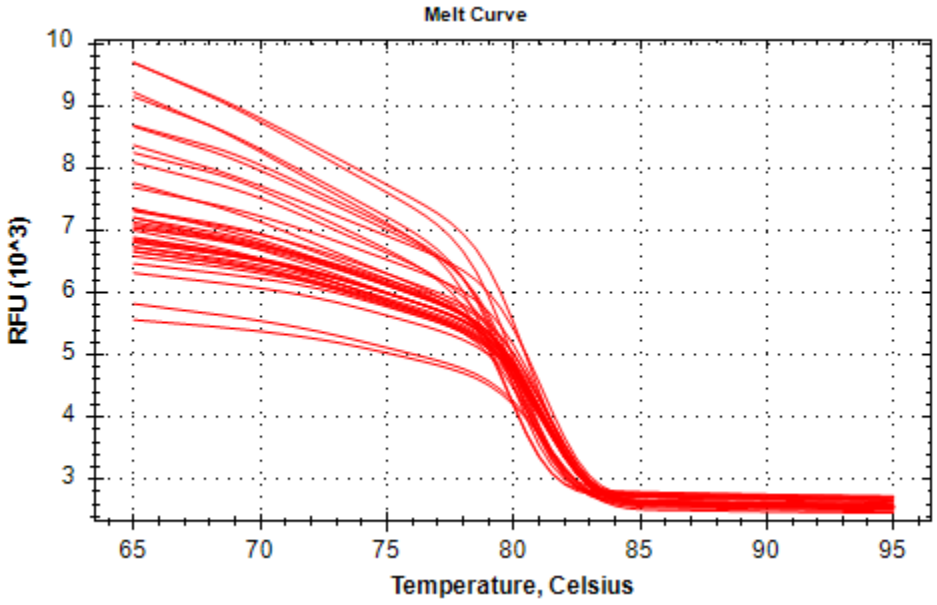

Melt Peak
-d(RFU)/dT
1000
800
600
400
200
0
65
70
75
80
85
90
95
Temperature, Celsius

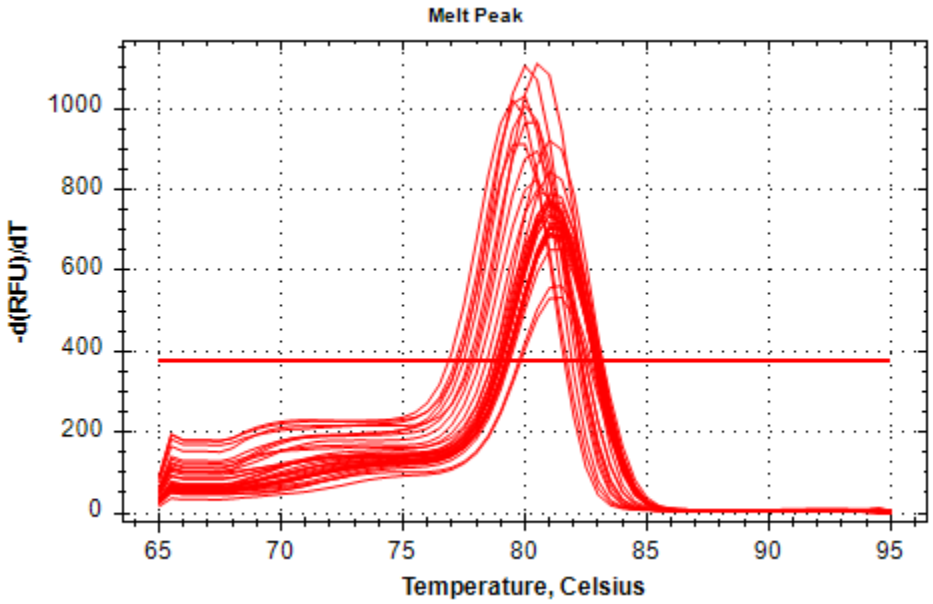
**FGF2**


Amplification
RFU
6000
5000
4000
3000
2000
1000
0
0
10
20
30
40
50
Cycles


Standard Curve
Cq
36
35
34
33
32
31
6.0
6.5
7.0
7.5
8.0
Log Starting Quantity
Standard
Unknown
SYBR E=134.5% R^2=0.977 Slope=-2.702 y-int=52.529

**FGF2**


Melt Curve
RFU (10^3)
10
9
8
7
6
5
4
3
65
70
75
80
85
90
95
Temperature, Celsius


Melt Peak
-d(RFU)/dT
1000
800
600
400
200
0
65
70
75
80
85
90
95
Temperature, Celsius

## Appendix E: Qiagen® RT$^2$ Profiler PCR Array-Human cancer pathway finder

| GenBank | Symbol | Description |
|---|---|---|
| NM_001096 | ACLY | ATP citrate lyase |
| NM_004458 | ACSL4 | Acyl-CoA synthetase long-chain family member 4 |
| NM_001124 | ADM | Adrenomedullin |
| NM_001146 | ANGPT1 | Angiopoietin 1 |
| NM_001147 | ANGPT2 | Angiopoietin 2 |
| NM_001160 | APAF1 | Apoptotic peptidase activating factor 1 |
| NM_001668 | ARNT | Aryl hydrocarbon receptor nuclear translocator |
| NM_004046 | ATP5A1 | ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit 1, cardiac muscle |
| NM_003600 | AURKA | Aurora kinase A |
| NM_006538 | BCL2L11 | BCL2-like 11 (apoptosis facilitator) |
| NM_001165 | BIRC3 | Baculoviral IAP repeat containing 3 |
| NM_005180 | BMI1 | BMI1 polycomb ring finger oncogene |
| NM_001216 | CA9 | Carbonic anhydrase IX |
| NM_032982 | CASP2 | Caspase 2, apoptosis-related cysteine peptidase |
| NM_001227 | CASP7 | Caspase 7, apoptosis-related cysteine peptidase |
| NM_001229 | CASP9 | Caspase 9, apoptosis-related cysteine peptidase |
| NM_002982 | CCL2 | Chemokine (C-C motif) ligand 2 |
| NM_001759 | CCND2 | Cyclin D2 |
| NM_001760 | CCND3 | Cyclin D3 |
| NM_001255 | CDC20 | Cell division cycle 20 homolog (S. cerevisiae) |
| NM_001792 | CDH2 | Cadherin 2, type 1, N-cadherin (neuronal) |
| NM_003879 | CFLAR | CASP8 and FADD-like apoptosis regulator |
| NM_004255 | COX5A | Cytochrome c oxidase subunit Va |
| NM_000098 | CPT2 | Carnitine palmitoyltransferase 2 |
| NM_000107 | DDB2 | Damage-specific DNA binding protein 2, 48kDa |
| NM_004083 | DDIT3 | DNA-damage-inducible transcript 3 |
| NM_001363 | DKC1 | Dyskeratosis congenita 1, dyskerin |

| GenBank | Symbol | Description |
|---|---|---|
| NM_004415 | DSP | Desmoplakin |
| NM_001950 | E2F4 | E2F transcription factor 4, p107/p130-binding |
| NM_000799 | EPO | Erythropoietin |
| NM_000122 | ERCC3 | Excision repair cross-complementing rodent repair deficiency, complementation group 3 (xeroderma pigmentosum group B complementing) |
| NM_000123 | ERCC5 | Excision repair cross-complementing rodent repair deficiency, complementation group 5 |
| NM_005239 | ETS2 | V-Ets erythroblastosis virus E26 oncogene homolog 2 (avian) |
| NM_000639 | FASLG | Fas ligand (TNF superfamily, member 6) |
| NM_002006 | FGF2 | Fibroblast growth factor 2 (basic) |
| NM_002019 | FLT1 | Fms-related tyrosine kinase 1 (vascular endothelial growth factor/vascular permeability factor receptor) |
| NM_005251 | FOXC2 | Forkhead box C2 (MFH-1, mesenchyme forkhead 1) |
| NM_000402 | G6PD | Glucose-6-phosphate dehydrogenase |
| NM_006705 | GADD45G | Growth arrest and DNA-damage-inducible, gamma |
| NM_000408 | GPD2 | Glycerol-3-phosphate dehydrogenase 2 (mitochondrial) |
| NM_173849 | GSC | Goosecoid homeobox |
| NM_002133 | HMOX1 | Heme oxygenase (decycling) 1 |
| NM_000598 | IGFBP3 | Insulin-like growth factor binding protein 3 |
| NM_000599 | IGFBP5 | Insulin-like growth factor binding protein 5 |
| NM_001553 | IGFBP7 | Insulin-like growth factor binding protein 7 |
| NM_002253 | KDR | Kinase insert domain receptor (a type III receptor tyrosine kinase) |
| NM_000526 | KRT14 | Keratin 14 |
| NM_005566 | LDHA | Lactate dehydrogenase A |
| NM_002312 | LIG4 | Ligase IV, DNA, ATP-dependent |
| NM_000237 | LPL | Lipoprotein lipase |
| NM_002755 | MAP2K1 | Mitogen-activated protein kinase kinase 1 |
| NM_002756 | MAP2K3 | Mitogen-activated protein kinase kinase 3 |
| NM_001315 | MAPK14 | Mitogen-activated protein kinase 14 |
| NM_004526 | MCM2 | Minichromosome maintenance complex component 2 |
| NM_002417 | MKI67 | Antigen identified by monoclonal antibody Ki-67 |

| GenBank | Symbol | Description |
|---|---|---|
| NM_003946 | NOL3 | Nucleolar protein 3 (apoptosis repressor with CARD domain) |
| NM_002538 | OCLN | Occludin |
| NM_002626 | PFKL | Phosphofructokinase, liver |
| NM_002632 | PGF | Placental growth factor |
| NM_017884 | PINX1 | PIN2/TERF1 interacting, telomerase inhibitor 1 |
| NM_002690 | POLB | Polymerase (DNA directed), beta |
| NM_014330 | PPP1R15A | Protein phosphatase 1, regulatory (inhibitor) subunit 15A |
| NM_002575 | SERPINB2 | Serpin peptidase inhibitor, clade B (ovalbumin), member 2 |
| NM_002615 | SERPINF1 | Serpin peptidase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium derived factor), member 1 |
| NM_005983 | SKP2 | S-phase kinase-associated protein 2 (p45) |
| NM_006516 | SLC2A1 | Solute carrier family 2 (facilitated glucose transporter), member 1 |
| NM_005985 | SNAI1 | Snail homolog 1 (Drosophila) |
| NM_003068 | SNAI2 | Snail homolog 2 (Drosophila) |
| NM_178310 | SNAI3 | Snail homolog 3 (Drosophila) |
| NM_000454 | SOD1 | Superoxide dismutase 1, soluble |
| NM_006941 | SOX10 | SRY (sex determining region Y)-box 10 |
| NM_005563 | STMN1 | Stathmin 1 |
| NM_005994 | TBX2 | T-box 2 |
| NM_000459 | TEK | TEK tyrosine kinase, endothelial |
| NM_007110 | TEP1 | Telomerase-associated protein 1 |
| NM_017489 | TERF1 | Telomeric repeat binding factor (NIMA-interacting) 1 |
| NM_018975 | TERF2IP | Telomeric repeat binding factor 2, interacting protein |
| NM_012461 | TINF2 | TERF1 (TRF1)-interacting nuclear factor 2 |
| NM_003747 | TNKS | Tankyrase, TRF1-interacting ankyrin-related ADP-ribose polymerase |
| NM_025235 | TNKS2 | Tankyrase, TRF1-interacting ankyrin-related ADP-ribose polymerase 2 |
| NM_006003 | UQCRFS1 | Ubiquinol-cytochrome c reductase, Rieske iron-sulfur polypeptide 1 |
| NM_005429 | VEGFC | Vascular endothelial growth factor C |
| NM_003390 | WEE1 | WEE1 homolog (S. pombe) |
| NM_001167 | XIAP | X-linked inhibitor of apoptosis |

| GenBank | Symbol | Description |
|---|---|---|
| NM_001101 | ACTB | Actin, beta |
| NM_004048 | B2M | Beta-2-microglobulin |
| NM_002046 | GAPDH | Glyceraldehyde-3-phosphate dehydrogenase |
| NM_000194 | HPRT1 | Hypoxanthine phosphoribosyltransferase 1 |
| NM_001002 | RPLP0 | Ribosomal protein, large, P0 |

## Appendix F: Abbreviations

| ABBREVIATION | FULL NAME |
|---|---|
| AKT | Protein kinase B |
| AMPK | AMP-activated protein kinase |
| ANGPT2 | Angiopoietin 2 |
| ANOVA | Analysis of variance |
| APC | Adenomatous polyposis coli |
| ArH | Aryl hydrocarbon receptor |
| ARNT | Aryl Hydrocarbon Receptor Nuclear Translocator |
| ARPE-19 | Human Retinal Pigment Epithelium Cells |
| ATF3 | Activating transcription factors |
| ATR/ATRIP | ATM and Rad3-related/ATR interacting protein |
| Bcl-2 | B-cell lymphoma 2 |
| BRCA1 | Breast cancer gene |
| CCK-8 | Cell counting kit-8 |
| CCND1 | Cyclin D1 |
| Cdc25A | Cell division cycle 25 A |
| CDK | Cyclin dependent kinase |
| CDKN2A | Cyclin Dependent Kinase Inhibitor 2A |
| Chk1 | Checkpoint kinase 1 |
| CIN | Chromosomal Instability Pathway |
| COX-2 | cyclooxygenase-2 |
| CRC | Colorectal cancer |

| ABBREVIATION | FULL NAME |
|---|---|
| CTNNB1 | Catenin, beta-1 |
| CXCL12 | C-X-C motif chemokine 12 |
| EGCG | epigallocatechin-3-gallate |
| EGF | Epidermal growth factor |
| EGFR | Epidermal growth factor receptor |
| ErbB | Epidermal Growth Factor family of receptor tyrosine kinases |
| FBS | Fetal bovine serum |
| FGF2 | Fibroblast growth factor |
| GADD153 | Growth Arrest and DNA Damage-Inducible Protein |
| HCC | Hepatocellular carcinoma |
| HepG2 | human liver cancer cell line |
| Her2/neu | Human epidermal growth factor receptor 2 |
| HGF/MET | Human growth factor/Mesenchymal epithelial transition |
| HH/GL1 | Hedgehog/ glioma-associated oncogene |
| hMLH1 | human mutL homolog 1 |
| HSP70 | Heat shock protein 70 |
| IGF | Insulin like growth factor |
| IGFBP7 | Insulin Like Growth Factor Binding Protein 7 |
| K-ras | Proto-oncogene |
| MAPK | Mitogen activated protein kinase |
| MCF-7 | Michigan Cancer Foundation-7 (Breast cancer cell line) |
| MITF | Melanogenesis Associated Transcription Factor |
| MMPs | Matrix metalloproteinases |

| ABBREVIATION | FULL NAME |
|---|---|
| MSI | Microsatellite instability |
| MTORC1 | mammalian target of rapamycin complex 1 |
| MVD | mevalonate pyrophosphate decarboxylase |
| NF-kB | nuclear factor kappa B |
| NOS | Nitrogen species |
| NSCLC | Non-small lung cancer |
| P13 K/Akt | Phosphoinositide 3-kinase/Protein kinase B |
| PTHrp | Para Thyroid Hormone related protein |
| ROS | Reactive oxygen species |
| SERPINF1 | Serpin Family F Member 1 |
| STAT | Signal Transducer and Activator of Transcription |
| STAT-3 | Signal transducer and activator of transcription 3 |
| SW1417 | Human colorectal adenocarcinoma |
| TGF-β | Tumor Growth Factor- β |
| TP53 | tumor protein p53 |
| VEGF | Vascular endothelial growth factor |
| WHO | World Health Organization |
| WNT | Wingless-related integration site |