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Molecular Targets of Green Tea Catechin, EGCG, on Human Colorectal Carcinogenesis

Mugdha G. Sukhthankar
University of Tennessee - Knoxville

To the Graduate Council:

I am submitting herewith a dissertation written by Mugdha G. Sukhthankar entitled "Molecular Targets of Green Tea Catechin, EGCG, on Human Colorectal Carcinogenesis." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Comparative and Experimental Medicine.

Seung Joon Baek, Major Professor

We have read this dissertation and recommend its acceptance:

Michael F. McEntee, Claudia Kirk, Jay Wimalasena

Accepted for the Council:

Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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A Dissertation Presented for the Doctor of Philosophy Degree
The University of Tennessee, Knoxville

Mugdha G. Sukhthankar
December 2009

DEDICATION

This thesis is dedicated to my late grandmother, Vijaya K. Sukhthankar, as it was her dream that I should have a successful career in a field wherein I could give back something to the society, and to my grandfather, Kamalaksha B. Sukhthankar; father, Girish K. Sukhthankar; mother, Uma G. Sukhthankar, who has suffered from ulcerative colitis and had her large intestine removed because of future risks to colorectal cancer. Without their love, support, encouragement and blessings I would not even be in US. They are truly responsible for all my success.

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What to say and how much to say about my family members, my grandparents, my parents, Mothiaai, and my in-laws. I don't want to devalue the consideration, affection and all kinds of support they provided, by binding those in words. After all, I owe my very existence and my status to them. Also my best friend and my better half, Bhupesh, for his timely support, love and caring.

I express my sincere gratitude to the College of Veterinary Medicine, University of Tennessee for having inculcated in me the best qualities and spirit to do research and provided me with financial support for this study and also NIH for grant support.

Abstract

This thesis explores the effect of major green tea catechin, EGCG, on colorectal carcinogenesis particularly on target genes, *bFGF* and *NUDT6.* Cancer is a second leading cause and colorectal cancer is a third leading cause of deaths in the Western World. Recently there is a lot of persuasive epidemiological and experimental evidence that phytochemical-enriched diet may be involved in the prevention of colorectal cancer. EGCG exhibits beneficial effects like anti-tumorigenic, anti-oxidant, anti-angiogenic, and pro-apoptotic activity.

In studies presented here, we investigated the molecular mechanisms by which EGCG affects bFGF degradation (Chapter 3): EGCG increased the ubiquitination as well as trypsin like activity of 26S proteasome causing faster bFGF degradation. We also demonstrated that EGCG could decrease the tumor number and volume in mouse models of colorectal cancer.

Our microarray data from catechin-treated colorectal cancer cells revealed down regulation of a gene called *NUDT6*, an anti-sense of *bFGF*, which we have shown to regulate the bFGF function. NUDT6 is a mitochondrial protein whose biological function is unknown. EGCG affected the mRNA stability of *NUDT6* by decreasing the luciferase activity of its 3'UTR (Chapter 4). NUDT6 stable cells showed increase in cell proliferation and soft agar colony formation. The electrical impedance bio-analytical assay showed increased attachment of NUDT6 cells to the surface media and to each other. NUDT6 cells showed increased invasion rate and decreased Caspase 3/7 activity.

Injection of CONTROL and NUDT6 cells into nude mice complemented the *in vitro* data showing increase in tumor volume, mitotic index and decrease in caspase activity and apoptotic index. This was possibly due to binding of p53 to NUDT6 which led to degradation of p53.

NUDT6 was highly expressed in human colon tumor tissue sample compared to normal and NUDT6 could be used as a marker in colon tumorigenesis.

The findings presented in this thesis clearly show that EGCG is an effective chemopreventive dietary agent, decreasing the expression of pro-angiogenic and cell proliferation genes, *bFGF* and *NUDT6* in colorectal cancer cells. Further characterization of NUDT6 as a potential biomarker in colorectal carcinogenesis may provide a better prediction of disease progression in the future.

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List of Symbols and /or Abbreviations

TGF-β	**Transforming growth factor beta**
APC	**Adenomatous polyposis coli**
GSK3β	**Glycogen synthase kinase-3beta**
CK1	**Casein kinase 1**
Ser/Thr	**Serine/ Threonine**
TCF/LEF	**T-cell factor/ Lymphoid enhancing factor**
PPAR-δ	**Peroxisome proliferator activated receptor-delta**
SE	**Surface epithelium**
CC	**Colon crypts**
GC	**Goblet cells**
LP	**Lamina propria**
MM	**Muscularis mucosa**
K-RAS	**Kirsten rat sarcoma**
MLH1	**mutL homolog 1**
MSH2	**mutS homolog 2**
MAPK	**Mitogen-activated protein kinase**
MAPKKK	**MAPK kinase kinase**
MAPKK	**MAPK kinase**
ERK	**Extracellular signal-regulated kinase**
JNK	**c-JUN N-terminal kinase**
MNK	**MAPK signal-integrating kinase**
CREB	**cAMP response element binding**
CRE	**cAMP response element**
HNPCC	**Hereditary non polyposis colorectal cancer**
COX	**Cyclooxygenase**
PGE2	**Prostaglandin E2**
LOX	**Lipoxygenase**
PG	**Prostaglandin**
Tx	**Thromboxane**
PGG2	**Prostaglandin G2**
PGH2	**Prostaglandin H2**
ATM	**Ataxia telangiectasia mutated kinase**
ATR	**ATM and RAD3- related kinase**
Cdk2	**Cyclin dependent kinase 2**
PKC	**protein kinase C**
FAP	**Familial adenomatous polyposis**
IBD	**Inflammatory bowel disease**
AFAP	**Attenuated familial adenomatous polyposis**
MUTYH	**mutY homolog**
MMR	**Mismatch repair**
MSI	**Microsatellite instability**
DCC	**Deleted in colorectal cancer**

5-FU	**5- Fluorouracil**
EGCG	**Epigallocatechin-3-gallate**
EGC	**Epigallocatechin**
ECG	**Epicatechin-3-gallate**
EC	**Epicatechin**
NF-κB	**Nuclear factor- κB**
GTP	**Green tea polyphenols**
NAG-1	**Non-steroidal anti-inflammatory drug activated gene 1**
EGR-1	**Early growth receptor gene-1**
ATF3	**Activating transcription factor gene 1**
Min	**Multiple intestinal neoplasia**
bFGF	**Basic fibroblast growth factor**
NUDT6	**(nucleoside diphosphate linked moiety X)- type motif 6**
VEGF	**Vascular endothelial growth factor**
ECM	**Extracellular matrix**
NQO1	**NAD(P)H quinone oxidoreductase 1**
3'UTR	**3' untranslated region**
NR4A1	**Nuclear receptor subfamily 4 group A member 1**
ID2	**Member of Id helix-loop-helix family of protein**
ID1	**Inhibitory of DNA binding 1**

Introduction: Colorectal cancer and Green tea

CHAPTER 1

1.1 COLORECTAL CANCER

Cancer is a group of diseases which arises when a cell, for a variety of reasons, escapes from the normal constraints placed on its growth and is then characterized by unregulated cell growth, invasion and spread of cells from the site of origin, or primary site, to other sites in the body. Colorectal cancer is a type of cancer characterized by the development of malignant cells in the lining or epithelium of the colon and rectum and is an epithelial neoplasm.

1.1.1 NORMAL INTESTINAL EPITHELIUM

The epithelial cells lining the luminal surface are tightly regulated to ensure homeostasis in the intestine and different layers are shown in **Figure 1**. The renewal of epithelial cells is dependent upon the adult stem cell compartment localized at the bottom of the crypt (1, 2). After a transient proliferative phase when the cells start migrating upwards, the epithelium differentiates into specialized cell types including absorptive enterocytes, mucus secreting goblet cells, and enteroendocrine cells and the migratory process is finalized when cells reach the top of the crypt where they are exfoliated into the lumen upon apoptosis. In the upper gastrointestinal tract, all cells move upwards during differentiation whereas Paneth cells move downwards **(Figure 1.1)**. Intestinal subepithelial myofibroblasts are specialized stromal cells that form a continuous sheet directly localized underneath the epithelium. These myofibroblasts contribute to epithelial cell function by providing mechanical support and secreting key signaling ligands. Thus, the intimate interaction between the parenchyme and mesenchyme ensures proper tissue function, balancing cell renewal, and differentiation (3). Also tumor progression is driven not just by what is happening inside the tumor cell itself but also by what is happening around it (stroma). The key hallmarks of tumor cell dependent on microenvironment are angiogenesis and invasion (4).

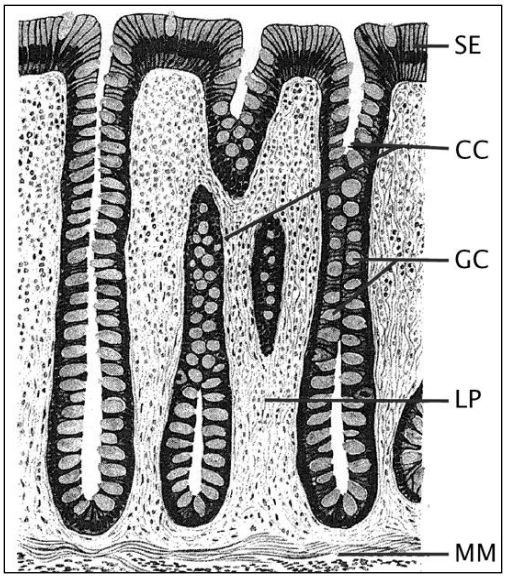

Figure 1. The morphology of normal colonic mucosa. Labels show lumen surface epithelium (SE), colon crypts (CC), goblet cells (GC), lamina propria (LP), and muscularis mucosa (MM). The crypts open to the surface epithelium (20).

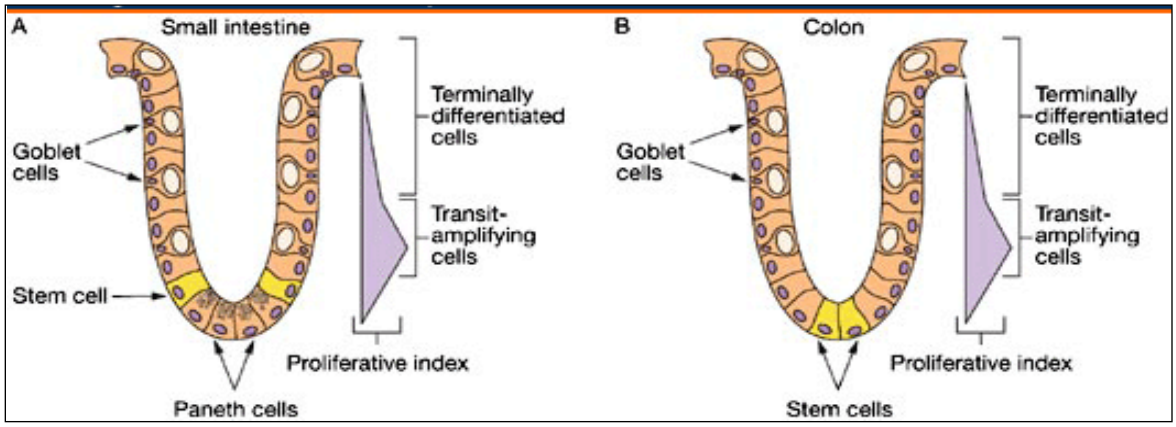

Figure 1.1: Schematic of the small intestinal and colonic crypts. (A) Within the small intestine, stem cells are thought to be located at position 4-5 distal to the Paneth cells. (B) Within the colon, they are at the base of the crypt. In the crypt, the majority of cell proliferation takes place in the transit-amplifying region and terminal differentiation usually occurs distal to this region. (Figure from reference (21)).

Overall, cell renewal, proliferation, and differentiation are coupled to positional localization along the crypt-to-villus axis. This positional regulation of proliferation and differentiation can be correlated to several signaling pathways known to govern stemness and differentiation, including Wnt/β-catenin, and transforming growth factor-β **(Figure 1.2)** [TGFβ; (5)]. Kirsten rat sarcoma (KRAS), a GTP-ase that controls cell proliferation, is mutated in 50–60% of cases of colorectal cancer, and expression of the cell-adhesion transmembrane glycoprotein E-cadherin is down regulated in a similar number of patients. Mutations in the mismatch-repair genes mutL homolog 1(*MLH1)* and mutS homolog 2 (*MSH2)* contribute to genetic instability. SMAD4 belongs to the SMAD family, which is involved in the TGF-β signaling pathway to suppress epithelial-cell growth. p*53* mutations tend to be late events and increase the resistance of cancer cells to apoptosis.

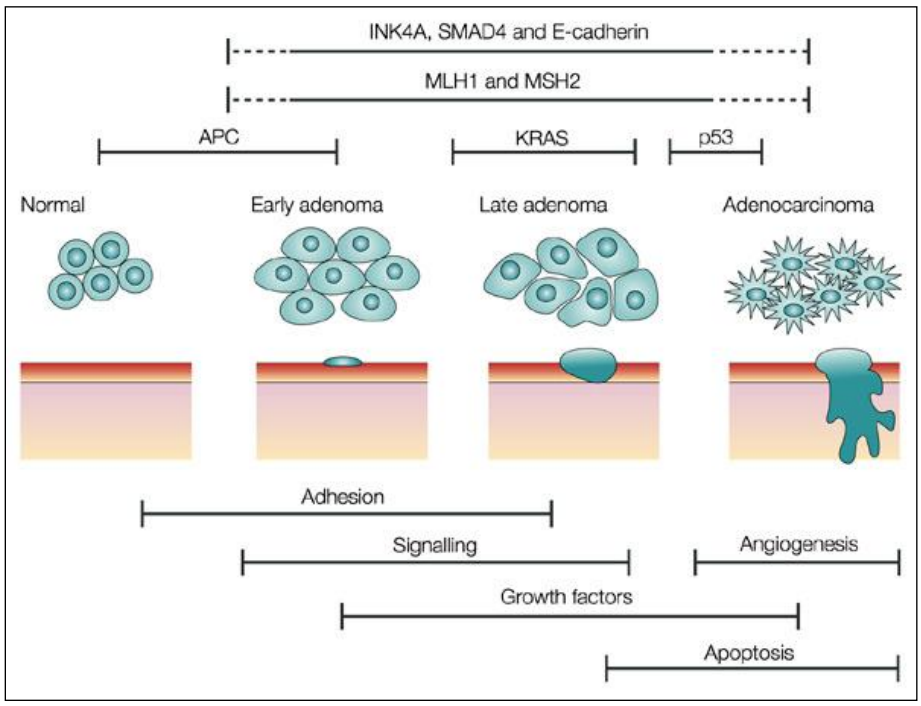

Figure 1.2: Transition from normal to cancer. There is a well-described sequence of mutational events that characterize the transition from normal colon epithelium to premalignant adenoma and then invasive adenocarcinoma. (Figure from reference (22)).

1.1.2 SIGNALING PATHWAYS IN COLORECTAL CARCINOGENESIS

WNT pathway: The over –activation of the Wnt signaling route disturbs intestinal homeostasis paving the way for pathogenesis. This pathway demonstrates a complex network of proteins most well known for their roles in embryogenesis and cancer, but also involved in normal physiological processes in adult animals **(Figure 1.3)** (6). Indeed, the vast majority of sporadic colorectal cancer cases are caused by constitutive Wnt activation due to mutations in either the adenomatous polyposis coli (*APC)* tumor suppressor or the β-catenin (*CTNNB1*) oncogene (7). *APC* is one of the early and frequently mutated genes in colorectal cancer, designated as a `gatekeeper' gene (8). Loss of APC function and upon the binding of a Wnt peptide to its receptor (Frizzled) (9), a signal is instigated, which through the disheveled gene product leads to destabilization of the 'destruction complex', a multiprotein complex (10), encompassing three scaffold proteins, APC, Axin1, and Axin2 (conductin), and two kinases, glycogen synthase kinase-3β (GSK3β) and casein kinase 1 (CK1). The complex binds and phosphorylates β-catenin at serine and threonine residues, thus targeting it for ubiquitination and proteolytic degradation. In contrast, oncogenic mutations in β-catenin render it resistant to serine/ threonine (Ser/Thr) phosphorylation and proteolytic degradation. Upon its cytoplasmic stabilization and subsequent nuclear translocation, β-catenin binds to members of the T-cell factor/ Lymphoid enhancing factor (TCF/LEF) family of transcription factors, thus modulating expression of a broad range of target genes. One gene is *Cyclin D1* which has crucial role in cell growth, proliferation and differentiation, and is inappropriately activated in colon cancer (11). Another gene which is activated in a deranged Wnt signaling system is *c-myc* (12, 13). *C-myc* codes for a transcription factor regulating a large set of genes including *p53* and exert diverse overall effects, such as

activation of the cell cycle, induction of apoptosis, and inhibition of differentiation. This shows the importance of Wnt signaling pathway in normal function of a colonic epithelial cell. Yet another gene is peroxisome proliferator activated receptor-delta (*PPAR-δ)* which is an APC regulated gene. PPAR-δ expression is found to be elevated in colorectal cancer and is repressed by APC (14). This repression was mediated by β-catenin/Tcf-4-responsive elements in the PPARdelta promotor (15). Tumor cells located at the invasive front and those migrating into the adjacent stromal tissue are characterized by nuclear β-catenin accumulation (16, 17). Hence, different levels of Wnt/β-catenin-signaling activity are likely to reflect tumor heterogeneity and to underlie malignant behavior (18, 19).

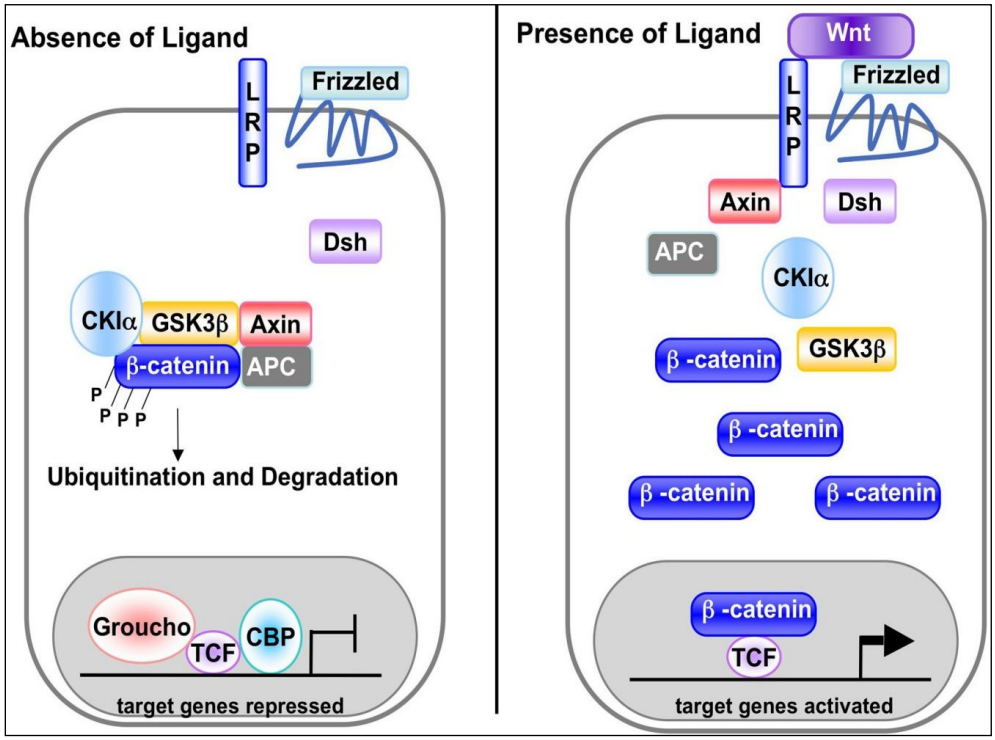

Figure 1.3: Wnt signaling pathway. The events ocurring in presence and absence of Wnt ligand binding. Details in text. Figure from reference (23).

K-RAS and MAPK pathways: Mitogen-activated protein kinase (MAPK) is a signal transduction pathway which involves serine/threonine specific protein kinases that respond to extracellular stimuli (mitogens). They regulate various cellular activities, such as gene expression, mitosis, differentiation, proliferation, and cell survival/apoptosis (24). The cascade of this pathway begins with activation of RAS oncogene and mutation of K-RAS is important in colorectal adenoma progression **(Figure 1.4)**. There have been reports wherein MAPK activities have been shown to increase in the adenoma-to-carcinoma sequence suggesting its role in colorectal tumor progression **(Figure 1.2)** (25). At the core of each MAPK cascade, there is a three-kinase module consisting of a MAPK kinase kinase (MAPKKK), a MAPK kinase (MAPKK) and a MAPK **(Figure. 1.3)**. Activation of MAPK is initiated by phosphorylation of MAPKKK leading to a cascade of site-specific phosphorylations of Thr and Tyr residues within catalytic domains of the enzymes transmitted through the module (26). According to the sequence homology **(Table 1),** MAPKs are divided into subfamilies, of which extracellular signal-regulated kinase (ERK or ERK1/2), p38 MAPK (p38) and c-Jun N-terminal kinase (JNK) have been under intensive investigation (27, 28). Several transcription factors are regulated by MAPK. MAPK phosphorylates and activates MAP kinase signal-integrating kinase (MNK) which in turn phosphorylates cAMP response element binding (CREB) (29). CREB is a protein that is a transcription factor which binds to certain DNA sequences called CRE or cAMP response elements and thereby increase or decrease the transcription and expression of certain genes (30). MAPK also regulates the transcription of the c-fos gene (31). This gene is a transcription factor which is a cellular proto-oncogene belonging to the family of immediate early genes. By altering the levels and activities of transcription factors, MAPK leads to altered transcription of genes that are important for the cell cycle.

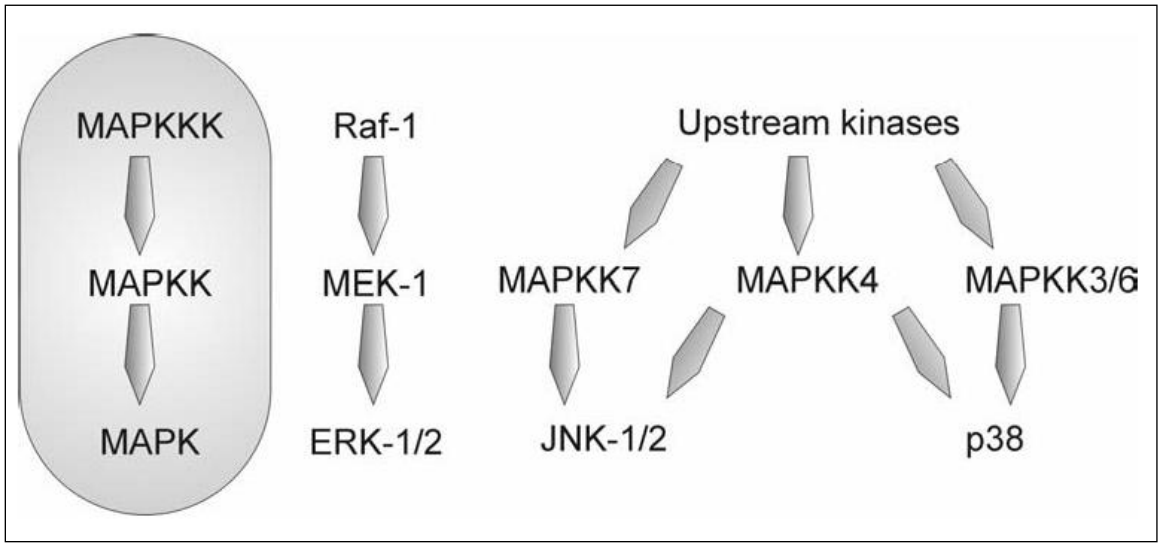

Figure 1.4: MAPK module for ERK, P38, and JNK pathways (28).

Table 1: Phosphorylation motifs. The phosphorylations of specific motifs activate the three major subfamilies of MAPK cascade. The sequence motif of Thr-glutamine (Glu)-Tyr is phosphorylated in case of ERK pathway. The sequence motif of Thr-glycine (Gly)-Tyr is phosphorylated in case of p38 pathway. The sequence motif of Thr-proline (Pro)-Tyr is phosphorylated in case of JNK pathway leading to different activities of cell proliferation and cell differentiation.

SUBFAMILIES	SEQUENCE MOTIF PHOSPHORYLATED	ACTIVATION	ACTIVITIES
ERK	Thr-Glu-Tyr	Growth factors, phorbol esters and viral transformation	Cell proliferation and cell differentiation
P38	Thr-Gly-Tyr	osmotic stress and also pro-apoptotic stimuli	Cell differentiation
JNK	Thr-Pro-Tyr	ultraviolet irradiation	Cell differentiation

TGF-β pathway: TGF-β is part of a superfamily of proteins known as the transforming growth factor β superfamily (32). TGF- β initiates signaling by formation of heteromeric complexes of type I and type II serine/threonine kinase receptors (33). The role of TGF-β in tumorigenesis is quite complex, but it appears that TGF-β functions both as a tumor suppressor in early tumor onset (34, 35) and as an oncogene during late tumor progression (35). The loss of suppression activity of TGF- β receptor II due to somatic mutations are found in patients with hereditary non polyposis colorectal cancers (HNPCCs). (36) **(Figure 1.5)**. The tumor suppressor gene involved in 18q deletions known for poor prognosis in colorectal cancer patients, is *SMAD-4*. The *SMAD-2* gene, coding for another component of the TGF-β signaling pathway might be deleted in another group of colorectal cancers. SMAD-2 binds to SMAD-4 and the complex translocates to nucleus causing transcription of genes involved in tumor suppression or promotion.

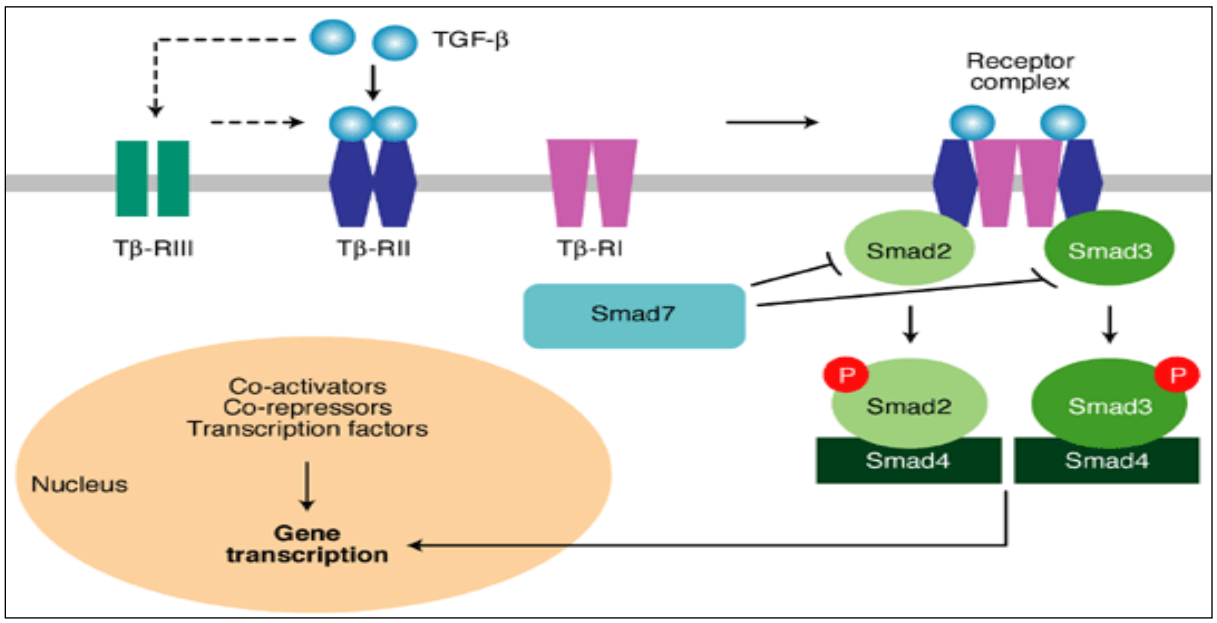

Figure 1.5 TGF-β signaling pathway. TGF-β binds to the receptor Tβ -RII; this binding might be enhanced by the presence of Tβ-RIII. After binding to TGF-β, Tβ-RII recruits and phosphorylates Tβ-RI, leading to activation of Smad2 and Smad3 by phosphorylation (P). This process is inhibited by SMAD7. Activated SMAD2 and SMAD3 form heterodimers with SMAD4 and translocate to the nucleus. Together with co-activators, co-repressors and other transcription factors, the SMAD complex regulates gene expression (37).

P53 mutations: The tumor suppressor gene, *p53* plays a crucial role at the crossroads between cellular proliferation and apoptosis, as the guardian of the integrity of the genome, and as cell invasion inhibitor (38) . The loss of p53 activity can be dangerous in relation to cancer as it allows faulty mutant cells to continue through the cell cycle, these cells escape apoptosis, and it leads to the genetic instability characteristic of cancer cells, allowing further cancer promoting mutations to accumulate as they divide.

The tumor suppressor protein p53 is one of many proteins that contribute to the activation of the intrinsic apoptotic signaling pathway (39, 40). The *p53* tumor suppressor gene product is a primary regulator of cellular responses to stressors that contribute to oncogenesis (41-43). It functions as a sequence- specific transcription factor (44). The human genome contains more than 200 genes that are directly responsive to p53 (45). Promoter mapping and microarray studies have already implicated p53 in the regulation of over 100 genes involved in cellular processes such as DNA repair, angiogenesis, signal transduction, and oxidative stress (46)

However the most intensively studied outcomes of induction of p53 are cell cycle block (47) at different stages of cell cycle (48) and activation of apoptosis (49-53), thus helping to prevent DNA damage and blocking the propagation of cells that have experienced damage.

The mechanisms underlying p53-mediated apoptosis are not as clearly defined, although lots of p53 target genes have been implicated in this process (54). p53 initiates cell cycle arrest and apoptosis in various ways among different cell-types (55, 56). Different mechanisms are involved in regulation of the two major aspects of the p53 response, activation and accumulation (57, 58), so that transactivation by p53 of genes required for cell cycle arrest is not accompanied necessarily by an increase in the amount of p53 protein (58). Primary cause of accumulation of p53 is by an increase in its half-life (59). p53 degradation is mediated by ubiquitin-dependent

proteolysis (60). Posttranslational modification is the major mechanism of p53 regulation. Several different protein kinases can phosphorylate distinct domains of p53 *in vitro* (61). These phosphorylation events affect the transactivation function of p53. The phosphorylation sites of p53 and the different kinases involved in phosphorylation are demonstrated in **Figure 1.6**. Mutation in p53 gene has shown to cause increase cell invasion (62).

Cyclooxygenase Pathway (COX): Cyclooxygenases (COXs), key enzymes responsible for eicosanoid production, can profoundly influence cancer development, progression, metastasis and therapeutic response especially COX-2 and its enzymatic product prostaglandin E2 (PGE2) (63).

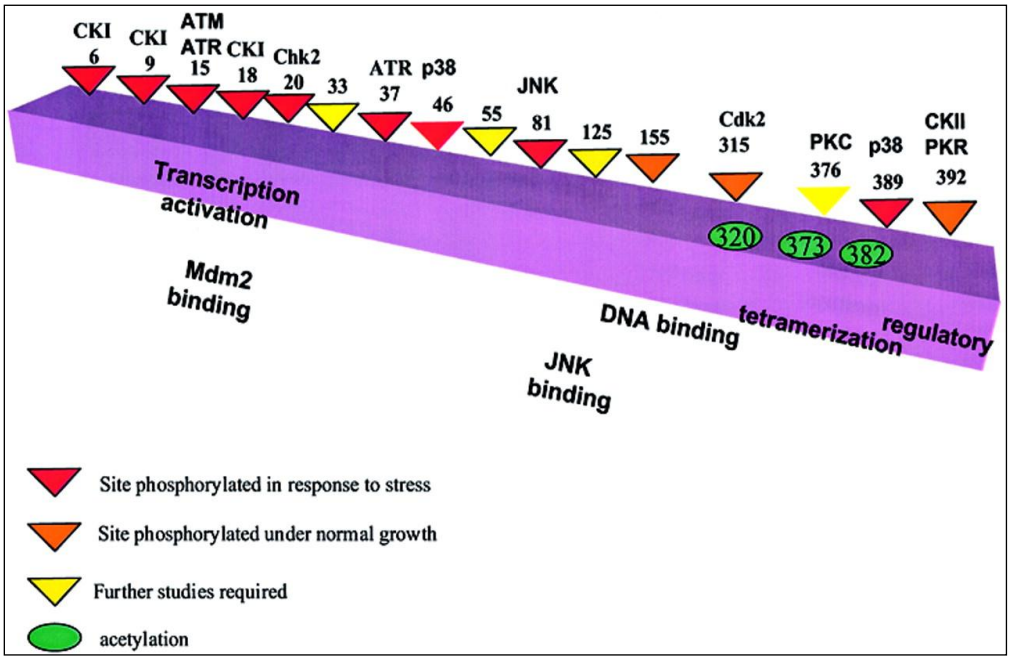

Figure 1.6: Phosphorylation sites for p53. p53 is phosphorylated at different serine residues by different kinases. Serine 15 site is phosphorylated by ataxia telangiectasia mutated kinase (ATM) and ATM and Rad3-related kinase (ATR) and is extensively studied. The other known kinases are casein kinase I (CKI) phosphorylating serine 6, 9, and 18. Checkpoint homolog 2 kinase (Chk2) phosphorylates serine 20. P38 MAPK phosphorylating serine 46 and 389. JNK phosphorylating serine 81. Cycling dependent kinase 2 (Cdk2), protein kinase C (PKC), and casein kinase II (CKII) phosphorylate serine 315, 376 and 392 respectively.

This pathway is more commonly known as arachidonic acid pathway **(Figure 1.7)**. Metabolism of arachidonic acid is caused either by the COX or Lipoxygenase (LOX) pathway generating eicosanoids which have been shown to play an important role in pathogenesis of variety of human diseases, like cancer and by its promotion, progression and metastasis (64). COX is the first enzyme in the pathway for producing prostaglandins (PG) and thromboxane (Tx) from Arachidonic acid. It occurs in two isoforms, COX-1 and COX- 2 (65). Arachidonic acid is released from cell membrane phospholipids by phospholipase A2 and is then acted on by COX-1 or COX-2 to produce the intermediate metabolites, prostaglandin G2 ($PGG_{2)}$ and prostaglandin H2 ($PGH_{2)}$. COX-1 occurs in tissues and its function is to protect the cell (66). COX-2 is present in stroma and takes part early in neoplastic process (67). COX-2 is highly inducible by various cytokines, growth factors, and tumor promoters (68). Elevated COX-2 levels are associated with inflammation, neoplastic transformation, metastasis, and increased expression of mRNA and protein is found in the great majority of colorectal cancers (69).

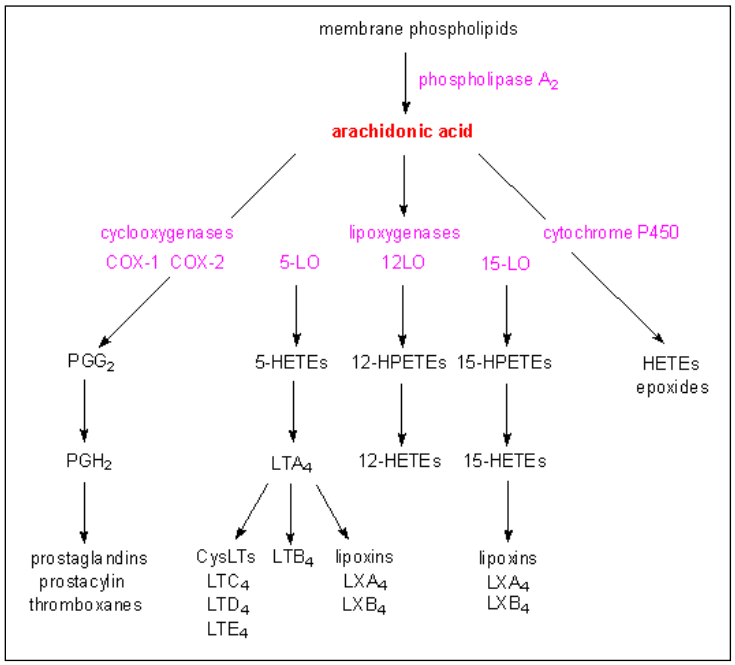

Figure 1.7: The arachidonic acid metabolic pathway (74)

LOX is the first enzyme in the pathway that produces leukotrienes from arachidonic acid. The isoenzymes of LOX include 5-LOX, 12-LOX, and two 15-LOX (1 and 2). They are involved in formation of the biologically active compounds such as leukotrienes and hydroxyl-eicosatetraenoic acids (66).

Leukotrienes belong to a key group of pro inflammatory mediators that are synthesized from arachidonic acid via the 5-LOX pathway. 5-LOX leads to formation of unstable LTA_4, which can be converted to LTB_4 or LTC_4, LTD_4 and LTE_4. 5-LOX, 12-LOX and 15-LOX-1 have shown to have pro-tumorigenic effects but 15-LOX-2 has anti-carcinogenic effects (70).

1.1.3 VOGELSTEIN MODEL

Fearon and Vogelstein model (1990) of colorectal carcinogenesis is primarily defined as sequences of mutations associated with progression of tumors (71) **(Figure 1.8)**. According to this model every colorectal carcinoma is likely to have developed through the same progression of histological stages, but the underlying genetic changes are much less predictable. The sequences of events that have been shown in this model correspond to the changes frequently involved in particular stages of progression.

In HNPCC (discussed later), the early stages probably follow an alternative pathway. After loss of the wild-type mutS homolog 2 (MSH2) or mutL homolog 1 (MLH1) allele, which produces cells with deficient mismatch repair, specific genes including the *TGFβ receptor II* gene show frame shift mutations. The 100–1000-fold increase in mutation rate in these tumors must also speed progression through the later stages. These are not the only changes seen in colorectal carcinomas, but they are the ones which can be most readily associated with specific stages.

The *MSH2* and mutS homolog 1 (*MSH1)* genes in HNPCC are not directly involved in this pathway.

Microsatellites are repeated sequences of DNA and several genes have been identified that are frequently mutated in cancers with microsatellite instability (*TGFBR2, RAS, E2F-4, BAX*, etc.) that is appearance of abnormally long or short microsatellites in an individual's DNA due to defects in normal DNA repair, and it seems likely that the mutator phenotype can either accelerate progress along the pathway, or facilitate alternative routes to malignant transformation.

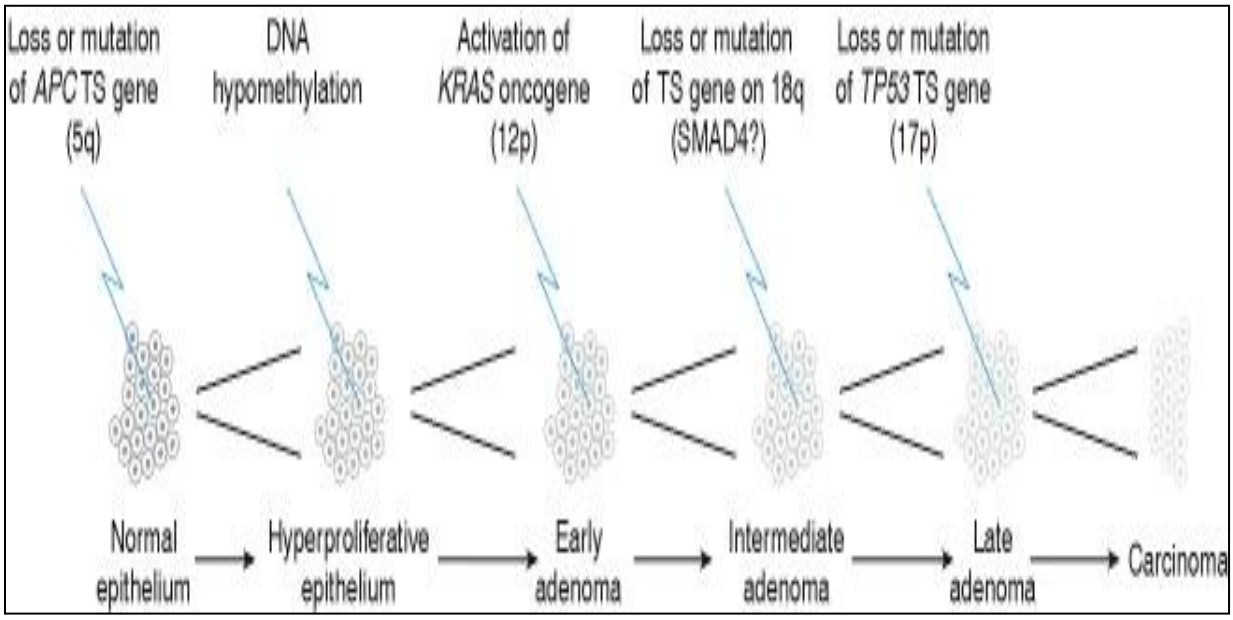

Figure 1.8. Fearon and Vogelstein model of colorectal carcinogenesis. *APC* gene mutation occurs at chromosome 5q which is associated with FAP in humans (112). Loss of APC initiates the colorectal cancer formation in normal epithelium. Hyperproliferation of epithelium is seen with DNA hypomethylation. KRAS gene activation at chromosome 12p causes formation of early adenomas which then become cancerous following loss of other tumor suppressor genes such as SMAD4 and p53 on chromosomes 18q and 17p respectively.

1.1.4 COLORECTAL CANCER STATISTICS

Colorectal cancer is one of the most common cancer in the United States ranking third in frequency behind lung and prostate cancer in men in the United States and behind lung and breast cancer in women with about 148,810 new cases and 49,960 deaths expected in 2008 (72). It develops slowly over many years and usually begins as a polyp: a benign growth of tissue that starts in the lining and grows into the lumen of the colon or rectum. About 72% and 28% cases arise in the colon and rectum respectively. Early detection and removal of polyps may prevent formation of adenocarcinomas, which account for over 95% of colorectal cancers. Colorectal cancer accounts for about 10 percent of all new cases of cancer each year in the United States. It is also responsible for about 10 percent of all deaths from cancer. The lifetime risk of being diagnosed with colorectal cancer is 5.5% for men and 5.1% for women in the US (73).

1.1.5 TYPES OF COLORECTAL CANCER

Broadly colorectal cancer is divided into two major types: hereditary and sporadic **(Figure 1.9).**

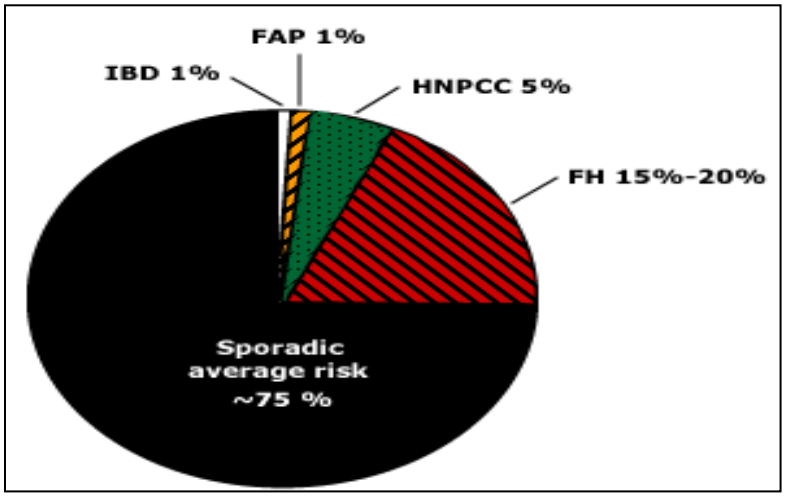

Figure 1.9: Pie-chart showing risks due to different types of colorectal cancers. Sporadic cancers risk is seen in men and women over 50 years and more having no special risk factors. Inflammatory bowel disease, IBD accounts for 1% risk. The hereditary cancers accounts for almost 25%.

Hereditary cases account for almost 25% of total colorectal cancer incidence and include familial colorectal cancer or family history, familial adenomatous polyposis (FAP), HNPCC, inflammatory bowel disease (IBD), and others. The sporadic cancer accounts for almost 75% (75). Familial colorectal cancer or family history accounts for 15-20% of hereditary or inherited colorectal cancer. It is acquired in autosomal dominant manner **(Figure 1.9)**. However, major emphasis is given to FAP and HNPCC in considering inherited forms of colorectal cancer **(Figure 1.10)**.

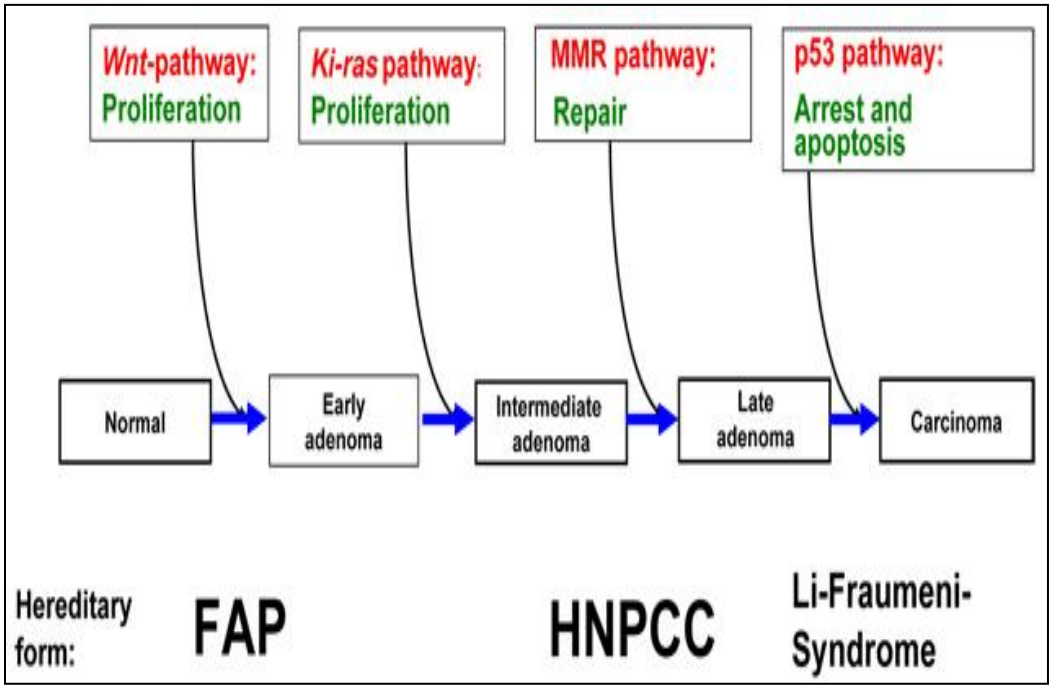

Figure 1.10: Different forms of colorectal cancer. They occur due to stepwise loss of signaling pathways and processes. The genes involved in formation of colorectal cancer belong to both oncogenic as well as tumor suppressor pathways. The initial pathways playing a role are oncogenic pathways like WNT and Ki-RAS. In case of WNT pathway, the tumor suppressor gene, APC is mutated and in Ki-RAS pathway, KRAS gene is activated. This is seen in case of inherited familial adenomatous polyposis syndrome (FAP) which is identified based on adenomas. Later on the genes belonging to mismatch repair (MMR) pathway are mutated. Finally the guardian of genome, p53 tumor suppressor gene is mutated causing cell proliferation and cell invasion. This is seen in case of another inherited disease, hereditary non polyposis colorectal cancer (HNPCC) wherein adenocarcinoma is seen. Li-Fraumeni syndrome is a rare autosomal dominant hereditary disorder due to p53 mutation. (Figure from reference (71)).

Familial adenomatous polyposis (FAP): FAP is very rare inherited disorder (76). It causes less than 1% of all colorectal cancer cases that is the incidence rate is 1 in 7,000 to 1 in 22, 000 (77). People with classical FAP may begin to develop multiple noncancerous (benign) growths (polyps) in the colon as early as their teenage years. Unless the colon is removed, these polyps will become malignant (cancerous).

The number of polyps' increases with age in classic FAP cases and hundreds to thousands of polyps can develop in the colon. In both classic familial adenomatous polyposis and its attenuated variant, benign and malignant tumors are sometimes found in other places in the body, including the duodenum (a section of the small intestine) (78), stomach (79), bones, skin (80), and other tissues (81, 82). Adenomatous polyposis gene or *APC* tumor suppressor gene plays an important role and its mutations can cause both classic and attenuated familial adenomatous polyposis (83, 84). Around five hundred *APC* mutations have been identified so far (85). With *APC* gene mutation, FAP is inherited in an autosomal dominant pattern.

Attenuated familial adenomatous polyposis (AFAP): Some people have a variant of the disorder, called attenuated familial adenomatous polyposis, in which polyp growth is delayed. The average age of colorectal cancer onset for attenuated familial adenomatous polyposis is 55 years (86). This milder presentation of FAP is characterized by fewer polyps and later age of onset than classic FAP (87). In AFAP, typically fewer than 100 polyps are observed. Polyps of the gastric fundus and duodenum also occur; however, many of the extra colonic manifestations commonly observed in FAP (e.g., epidermal cysts, dental abnormalities, congenital hypertrophy of retinal pigmented epithelium, desmoid tumors) may be absent in AFAP. Polyps and colon

cancers associated with AFAP do not usually exhibit microsatellite instability. AFAP is caused by mutations in the *APC* gene and is inherited in an autosomal dominant manner (88).

MYH- associated polyposis: mutY Homolog (E. coli) *(MUTYH)* is a human gene (89) that is involved in formation of enzyme (MUTYH glycosylase) required in the DNA repair. With mutations in the mutY Homolog (E. coli) (*MUTYH)* human gene, colorectal cancer is inherited in autosomal recessive pattern (90). Mutations in this gene prevent the enzyme from correcting mistakes that are made during DNA replication in preparation for cell division. As these mistakes build up in a person's DNA, the likelihood of cell overgrowth increases, leading to colon polyps and the possibility of colon cancer. People with the autosomal recessive type of this disorder have fewer polyps than those with the classic type (91)

Hereditary non-polyposis colon cancer (HNPCC): HNPCC, also known as Lynch syndrome (92), accounts for about 2 to 7% of all colorectal cancers. It is an inherited colorectal cancer syndrome and also results in neoplasia in at least 7 other organs (93). HNPCC is caused by an inherited mutation in one of the DNA mismatch repair (MMR) genes (94). Genes in the MMR pathway are responsible for identifying and repairing single nucleotide mismatches and insertion or deletion loops that occur as cells grow and divide. Defects in the genes involved with mismatch repair lead to an accumulation of somatic mutations in a cell, which may result in the cell becoming malignant. There are at least 5 genes that have been found to cause HNPCC. Individuals with HNPCC have an approximately 80% lifetime risk for colon cancer (73). The average age of colorectal cancer diagnosis is 61 years. The *hMSH2* and *hMLH1* are most commonly mutated *MMR* genes in HNPCC, and the other genes involved are *hMSH6*, *PMS1* and *PMS2* (95). 10- 20% of colon cancers occurring in individuals who are not known to have or

suspected of having HNPCC have microsatellite instability (MSI) caused by silencing of the *MLH1* gene by methylation or caused by somatic mutations of the mismatch repair genes (96). The aberrant operation of DNA mismatch repair systems lead to MSI and cause naturally occurring, highly repeated short DNA sequences, called microsatellites, to get shorter or longer than expected. Microsatellites have a repetitive sequence of nucleotides (e.g., AAAAA or CGCGCGCG) that are particularly susceptible to acquiring errors when mismatch repair gene function is impaired and are a good marker to distinguish HNPCC from other forms of inherited colorectal cancers. The other rare syndromes include:

Li-Fraumeni syndrome: There is also another rare syndrome which occurs due to germ line mutations of the p53 tumor suppressor gene (97) **(Figure 1.10)**. This hereditary disorder is called as Li-Fraumeni syndrome named after Frederick Pei Li and Joseph F.Fraumeni, Jr. who were both American Physicians. They first described that this syndrome greatly increases susceptibility to cancer. They discovered that mutations are either inherited or they can arise de novo early in embryogenesis or in one of the parent's germ cells. This syndrome also develops when people are relatively young.

Turcot syndrome: This is a rare inherited condition in which people are at increased risk of adenomatous polyps and colorectal cancer, as well as brain tumors (98). There are actually 2 types of Turcot syndrome; one can be caused by gene changes similar to those seen in FAP, in which cases the brain tumors are medulloblastoma, and the other can also be caused by gene changes similar to those seen in HNPCC, in which cases the brain tumors are glioblastomas.

Peutz-Jeghers syndrome: People with this rare inherited condition tend to have freckles around the mouth, sometimes on the hands and feet, and large polyps in their digestive tracts (99). They are at greatly increased risk for colorectal cancer, as well as several other cancers, which usually appear at a younger than normal age. Families with this inherited syndrome should be identified and members should be screened regularly from an early age.

Sporadic Colorectal cancer: The vast majority of colorectal cancers are sporadic, they occur without an inherited mutation predisposing them to colorectal cancer or a family history of it (100). However a series of mutations is needed for colorectal cancer to develop. It often takes many decades for these mutations to accumulate –hence the majority of colorectal cancer cases occur in the elderly. Mutations in *APC* are seen in 70 to 80% of sporadic tumors and often occur early in the development of colorectal cancer (101). During early stages of colorectal tumorigenesis, K-RAS gene is mutated (102-104).The *p53* gene is another tumor suppressor gene that is known to be mutated (105, 106). The *p53*-encoding gene mutations in colorectal cancer occur in specific conserved regions of the gene and might be present in over 50% of the colorectal cancers (102, 107). In addition, the *DCC* (deleted in colorectal cancer) gene is mutated in 70% of colorectal cancers (108, 109), and *MMR* genes are inactivated in around 15% of sporadic cases (8, 110).

1.1.6 RISK FACTORS FOR COLORECTAL CANCER

Any factor increasing a chance of getting diseases like cancer is a risk factor. Relative risk (RR) is a ratio of the probability of the event occurring in the exposed group versus a non-exposed group (111) **(Table 2**).

Table 2: Selective risk factors for colorectal cancer. A relative risk of 1 means there is no difference in risk between the two groups. An RR of < 1 means the event is less likely to occur in the experimental group than in the control group. An RR of > 1 means the event is more likely to occur in the experimental group than in the control group.

NO.	RISK FACTORS	RELATIVE RISK	REFERENCES
I	Family and medical histories:		
1.	Family history (first degree relative)	1.8	**(73, 113, 114)**
2.	Inflammatory bowel disease (diagnosed ≥10 years)	1.5	**(115)**
II	Modifiable factors that increase risk		
1.	Obesity (Body mass index ≥ 30)	1.5- 2.0	**(116-118)**
2.	Red meat (≥ 7 servings/ week vs 1 serving/ month.	1.5	**(119, 120)**
3.	Cigarette use (current vs never)	1.5	**(121-124)**
4.	Alcohol (≥ 4 drinks/week vs none)	1.4	**(125)**
III	Modifiable factors that decrease risk		
1.	Physical activity (more than 3 h/ week vs none)	0.6	**(126-129)**
2.	Vegetable and fruit consumption (≥ 5 vs <3 servings/ day	0.7	**(128, 130-133)**

1.1.7 CHEMOTHERAPY REGIMEN AND SIDE EFFECTS

Chemotherapy is a referral term for drugs that prevent cell proliferation or kill cancer cells or both. There are some specific drugs used at specific dosages and schedules for a specific type of cancer. Chemotherapy is currently the main treatment for metastatic disease (134) and used;

1) In advanced stages of colorectal cancer that has already metastasized to other parts of the body and there is no option for surgery.

2) Given after cancer is surgically removed in order to make sure that all cancer cells are killed that might have been missed during surgery like the metastasized ones.

3) Tried before surgery to shrink the big tumors to facilitate easy removal.

The first choice of drug for colorectal cancer for many years has been 5-Fluorouracil (5-FU) (135). It is given intravenously. Recently, a pill form of 5-FU has also been developed, called Xeloda, which is used for metastatic colorectal cancer. Certain new drugs like Camptosar, Eloxatin and Avastin have been given along with 5-FU for metastatic colorectal cancer.

Patients with mutations in KRAS do not respond to drugs inhibiting epidermal growth factor receptor (EGFR) (136) like Erbitux (cetuximab) and Vectibix (panitumumab) unlike patients having KRAS wild type expression.

Radiation therapy is not very commonly used as it is difficult to target specific portions of the colon and also it could lead to radiation enteritis (137). But sometimes chemotherapy agents are used to increase the effectiveness of radiation by sensitizing tumor cells if present (138).

This chemotherapeutic regimen even though effective is not desirable because of the severe side effects that accompany it (139). The side effects mainly occur as the mechanism of these drugs is to kill rapidly dividing cancer cells but in that process they also rapidly kill healthy dividing cells lining the mouth, gastrointestinal tract, hair follicles and the bone marrow.

Some of the side effects are nausea, vomiting, loss of appetite, diarrhea, hair loss, mouth sores, increased risk of infection, bleeding, anemia due to low white and red blood cells. The effective cancer drug Cisplatin is known for severe nephrotoxicity and ototoxicity (140). A preferable option would be to take advantage of the natural characteristics of dietary components in tackling cancer.

1.2 DIETARY COMPOUNDS

Epidemiological studies have suggested that nutrition plays an important role in carcinogenesis. Approximately 30% of cancer morbidity and mortality might be prevented with proper adjustment of diets (141-143) and one-third of all the cancers in the United States might be avoided by introducing fruits and vegetables in the daily diet (144). The relative risk of colorectal carcinogenesis is decreased with diets rich in vegetables and fruits **(Table 2)**. The basic theory of chemoprevention is to reduce the occurrence of cancer either by slowing, blocking, or reversing the development of the disease by the administration of natural or synthetic compounds (145). Although epidemiologic studies suggest a significant dietary influence on carcinogenesis, the molecular mechanisms responsible for potential human health benefits derived from dietary components must be studied *in vitro* and validated in pre-clinical studies in animal models before strong support can be given for more extensive clinical trials.

Some foods contain phytochemicals or flavanoids which are anti-oxidants, anti-inflammatory and anti-cancer agents (146). There are epidemiological studies carried out which clearly suggest that higher intake of fruits, vegetables, whole grains and plant proteins in comparison to typical American diet is associated with a markedly reduced risk of cancer, heart disease, and some other chronic diseases (147). These dietary patterns are also termed as low-fat, high-fiber diets. The diet rich in plant foods not only has essential vitamins and minerals but also over 25,000 phyto-chemicals which is not available in a typical Western pattern based on refined grains, added oils, sugar, and salt (148). Few examples of the dietary agents with active components are as seen in **Figure 1.11.** We decided to focus on green tea since it has attracted significant attention, both in the scientific and in consumer communities for its health benefits for a variety of disorders, ranging from cancer to heart disease to weight loss (149-151). Not only has the intake increased over a period of years but also it is been now seen as a featured ingredient in several nutritional supplements, including multivitamin supplements (152).

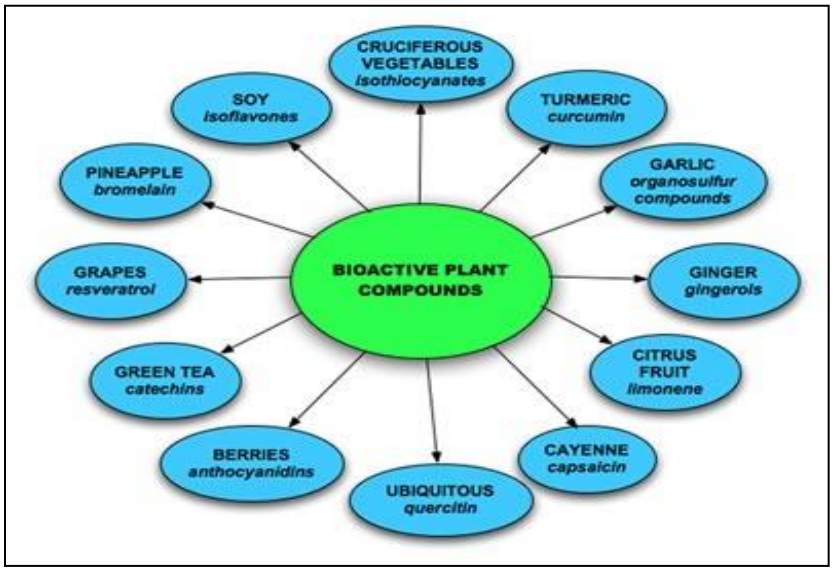

Figure 1.11: Important bioactive plant compounds in different dietary agents. All these Polyphenols are potent antioxidant agents and have effect on cancer at one or other stages of initiation, promotion and progression (159).

1.2.1 TEA

Tea is the most widely consumed popular beverage second to water, well ahead of coffee, beer, wine, and carbonated soft drinks (153, 154). Tea is cultivated in approximately 30 countries worldwide and is consumed globally. About three billion kilograms of tea is produced and consumed yearly (155). Successful tea cultivation requires moist humid climates available in the slopes of northern India, Sri Lanka, Tibet, and southern China which have the ideal growing conditions (156).

1.2.2 TEA PROCESSING AND MANUFACTURING

Tea, Camellia sinensis, is manufactured in four basic forms - White tea, green tea, oolong tea and black tea **(Figure 1.12)**. White tea is made from new growth buds and young leaves that have been steamed to inactivate polyphenol oxidation and then dried. The young buds are usually shielded from direct sunlight to prevent formation of chlorophyll. Green tea is produced by an initial heating process or steaming, which kills the enzyme polyphenol oxidase (157), which is responsible for the conversion of the flavanols in the leaf into the dark polyphenolic compounds that color black tea. All the active ingredients in tea called polyphenols or catechins are intact as green tea is not fermented. Oolong tea is partially oxidized product. In case of manufacturing black tea, the tea leaves are crushed to allow the polyphenol oxidase to catalyze the oxidation, leading to polymerization of catechins forming theaflavins and thearubigins. Of the tea produced worldwide, 78% is black tea, which is usually consumed in the Western countries and Asian countries like India, 20% is green tea, which is commonly consumed in Asian countries, and 2% is Oolong tea which is consumed mainly in Southern China (158).

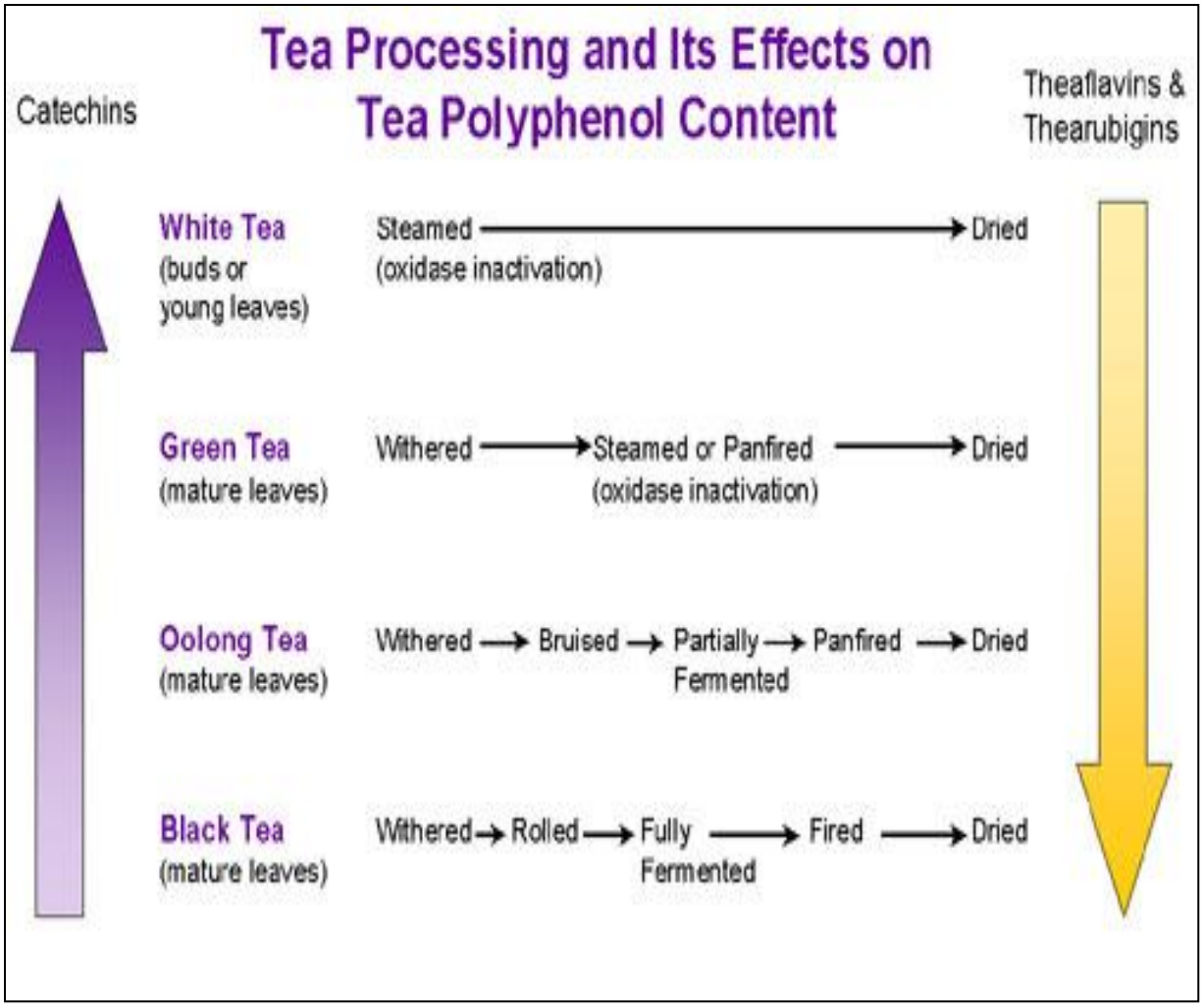

Figure 1.12: Processing of tea. Camellia sinensis tea plant is processed in different ways to obtain four main kinds of teas: white tea which is just steamed dried, green tea; which is withered, steamed and dried, oolong tea; which is partially fermented, and black tea which is fully fermented and this fermentation destroys most of the active components of tea. The purple arrows designate increase in catechin content as the teas are less fermented. The yellow arrow shows increase in theaflavins and thearubigins when the teas undergo fermentation. White tea has maximum catechin content but is very expensive and not readily available.

1.2.3 CHEMICAL COMPOSITION OF GREEN TEA

The chemical composition of green tea varies with climate, season, horticultural practices, and position of the leaf on the harvested shoot. The chemical composition of green tea is complex. Mainly it contains: proteins (15– 20% dry weight); carbohydrates (5–7% dry weight); vitamins (B, C, E); xanthine bases such as caffeine and theophylline; minerals and trace elements (5% dry weight) (157). Polyphenols constitute the most interesting group of green tea leaf components, particularly flavonoids (phenol derivatives) and are widely distributed in plant World (160). The main flavonoids present in green tea include catechins (flavan-3-ols). The four major catechins are (-) - epigallocatechin-3-gallate (EGCG), that represents approximately 59% of the total of catechins; (-)-epigallocatechin (EGC) (19% approximately); (-)-epicatechin-3-gallate (ECG) (13.6% approximately); and (-)-epicatechin (EC) (6.4% approximately) (161) **(Figure 1.13)**.

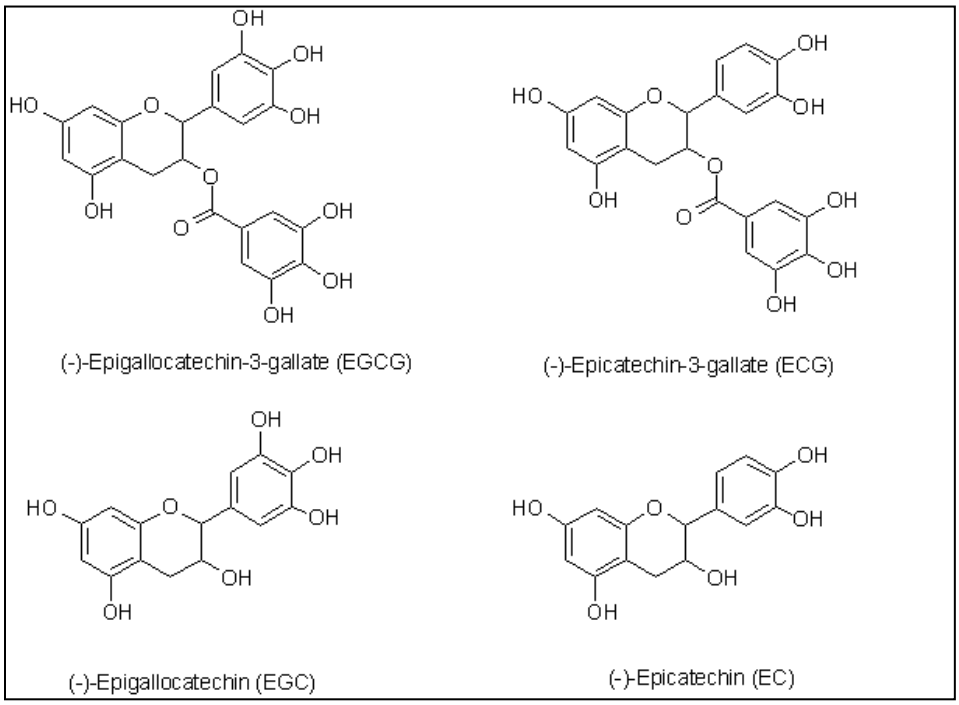

Figure 1.13: The chemical structures of four different catechins. The hydroxyl groups present on the catechins give them their anti-oxidant properties. EGCG is the most abundant of the total catechins in green tea and is the strongest antioxidant agent.

Green tea also contains gallic acid (GA) and other phenolic acids such as chlorogenic acid and caffeic acid, and flavonols such as kaempferol, myricetin and quercetin (162). The relative catechin content of green tea as mentioned earlier depends on how the leaves are processed before drying, a certain grade of fermentation and heating of tea leaves during the manufacturing process. Other factors influencing catechin content are the geographical location and growing conditions (soil, climate, agricultural practices, fertilizers), the type of green tea (e.g., blended, decaffeinated, instant), and the preparation of the infusion (e.g., amount of the product used, brew time, temperature). A cup of green tea (2.5 g of green tea leaves/200 mL of water) may contain 90 mg of EGCG (163, 164). The catechins usually account for 30-42% of the dry weight of the solids in brewed green tea (165). Catechin and epicatechin are epimers, with (-)-epicatechin and (+)-catechin being the most common optical isomers found in nature (166). Epigallocatechin and gallocatechin contain an additional phenolic hydroxyl group (167), which makes them stronger anti-oxidant agents compared to epicatechin and catechin, respectively. Catechin gallates are gallic acid esters of the catechins, such as EGCG (epigallocatechin gallate).

1.2.4 PHARMACOKINETICS OF GREEN TEA

The possible influence of green tea on cancer in humans has been difficult to interpret due to confounding factors, such as diversity in types of tea used, preparation methods, including temperature of infusion, frequency of tea drinking and other environmental factors**.** The potential health benefits of catechins depend on the amount consumed and on their bioavailability. The bioavailability appears to be very variable (168). Bioavailability in two early studies found plasma levels at 0.2%–2% of the ingested amount of EGCG (up to 4μmol/L), but higher plasma concentrations have since been reported in fasting patients compared to those who consumed

catechins with food (169-171). The oral administration in human patients resulted in high plasma clearance levels and volume distribution, suggesting that the bioavailability of EGCG in the blood may be low.

In a study conducted on rats, following oral administration of tea catechins, the four principal catechins (EC, ECG, EGC, and EGCG) have been identified in the portal vein, indicating that tea catechins are absorbed intestinally (172). The study carried out in rats and mice indicated species differences in the bioavailability of EGCG and adaptive response of plasma catechin levels to catechin ingestion over time in mice and rats (173). Catechin levels in human plasma reach their peak 2 to 4 h after ingestion (174). After a single dose (1.2g/ 200ml water) of green tea or green tea extract, the highest concentrations of individual catechins measured in human plasma were slightly greater than 1 μM (175).

EGCG Metabolism: EGCG is about 25-100 times more potent than Vitamin C in its antioxidant property (176). EGCG is mostly present in free form in plasma (177). EGCG levels are higher in the esophagus, small intestine and large intestine, but lower in other organs, likely due to poor systemic absorption of EGCG. EGCG is mainly excreted through the bile (157). EGCG may be metabolized into simpler compounds by colonic bacteria, absorbed, and ultimately excreted in the urine (178). Determination of the actual bioavailability of metabolites in tissues may be much more important than knowledge of their plasma concentration (179). A study that examined salivary levels of tea catechins in humans found that holding an EGCG solution in the mouth resulted in EGCG in the saliva (180). Additionally, saliva was found to have catechins esterase activity, suggesting that EGCG may be degalloylated in the mouth and esophagus (181). Although phase I transformation reactions for catechins have not been well defined, phase II

reactions, including glucuronidation, sulfation, and *o*-methylation, have been reported in rodents and humans after oral, i.v., and i.p. administration (182, 183). Enzymes involved in polyphenol metabolism include catechol-o-methyltransferase (COMT) having highest activity in liver, kidney, and jejunum (184), UDP-glucuronosyltransferase (UGT) having highest activity in mucosa of the small and large intestine (185), and phenolsulfotransferase (SULT) having highest activity in the liver (186).

Physiologically relevant concentration of EGCG in humans: The plasma bioavailability of EGCG, whether administered as tea or a pure compound, is in the range of 0.1–7 μmol/L in humans, with concentrations over 100 μmol/L observed in saliva. No significant excretion occurred in urine (generally <0.1% of dose) (187).

1.2.5 HEALTH BENEFITS OF GREEN TEA

Green tea is known for its medicinal benefits on almost all parts of the body, we will be mainly focusing on its anticancer benefits. Health benefits of Green tea are mainly attributed to it abundant catechin, EGCG. Some of the molecular targets of EGCG in vitro are mentioned in **Table 3.**

Anticancer health benefits: Abundant experimental and epidemiological evidence accumulated mainly in the past decade from several centers worldwide provide a convincing argument that polyphenolic antioxidants present in green tea can reduce cancer risk in a variety of animal tumor bioassay systems (188-190).

Table 3: Molecular targets of EGCG. There are many signaling molecules which are targets of EGCG, few of which are mentioned in the table below. Table is based on information from reference (187)

TARGET SIGNALING MOLECULES	**INDUCTION (↑) OR INHIBITION (↓)**
Growth factor receptors (EGFR**,** platelet derived (PDGFR), fibroblast (FGFR), vascular endothelial (VEGFR)	↓
Survival signaling pathway components (extracellular signal regulated kinase (ERK), p38, activating protein-1 (AP-1), signal transducer and activator of transcription (STAT), PI3K, Akt, and nuclear factor, NF-κB]	↓
cell cycle regulators [cyclin D1, cyclin-dependent kinases (cdk)2/4/6] p21, p27, phosphorylated retinoblastoma (pRb)	↓ ↑
Regulators of apoptosis (Bcl2) Bax, Bad, caspases-3/7/8/9, and poly (ADP ribose) polymerase (PARP)].	↓ ↑
Cell junction proteinases (MMP-9, uPAR)	↓

Tea consumption also assures protection against cancers induced by chemical carcinogens that involve the lung, fore stomach, esophagus, duodenum, pancreas, liver, breast, colon, and skin in mice, rats, and hamsters (188, 191). An empirical link between green tea and its cancer prevention properties was made in the late 1980s (192). Molecular mechanisms, including

catechin-mediated induction of apoptosis and cell cycle arrest (193-196), inhibition of transcription factors NF-κB and AP-1 (195, 197) and reduction of protein tyrosine kinase activity (198) and *c-jun* mRNA expression have also been suggested as relevant chemo preventive pathways for tea (199). Some epidemiological studies also support a protective role of tea against the development of cancer (161). Importantly, the putative chemo preventive effect of tea also varies by the specific type of cancer.

Lung cancer: Studies of the effects of green tea on the development of lung cancer in mice could have promising implications for humans. Green tea infusions have also been shown to inhibit the spontaneous formation of lung tumors and rhabdomyosarcomas in A/J mice (200). In a population- based case-control study in Shanghai, China, consumption of green tea was associated with a reduced risk of lung cancer among non-smoking women and the risks decreased with increasing consumption (201). In a case control study in Uruguay, tea drinking is associated with reduced risk of lung cancer in male cigarette smokers (202).

Breast cancer: Dietary habits play a role in breast cancer risk and prevention as well. Recently, EGCG was found to suppress Wnt signaling in invasive breast cancer cells (203). Green tea polyphenols (GTPs) have been reported to have effect on growth and metastasis of highly metastatic mammary carcinoma cells in vitro and in vivo systems. Metastasis of tumor cells to lungs was inhibited and survival period of animals was increased after treatment with GTP (204). Green tea or EGCG exhibited chemo preventive action on DMBA-induced mammary carcinogenesis only when given in the post initiation stage, and the effect was not dose dependent (205).

Prostate cancer: There is low incidence of prostate cancer among Japanese and Chinese populations with a high intake of green tea (206). In studies using the transgenic adenocarcinoma of the mouse prostate (TRAMP), an animal model that mimics progressive forms of human prostatic disease, GTP consumption was found to significantly inhibit prostate cancer development and metastasis (206). Studies on cell-culture systems using human prostate cancer cells DU145 (androgen-insensitive) and LNCaP (androgen-sensitive) showed that EGCG induces apoptosis, cell-growth inhibition and cell-cycle dysregulation (207).

Gastrointestinal tract cancers: We and numerous others have reported the colorectal cancer preventive activities of EGCG: green tea induced pro-tumorigenic proteins, NAG-1, EGR-1, and ATF3 in colorectal cancer cells to induce growth inhibition and apoptosis (208, 209). Oral green tea administration inhibited intestinal tumor formation in the APC mutant Min mice (210-212). EGCG was found to inhibit growth and activation of the epidermal growth factor receptor and human epidermal growth factor receptor-2 signaling pathways in human colon cancer cells (213).Green tea also caused inhibition of angiogenesis and prevention of cancer metastasis (212), inhibition of inflammation (214), translocation of different genes (215), cell cycle regulation (216), inhibiting activation of IGF-1 (217) and many more functions.

1.2.6 IN VIVO EFFECTS OF EGCG

The topical application of EGCG to SKH-1 hairless mice that had been pretreated twice weekly with UVB light decreased the skin tumors by 44%– 72% and increased the apoptotic index by 56%–92%, again measured by increased caspase-3 activity (218). Fang and colleagues, found that liposomal delivery system of EGCG led to increased antiproliferative activity in basal

carcinoma cells *in vitro*, where the EGCG concentration in the liposomes was 21.3 μmol/L (219). EGCG inhibited AP-1 in UVB-treated transgenic mice carrying a luciferase reporter gene with an AP-1 binding sequence (220). Green tea consumption in 2 study groups, 1 in China and 1 in the USA, decreased oxidative DNA damage (8-hydroxydeoxyguanosine in white blood cells and urine), lipid peroxidation (malondialdehyde in urine), and free radical generation (2,3-dihydroxy benzoic acid in urine) in smokers. Non-smokers (USA group) also exhibited a decrease in overall oxidative stress, which was correlated to decreased levels of free radicals (221). A recent clinical trial involving 60 volunteers with (premalignant) prostate intraepithelial neoplasia, conducted by Bettuzzi *et al*, showed that after 1 year, only 1 man (3%) in the group receiving 600 mg/d green tea compounds in (oral) capsule form, presented with cancer compared to 9 (30%) from the placebo group (222). No significant side-effects were reported.

1.2.7 EGCG AND OTHER DRUGS/COMPOUNDS

There are a number of reports documenting an enhanced chemopreventive effect when EGCG is used in combination with another chemopreventive agent or a therapeutic drug. One study showed that epicatechin significantly enhanced the uptake of labeled EGCG into human lung PC-9 cells (223) and the proapoptotic effects of EGCG were also increased by tamoxifen or sulindac. Another study using the prostate cancer cell lines PC-3, LNCaP, and CWR22Rv1 showed that while 10 μmol/L EGCG only resulted in a 12%–21% inhibition in cell viability, the addition of 10 μmol/L NS-398 (a COX-2 inhibitor), resulted in a 44%–49% inhibition, greater than the additive effect of either agent alone due to decrease in Bcl-2, procaspases-6 and -9, phospho-p65 and peroxisome proliferator-activated receptor (PPAR)γ, and increase in Bax and PARP (224). EGCG at 0.1 μg/mL (equivalent to serum concentrations) markedly enhanced the

growth inhibitory effects of 5-fluorouracil in head and neck squamous carcinoma cells (225). A combination of EGCG and sulindac was also found to be efficacious in preventing azoxymethane-induced colon cancer in rat, where the combination synergistically enhanced apoptosis (226).

1.3 SUMMARY

In this thesis, the role of green tea catechin, EGCG in colorectal carcinogenesis will be discussed. I hypothesized that EGCG would suppress the pro-angiogenic factor, basic fibroblast growth factor (bFGF) and inhibit angiogenesis leading to suppression of tumor nourishment, progression, and metastasis. The objective of this study was to see the effect of EGCG on bFGF suppression, mediated by post-translational regulation based on our preliminary data. The involvement of ubiquitin proteasomal pathway in degradation of bFGF by EGCG would be elucidated (Details in chapter 3).

Next, data related to an EGCG effect on the anti-sense gene of *bFGF*, *NUDT6* will be discussed (Details in chapter 4). *NUDT6* was one of the down regulated genes in HCT-116 human colorectal cells as assessed by microarray experiment by green tea. Overall, the data presented in this thesis suggests that green tea suppresses bFGF and NUDT6 expression, thereby mediating the effects of this important dietary component as an anti-tumorigenic agent.

Materials and experimental procedures

CHAPTER 2

2.1 CELL CULTURE

All human colorectal and non-colorectal cancer cell lines were purchased from the American Type Culture Collection (Manassas, VA), except the head and neck cancer cell line, SPCCY-1, which was obtained from Dr. Dong M. Shin (Emory University, Atlanta, GA). The human colorectal cancer cells HCT-116 and HT-29 were grown in McCoy 5A media. The human colorectal cancer cells; LoVo, the lung cancer cell line; A549, and the prostate cancer cell line; PC-3, were grown in Ham's media. SW480, H292, and SPCCY-1 which are human colorectal cancer cell, lung cancer cell, and head and neck cancer cell respectively were all maintained in RPMI 1640 media. The breast cancer cell line, MCF-7 was maintained in Dulbecco's Modified Eagle medium, DMEM. The brain cancer cell line, T98G, was grown in EMEM medium. All the media were supplemented with fetal bovine serum (10% v/v), L-glutamine (2mM), 100U/ml penicillin and 100ug/ml streptomycin at 37^0C in an environment of 5% CO_2.

For treatment of the cancer cells, serum free media were used. The cells were then treated accordingly either with green tea catechins or pretreated with other compounds, followed by green tea catechins treatment, as per the experiments and are mentioned below under specific methods. The green tea catechins were prepared in 100% DMSO (Fisher Scientifics, Fairlawn, NJ) and DMSO was used as a vehicle control unless otherwise specified.

2.2 REAGENTS

The green tea catechins, EGCG, ECG, EGC, EC and glutathione synthetase were purchased from Sigma (St. Louis, MO). The kinase inhibitors used are as follows: ERK inhibitor (UO126), JNK inhibitor (SP600125) and PI3K inhibitor (LY294002) were purchased from Promega (Madison, WI); NF-κB inhibitor (MG-132) was obtained from Calbiochem (San Diego, CA);

Staurosporine was purchased from Biomol International (Plymouth Meeting, PA); proteosomal inhibitor (Lactacystin) was obtained from Tocris Biosciences (Bristol, UK). The other proteasomal inhibitors; Epoxomicin, and AW9155, were obtained from Biomol International (Plymouth meeting, PA). All the inhibitors were dissolved as concentrated stock solutions in DMSO or PBS as mentioned by the manufacturer. They were stored at -80^0C and diluted at the time of use in culture media.

Primary antibodies for bFGF and β-catenin were purchased from BD transduction laboratories (San Jose, CA). VEGF, Bcl2, Bax, p53 and actin primary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), cyclin D1, phosphor p53 serine 6 and 15 antibodies from Cell Signaling Technology (Beverly, MA), and V5 antibody from Invitrogen (Carlsbad, CA). NAG-1 antibody was described previously (ref). All the secondary antibodies were obtained from Amersham Biosciences (Piscataway, NJ). Chemiluminescence kit for protein detection was obtained from Pierce (Rockford, IL). All chemicals were purchased from Fischer Scientific, unless otherwise specified.

2.3 REVERSE TRANSCRIPTION PCR-ANALYSIS

The cells were grown till 60-70% confluent in respective growth medium as mentioned above. The cells were then treated accordingly as per the experiment with various reagents or green tea catechins in serum free media for 24 hours. The cells were washed once with 1x phosphate buffer saline and scraped using cell scraper and RNA extraction was performed using Trizol (Invitrogen, Carlsbad, CA) according to manufacturer's protocol and quantified by spectrophotometry. RT reaction was done using total RNA as a template and a cDNA i-script synthesis kit (Bio-Rad Laboratories, Hercules CA). The PCR primers used for amplification are

mentioned in **Table 4**. The *GAPDH* levels were used for the normalization of RNA using these primers. The thermal cycling conditions used on a Master Cycler Gradient (Eppendorf) for human gene expression detection included an initial denaturation at 94^{o}C for 2 min, followed by 25-29 cycles of denaturation at 94^{o}C for 30 sec, annealing at 55^{o}C for 30 sec, and extension at 72^{o}C for 1 min. Aliquots (10 µL) of the amplification products (25-30 cycles) were separated by electrophoresis through a 1.2% agarose gel, and the bands were visualized using ethidium bromide as an intercalating agent. The intensity of each band was quantified using Scion Image software (Scion, Frederick, MD). Results for intensity of each detected band were normalized with *GAPDH* band intensity values.

TABLE 4: Human sense and antisense primers. *GAPDH* is used as a control house keeping gene for standardization.

HUMAN PRIMERS	**FORWARD (Sense)**	**REVERSE (Anti-sense)**
bFGF	5'- agagcgaccctcacatcaag-3'	5'-actgcccagttcgtttcagt-3'
NUDT6	5'- catcctccaaagccgattta-3'	5'-aacttctcgaaccgctgtgt-3'
NAG-1	5'-ctccagattccgagagttgc-3'	5'-agagatacgcaggtgcaggt-3'
ATF3	5'-gtttgaggattttgctaacctgac-3'	5'-agctgcaatcttatttctttctcgt-3'
NR4A1	*5'*-cacagcttgcttgtcgatgt-3'	5'-tcttgtcaatgatgggtgga-3'
ID1	5'-cggatctgagggagaacaag-3'	5'-ctgagaagcaccaaacgtga-3'
ID2	5'-cgtgaggtccgttaggaaaa-3'	5'-atagtgggatgcgagtccag-3'
P53	5'- gcgcacagaggaagagaatc-3'	5'- tgagtcaggcccttctgtct-3'
GAPDH	5'-gggctgcttttaactctggt-3'	5'- tggcaggtttttctagacgc-3'

2.4. ANALYSIS OF PROTEIN EXPRESSION BY WESTERN BLOTS

The cells were grown to 60-80% confluence in 6-cm plates followed by 24 h treatment in the presence of indicated compounds. The cells were then washed once with phosphate buffered saline and lysed using cold RIPA buffer (1 ×PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitors (1 mM PMSF, 5 μg/ml aprotinin and 5 μg/ml Leupeptin) and phosphatase inhibitors (1 mM Na_3VO_4 and 1 mM NaF). Cell lysates were collected from culture plates using a cell scraper, and were kept on ice for 30 minutes followed by protein collection by centrifugation at 4^0C. The protein concentration was determined by the BCA protein assay (Pierce, Rockford, IL) using BSA as the standard. Equal aliquots of 50 μg protein were boiled in 2x loading buffer (0.1 M Tris-Cl, pH 6.8, 4% SDS, 0.2% Bromophenyl blue, 20% glycerol) for 5-10 minutes, and then resolved by electrophoresis on SDS-PAGE (8-14% gels). They were transferred to nitrocellulose membranes (Osmonics, Minnetonka MN) for 1 h. The blots were blocked for 1 h with 5% skim milk in TBS/Tween 0.05% (TBS-T), and probed with a specific primary antiserum in Tris buffered saline (TBS) containing 0.05% Tween-20 (TBS-T) and 5% non-fat dry milk at 4°C overnight. After washing with TBS-T, the blots were treated with horseradish peroxidase-conjugated secondary antibody for 1 h and washed several times. Proteins were detected by the enhanced chemiluminescence system (Pierce, Rockford, IL). Antibodies for bFGF, VEGF, V5, p53, phosphor p53 (serine 6 and 15), NAG-1, Blc2, Bax and Cyclin D1 antibodies were used in these studies. Membranes were probed with an antibody for Actin (Santa Cruz) to ensure equal loading of protein between samples.

2.5. CLONING OF EXPRESSION VECTORS

The full-length human *bFGF* cDNA (18 kDa) was isolated by RT-PCR from LoVo cells using forward (5'-atggcagccgggagcatcac-3') and reverse (5'-gctcttagcagacattggaagaaaa-3') primers, obtained from the reported human *bFGF* cDNA sequence (GenBank #NM_002006). Amplified PCR products were then cloned into the pcDNA3.1/V5-His-TOPO vector (Invitrogen). The sequence and orientation were confirmed by DNA sequencing analysis at Molecular Biology Resource Facility of University of Tennessee.

The luciferase-NUDT6 3'-untranslated region (3'UTR) hybrid construct was generated by PCR using two primers designed to contain the XbaI restriction enzyme site (underlined) as follows: Forward: 5'- gctctagattcacatttatatgtttag-3' and Reverse: 5'-gctctagacgaaaagaggcttttaaaat-3'. A PCR product using cDNA from HCT-116 cells was digested with Xba1 enzyme, followed by ligation into pGL3-promoter vector digested with XbaI. The full-length human *NUDT6* cDNA (915 bp) was isolated by RT-PCR from HCT-116 cells using forward (5'-ggacgaattaagcggcgtggaga-3') and reverse (5'-atcaattcctttcatagttttat-3') primers, obtained from the reported human *NUDT6* cDNA sequence (GenBank #NM_007083). Amplified PCR products were then cloned into the pcDNA3.1/V5-His-TOPO vector (Invitrogen, Carlsbad, CA) and named as pcDNA 3.1/NUDT6 for correct orientation and pcDNA 3.1/CONTROL for reverse orientation.

2.6 IMMUNOPRECIPITATION AND HISTIDINE PULL-DOWN EXPERIMENTS

LoVo cells were transiently transfected with either bFGF expression vector (pcDNA3.1/V5-His-TOPO/bFGF) or control vector (pcDNA3.1/V5-His-TOPO/LacZ) using Lipofectamin2000 (Invitrogen), according to the manufacturer's protocol**.** The bFGF proteins

containing six histidine residues at their C-termini were purified using ProBond nickel-chelating resin (Invitrogen), using native condition buffer (50 mM NaH_2PO_4/pH 8.0, 500 mM NaCl, 1 mM PMSF, 1 µg/ml aprotinin, 1 µg/ml leupeptin), and subsequently incubated with 50 µL of ProBond Resin (50% Slurry in 20% ethanol) for 30 min at 4°C. Beads were then washed three times, boiled for 5 min, and loaded on SDS/PAGE for Western blot analysis.

For the antibody immunoprecitation assay, the stable NUDT6 cell lines were grown till 80-90% confluent in 10cm plates. Next day the cells were washed twice with ice cold phosphate buffer saline. About 3 ml of 0.1M tris chloride (pH 7.4) supplemented with protease inhibitors (1 mM PMSF, 5 µg/ml aprotinin and 5 µg/ml leupeptin) and phosphatase inhibitors (1 mM Na3VO4 and 1 mM NaF) was added in the plates and cells were then scraped and collected in 15ml tubes on ice. Centrifugation was carried out at 4^0C for 10 minutes @ 1000 rpm. The supernatant was removed and cells were resuspended in the Tris chloride and protein concentration was measured using BCA. About 500-1000 µg of protein was used for immunoprecipitation. About 50 µl of protein A/G agarose resin beads is washed with Tris chloride and stored on ice. The samples are prepared using Tris chloride supplemented with protease inhibitors making a final volume of 500 µl. About 5-10 µg of desired antibody is added on the samples and incubated for 1-2 hours on rocking shaker at 4^0C. After 1-2 hours the protein samples with antibody are placed on the premade resin and incubated overnight at 4^0C on rocking shaker. Next day the samples are centrifuged at 1000 rpm for 1 min and supernatant is stored for later use. Around 5-10 washings are given to the protein beads using Tris Chloride making sure that the supernatant are carefully removed to prevent any background. After the last wash, 50 µl of 2x loading buffer is added on the beads and boiled at 95-100°C for 5-10 minutes to denature the protein and separate it from the protein-A/G beads, then centrifuged and run on SDS gel for Western Blot analysis.

2.7 PROTEIN DEGRADATION STUDY

The cells were grown to 60-80% confluence in 6-cm plates and pretreated with vehicle (DMSO) and EGCG 50 µM for 24 hours. In all the experiments that were carried out for this thesis, the cells were then treated with cycloheximide (10 µM) for 1, 3, 6, 12, 24 hours. However for the p53 protein degradation experiment in NUDT6 stable cell lines, puromycin hydrochloride 50 µM was used for 1, 3, 6, 12, and 24 hours. Total cell lysates were harvested and subjected to Western blot as mentioned earlier.

2.8 ELISA ASSAY FOR bFGF

ELISA was done using tissue lysates from the green tea treated $APC^{Min/+}$ mice and lysates from HCT-116 cells. Tissue samples and cells were homogenized in RIPA buffer, and then sonicated at 4^0C, followed by centrifugation for 20 min at 14,000 rpm. The supernatant was collected and subjected to ELISA. Quantikine FGF basic Immunoassay kit (R&D systems, Minneapolis, MN) was used for mouse tissue samples, and the Raybiotech kit (Norcross, GA) was used for cell lysates. All tests were carried out in triplicate. Optical densities were read immediately using a micro-titer plate reader (Bio-Tek Instruments) at 450 nm. The amount of bFGF protein was normalized to ng of bFGF per mg total protein.

2.9 UBIQUITIN ASSAY FOR bFGF

An ubiquitin enrichment kit (Pierce, Rockford, IL) was used according to the manufacturer's protocol. All the samples were adjusted to equal volume with TBS buffer. The polyubiquitin affinity resin (20 µl) was added to spin columns with samples and incubated at 4^0C for 2 hours.

The columns were then centrifuged and washed with wash buffer twice. The SDS-PAGE sample-loading buffer was added to the columns and placed in a heat block for 10 min and centrifuged to obtain ubiquitin enriched fraction ready for western blot analysis. Gradient gel 4-20% was used for the western blot with anti-ubiquitin antibody (1:10,000) for overnight incubation, followed by adding anti-rabbit secondary antibody.

2.10 20S PROTEASOME ASSAY

A Proteasome-Glo Assay Systems Kit from Promega (Madison, WI) was used to detect the 20S proteasomal activity. This kit allows three separate assays that differ in their ability to detect different protease activities based on their substrate components. The LoVo cells were treated with vehicle (DMSO) and EGCG (50 µM). Samples were prepared in triplicate, and 30 µg of each sample was loaded in a white-walled 96-well plate, to which equal amounts of chymotrypsin-like, trypsin-like and caspase-like substrates were added. The plate was incubated for 1 hr at room temperature in the dark, and the luminescence for the three activities was recorded on a multi-detection microplate reader (BioTek, Winooski, VT). Relative luminescence units (RLU) were analyzed.

2.11 IMMUNOSTAINING OF FACTOR VIII FOR bFGF

Formalin-fixed, paraffin-embedded tissue sections were deparaffinized and rehydrated. Antigen retrieval was done with EDTA buffer (10 mM Tris/1mM EDTA/0.05% Tween20, pH 9.0) at 95°C for 20 min in a steamer. The slides were then soaked in TBS/pH 7.6 for 5 min, and loaded onto a Dako Autostainer (Carpentaria, CA). Samples were blocked with 5.3% hydrogen peroxide and serum-free protein for 5 min, respectively. Factor VIII polyclonal antibody (1:4,000, Dako)

was then applied, followed by Envision Polyclonal HRP (Dako) both for 30 min. DAB/chromogen was applied for 10 min, and counterstaining was carried out with hematoxylin followed by dehydration and cover-slipping.

2.12 ANIMAL STUDY

Min mice study: The $APC^{Min/+}$ mice were on C57/B6 background. The strain was originally obtained from Jackson laboratory and since maintained in our breeding colony. The mice were randomly divided into 3 groups of 9 mice to receive vehicle, EGCG, or ECG. The $APC^{Min/+}$ mice were maintained at 22 ± 2ºC on a 12 h light/dark cycle and with free access to standard rodent chow and water. $APC^{Min/+}$ mice were provided EGCG or ECG (0.01%) in their drinking water for 2 months starting at 6-7 weeks of age. Sucrose (3%) was added to the drinking water of all groups, including controls, to increase palatability. Twenty-four hrs after final treatment, the mice were euthanized, and the intestinal tract was isolated and washed with PBS. Tumor numbers and sizes in the small intestine were assessed with a stereoscopic microscope as previously described (227). All animal research procedures were approved by the University of Tennessee Animal Care and Use Committee and were in accordance with NIH guidelines.

Nude mice study: The male nude mice were obtained from Taconic (NCRNU-M, homozygous). The mice were kept in quarantine for a week after receiving them and were maintained at 22 ± 2^0C on a 12 hour light/dark cycle and with free access to standard rodent chow and water. The mice were fed with 3% sucrose water. Each mouse was injected with CONTROL and NUDT6 stable cell lines (mentioned below) on right and left flank respectively. The water was changed two times a week and tumor length and width were measured at the same time. The mice were sacrificed due to humane reasons after the tumors grew more than $1cm^3$. Tumor

volume was calculated using following formula: tumor volume= (l+w) *0.5*l*w where l=length, w=width. All animal research procedures were approved by the University of Tennessee Animal Care and Use Committee and were in accordance with NIH guidelines.

2.13 RNA STABILITY

For the mRNA stability experiment, HCT-116 cells were grown in 6-cm plates and then treated with DMSO and EGCG for 1 h. Actinomycin D (Fisher Bioreagents, Fairlawn, NJ) was added at the dose of 5 µM to terminate transcription. For the *de novo* protein synthesis experiment, HCT-116 cells were pretreated with 10 µg/ml cycloheximide or DMSO for 1 h in serum-free media followed by treatment with EGCG (50 µM) for 24 h. Total RNAs were harvested using Trizol as mentioned above. The cDNA was prepared, RT-PCR was carried out, and the products were analyzed on 1.2% agarose gel.

2.14. TRANSIENT TRANSFECTION AND LUCIFERASE REPORTER ASSAYS

HCT-116 cells were plated in 12-well plates at 2×10^5 cells per well and grown for 16 h. Plasmid mixtures containing 0.5 µg of reporter vector and 0.05 µg of pRL-null (Promega) were transfected by LipofectAMINE (Invitrogen) according to the manufacturer's protocol. After transfection, the media were replaced with serum-free media, and the indicated reagents were added. The cells were harvested in 1x luciferase lysis buffer, and luciferase activity was determined and normalized to the pRL-null luciferase activity using a Dual-Glo luciferase assay system (Promega).

2.15 DEVELOPMENT OF STABLE CELL LINES

HCT-116 cells were plated in 6-cm plates and transfected with either NUDT6 expression vector (pcDNA3.1/V5-His-TOPO/NUDT6) or CONTROL vector (pcDNA3.1/V5-His-TOPO/CONTROL) using LipofectAMINE (Invitrogen, Carlsbad, CA), according to the manufacturer's protocol. After 24 h, the cells were then transferred to a 10 cm plate with G418 (500 µg/ml) (Stratagene, Santa Clara, CA). Selection with G418 was carried out for 3 weeks, and then Western blot was carried out to check the stable cell lines.

2.16 CELL PROLIFERATION ANALYSIS

Cell proliferation was carried out using pcDNA3.1/NUDT6 and pcDNA 3.1/CONTROL cell lines with or without staurosporine (5 µM) using the Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI). Briefly, cells were seeded at a concentration of 1,000 cells/well in 96-well tissue culture plates in six replicates. The cells were then treated with 5 µM of staurosporine for 1, 2, 3 and 4 days. At 0, 1, 2, 3 and 4 days, 20 µl of CellTiter96 Aqueous One solution was added to each well, and the plate was incubated for 1 h at 37°C. Absorbance at 490 nm was recorded in an enzyme-linked immunosorbent assay (ELISA) plate reader (Bio-Tek Instruments, Winooski, VT).

2.17 SOFT AGAR ASSAY

Soft agar assays were performed to compare the clonogenic potential of the two stable cells in semi-solid medium as previously described (228). Cell colonies were visualized by staining with 0.5 ml of *p*-iodonitrotetrazolium violet (Sigma, St. Louis, MO) under the luminescent image analyzer (Fujifilm Co.). Colonies were counted using the Multi Gauge Program (Fujifilm Co.)

according to the instructions. For the impedance experiment, preliminary naked scans were performed to optimize sensitivity and to check for any electrode debris or defects.

2.18 ELECTRICAL IMPEDANCE MEASUREMENT ASSAY

Cells were kept viable using an incubator (Weather Station, Olympus) that kept the temperature (37°C), humidity, and CO_2 (5%) levels constant. These controlled conditions were crucial to keep cells alive during long time lapse measurements. The imaging system consisted of a 100X objective lens with a numerical aperture (NA) equal to 1.35, an Olympus Model IX-71 inverted microscope, and a Hamamatsu 14-bit electron multiplier (EM) cooled and intensified-CCD digital camera. Differential inference contrast microscopic (DICM) imaging has the additional optics such as a polarizer (IX-LWPO), a DIC prism (IX2-DIC100) in the DICM condenser (IX2-LWUCD, NA=0.55), a transmitted Nomarski prism (U-DICTS), an analyzer (IX2-AN), and a 100 W Halogen lamp house (U-LH100-3, Olympus), while current fluorescence microscopic imaging needs green cube (EX: 530~540, EM: 590~) and a xenon light source. Additionally, a mechanical shutter was synchronized with the CCD camera to minimize any effects the halogen lamp as well as the Xenon light source may have had on cell growth. A data acquisition and analysis system was implemented using Lab VIEW. A reference voltage source provided an ac 1 V_{rms} reference signal via a series 1 M Ω resistor, R_{cc}, to a gold electrode array (Applied Biophysics). A National Instruments SCXI-1331 switch made successive connections between the various working electrodes and the counter electrode of each array. The source voltage generator resistance, R_s, was 50 Ω. An SR830 lock-in amplifier with an input impedance equivalent to a parallel resistor, R_v, and capacitor, C_v, combination of 10 MΩ and 10 pF, respectively, measured the electrode voltage. Direct measurements of the cable parasitic

capacitances were made using an LCR meter and incorporated into a circuit model to estimate the impedance based on the lock-in amplifier voltage measurements.

Preliminary naked scans were performed to optimize the sensitivity and to check for any electrodes debris or defects. The electrodes were then inoculated with 400 μL McCoy5A, seeding NUDT6 or CONTROL stable cancer cells at a concentration of 2.4×10^4 cells/well. During the cellular micro-impedance scans, data were acquired at a rate of 32 Hz every 16 s using a 30 ms filter time constant and 12 dB/ decade roll off for approximately 100 h. Averages and standard deviation estimates were obtained from the 512 sampled data points over the 16 s time intervals. During the experiments, cell-inoculated electrodes were kept in a cell culture incubator at a temperature of 37°C and 5% CO_2.

2.19 MITOCHONDRIAL EXTRACTION PROTOCOL

Mitochondrial extraction kit (Pierce, Rockford, IL) was used to isolate the mitochondrial and cytoplasmic fractions. The CONTROL and NUDT6 stable cells were grown in 10 cm plates till 80-90 % confluent and washed with phosphate buffer saline, scraped and collected in 15ml tubes. The cells were centrifuged at 1000 rpm for 5 min in 4^0 C centrifuge machine. To the pellet, 800 μl of reagent A (provided in the kit with proteinase inhibitors) was added and the tubes were vortexed at maximum speed for 5 sec and tubes were incubated on ice for 2 min. Then 10 μl of reagent B was added and tubes were vortexed for 5 sec. Reagent C was added and tubes were inverted but not vortexed and centrifuged at 1500 rpm for 10 min. The supernatant is the cytosolic fraction and is collected in new 2ml tube and centrifuged again at 3000 rpm for 15mins and collected in new tube. The pellet at this stage contains the isolated mitochondria.

About 500 µl of reagent C is added to the pellet and centrifuged at 3000 rpm for 5 min. The supernatant is discarded and mitochondrial pellet is stored in -80^0 C till further processing.

2.20 IMMUNOCYTOCHEMISTRY

The stable cell lines were seeded in the culture plates @ 1000 cells/ well for 24 hours. The cells were then washed with phosphate buffered saline and introduce to serum free media. Briefly, cells were then incubated with 100 nM of mitotracker® Red FM probes (Molecular Probes, Eugene, OR) for 45 min in CO_2 incubator, and then washed with PBS. Fixation was carried out with 4% paraformaldehyde for 30 min. The cells were washed three times with PBS for 5 mins and blocked with 1% normal goat serum. The cells were incubated with anti-V5 FITC conjugated antibody (Invitrogen) for overnight at 4˚C. After three times washing with PBS, cells were stained with 0.5 mg/ml of DAPI for 10 min to counter stain the nucleus. The immunofluorescence was processed using a fluorescence microscope Olympus BX61 equipped with CC12 digital camera. The TIF images were captured using the program analySIS version 3 (Soft Imaging System Solutions, Lakewood, CO) with X400 magnification.

2.21 CASPASE ACTIVITY FOR NUDT6

The Caspase-Glo 3/7 Assay is a homogeneous, luminescent assay that measures caspase-3 and -7 activities. The cells were grown and cell lysates were collected in the similar manner as mentioned in Western blot protocol. The protein concentration was measured and 30 µg of protein was pipetted on a 96 well white walled plate making the final volume of 30µl each using Ripa Buffer. The blank well had ripa buffer. The blank reaction is used to measure background luminescence associated with the cell culture system and Caspase-Glo 3/7 Reagent. The blank

value will be subtracted from the experimental values. Then 30 µl of Caspase-Glo 3/7 Reagent was added in each well including blank and the plate was incubated at room temperature for 1-2 hours and the luminescence was recorded on a multi-detection microplate reader (BioTek, Winooski, VT). Relative luminescence units (RLU) were analyzed. Each sample was made in triplicates.

2.22 INVASION ASSAY FOR NUDT6

The stable cell lines (CONTROL and NUDT6) were grown till 60-70% confluent in 6cm plate for 24 hours. Matrigel was thawed at 4^0C overnight. Then it was diluted (5mg/ml to 1 mg/ml) in serum free-cold cell culture media (McCoy 5A). About 100 ul of the diluted matrigel was added into the upper chamber of 24-well transwell. The transwell was incubated at 37^0C for at least 4 to 5 h for gelling. In the meantime the stable cells were washed with phosphate buffered saline once and trypsinised from tissue culture plates using Trypsin/EDTA. The cells were resuspended using McCoy 5A media containing 10% FBS to make the final volume of 1×10^6 cells per ml per transwell. About 100 ul of the cell suspension was added onto the matrigel. The lower chamber of the transwell was filled with 600 ul of culture media containing 10%, as a growth agent. The plate was incubated at 37C for 24 to 48 hours. After 24 or 48 hours the transwells were removed from 24-well plates and stained with hematoxylin eosin stains. The noninvaded cells were scraped off from the top of the transwell with a cotton swab. The membrane was carefully removed and put on the slide covered with glass slip and invading cells were counted under a light microscope.

2.23 STATISTICAL ANALYSIS

SAS for Windows (9.1.3) (SAS Institute., Cary, NC) statistical analysis software was used. Tumor load, tumor polyps and ELISA for *in vivo* Min mice experiments were analyzed using ANOVA with the Dunnett's test, where $p<0.05$ was considered significant. For quantitative analyses in luciferase assay for 3'UTR of NUDT6, analysis of variance (ANOVA) with Tukey's multiple comparison test was used to compare mean values. The Student *t* test was used to analyze the differences between samples. $p<0.05$ was considered statistically significant and represented with one asterisk. Similarly, two and three asterisks were used to show $p<0.01$ and $p<0.001$, respectively.

Effect of EGCG on post translational regulation of Basic fibroblast growth factor in human colorectal cancer cells

CHAPTER 3

The results discussed in this chapter have been published in *Gastroenterology*, Volume 134, No. 7, pp 1972-1980 (2008) titled **"A green tea component suppresses posttranslational expression of basic fibroblast growth factor in colorectal cancer".**
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3.1 INTRODUCTION

Tumors require increasing blood supply for their sustained growth (229). All solid tumors in man and animals will have developed their own vascular supply by the time they are detected. Before the 1960s it was generally assumed that dilation of existing host vessels was the main basis for tumor vascularization (230). There were just two reports, in 1939 and 1945 which stated that tumors could induce new vessels from the host (231, 232). The idea that new vessels are necessary for continued tumor growth originated in the early 1960s from an experiment in which tumors were implanted in isolated perfused organs and they failed to vascularized beyond a few millimeters diameter. The capillary blood vessel proliferation did not occur in the isolated organs but neovascularization was induced when the tumors were re-implanted into their animal host (233-235). This showed that tumors had retained their "angiogenic" capacity and enlarged rapidly to more than thousand times compared to the tumors in the isolated organs and this led to the hypothesis that "tumor growth is angiogenesis-dependent" (229, 236-238).

Angiogenesis is the formation of new blood vessels. It is an important process during embryonic development and during tissue remodeling in the neonate and adults. It is also a key feature of wound healing and of pathological conditions such as growth and progression of solid tumors, where capillary invasion is a prerequisite of further growth of dormant tumors (239). The vasculature carries blood components which are absolutely necessary for cell life in various tissues. In normal cases, endothelial cells are generally non-proliferative stationary cells forming a non-thrombogenic luminal surface in intimate contact with the subendothelial extracellular matrix (240). Similarly for cultured cells to proliferate, they require nutrients, growth factors and appropriate substratum upon which they can attach and spread indicating that the substrate or

extracellular matrix is a decisive element in their proliferative and differentiation responses (241, 242). The extracellular matrix (ECM) contains collagens, proteoglycans, laminin, fibronectin, and elastin (243-245). Basement membranes are also of particular importance in the development of epithelial malignancies as they are the first barrier encountered in the invasive process of carcinomas (246).

The endothelium lines the interior surface of blood vessels forming the entire vasculature. The heparin binding growth factor family of polypeptides which control endothelial cell proliferation is considered an initiator of angiogenesis (247-250). This gene family includes mainly the prototypes aFGF (acidic fibroblast growth factor) and bFGF (basic fibroblast growth factor (248-251).

The basic fibroblast growth factor or bFGF protein (also known as FGF-2) is a member of the fibroblast growth factor family (252) and is a potent endothelial growth factor in vitro and capable of inducing angiogenesis in vivo (253). In normal tissue, basic fibroblast growth factor is present in basement membranes and in the sub endothelial extracellular matrix of blood vessels. The endothelial cells produce bFGF that remains cell-associated and stimulates their own growth. Basic FGF can be released from matrix by heparinase digestion or by trypsin and extracellular matrix might serve as a reservoir for bFGF (254). Basic FGF binds to cell surface heparan sulfate proteoglycan, an activity required to bind to high affinity FGF receptors (255). It is a single-chain polypeptide of 154 amino acids with molecular weight if about 18 kDa (239). However there are forms of bFGF containing more than 154 amino acids that have molecular weights of 22-25 kDa (17.8, 22.5, 23.1, and 24.2 kDa) (256-258) resulting from a canonical AUG (18 kDa) and three unusual CUG start codons (257, 258). The AUG initiated bFGF can stimulate cell migration; down-regulate its own receptor, and stimulate integrin synthesis. The

CUG initiated forms are involved in cell proliferation by a cell surface receptor independent pathway (259). bFGF is a primary inducer of mesoderm formation in embryogenesis. It modulates both cell proliferation and differentiation in vitro and in vivo. It is a mitogen and chemoattractant and hence a potent mediator of wound healing, angiogenesis and neural outgrowth. It is mostly known for its role as a pro-angiogenic factor. Cells transfected with a bFGF construct having a signal sequence undergo autocrine transformation and exhibit morphological and biochemical alterations characteristics of highly transformed cells (260). The signal peptide bFGF transformed cells have an accelerated proliferation rate, and are capable of anchorage-independent growth. Tumors induce blood vessel growth by secreting various growth factors that are pro-angiogenic such as bFGF which can induce capillary growth into the tumor and thereby supply required nutrients allowing for tumor expansion (261).

Another proangiogenic factor is vascular endothelial growth factor (VEGF) which is a chemical signal produced by cells that stimulates the growth of new blood vessels. The normal function of VEGF is to create new blood vessels during embryonic development, new blood vessels after injury, and new vessels (collateral circulation) to bypass blocked vessels. When VEGF is over expressed, it can contribute to disease. Solid cancers cannot grow beyond a limited size without an adequate blood supply; cancers that can express VEGF are able to grow and metastasize. VEGF is a sub-family of growth factors, and are important signaling proteins involved in both vasculogenesis (the new formation of the embryonic circulation) and angiogenesis (the growth of blood vessels from pre-existing vasculature).

These pro-angiogenic factors can be targeted to block angiogenesis and hence we focused on EGCG effects on bFGF. We found that EGCG can suppress bFGF at a post-translational level through an ubiquitin-dependent proteasomal pathway.

Ubiquitin is a 76 amino-acid residue protein found ubiquitiously in the body and hence the name. It is ligated through its C terminus to the lysine side chains of acceptor proteins in a process of post-translational modification called ubiquitination (262). Ubiquitination plays an important role in protein degradation maintaining protein homeostasis in the cell as well as in other biological processes, such as DNA repair, transcription, translation, signal transduction, organelle assembly, protein trafficking, and virus budding (262-264). The difference in activities lies in the type of ubiquitin chain – if the linkage to the target protein is via K48 on ubiquitin, this will lead to a series of events that culminate in degradation of the polyubiquitinated protein by the 26S proteosome. Also, there have to be a minimum of 4 ubiquitins attached in order to trigger degradation. However, if the polyubiquitin chains are linked via Lys63, this will direct protein-protein interactions via an ubiquitin binding domain on the interacting target protein. A major function of this type of ubiquination appears to be to allow proteins with this domain to assemble into multi-protein complexes, which might lead to access to substrates if kinases are also involved, but also regulates such processes as endocytosis and ribosomal protein synthesis. F-box motif is a protein structural motif of about 50 amino acids that mediates protein-protein interactions. The first identified F-box protein is one of three components of the Skp, Cullin, F-box containing complex (SCF complex) which is E3 ligase complex mediating ubiquitination of proteins targeted for degradation by the proteasome. The F-box motif interacts directly with the SCF protein Skp1 (265).

The biochemical steps in the ubiquitin pathway are illustrated in **Figure 3.** Briefly the C terminal Gly residue of ubiquitin is activated in an ATP-dependent manner by specific activating enzyme, E1. Activated ubiquitin is next transferred to an active site Cys residue of an ubiquitin-carrier protein, E2. The ubiquitin-protein ligase or E3 enzyme then links C-terminus of ubiquitin in an

amide isopeptide linkage to a ε-amino group of the substrate protein's Lys residues. (266, 267). The multiubiquitin chains are assembled by a family of ubiquitination factors (E4) that produces longer Ub-chains (268). The proteins containing polyubiquitin chains are recognized and degraded by the 26S proteasome complex that requires ATP hydrolysis for its action (269). The 26S proteasome consists of an ATP-dependent assembly of cylindrical 20S proteasome, a complex that contains the three proteolytic cleavage sites, and 19S cap or regulatory complexes **(Figure 3.1).** The catalytic core of the 20S proteasome is made up of 28 subunits, 14 *alpha* subunits and 14 *beta* subunits (270-272). The *alpha* subunits are arranged into two rings (each of which consists of 7 subunits) and comprise the ends of the 20S core. The *beta* subunits are also arranged in two 7-subunit-containing rings, making up the middle part of the 20S core. These four rings are stacked on top of one another in the arrangement: 7-*alpha*, 7-*beta*, 7-*beta*, and 7-*alpha*. The proteolytic activities of the proteasome are associated with the beta subunits **(Figure 3.1)**. In the eukaryotic 26S proteasome there are three known activities associated with these 7 beta subunits, which are chymotrypsin-like (cleavage after hydrophobic residues), trypsin-like (cleavage after basic residues), and caspase-like (cleavage after acidic residues) (271, 272). Because there are two sets of beta subunits, there are therefore two sets of each of these three activities **(Figure 3.1).** The 19S complexes contain several ATPase subunits and other subunits that are presumably involved in the specific action of the 26S proteasome on ubiquitinylated proteins (273). The proteasome's 19S regulatory cap binds the polyubiquitin chain, denatures the protein, and targets the protein into the proteasome's proteolytic core (274, 275). However recent reports have shown that proteasomal degradation does not always require ubiquitin. Asher et al showed that the proteasomal degradtion of tumor suppresssor proteins, p53 and p73, is carried out in an ubiquitin independent manner and regulated by NQO1 enzyme (276).

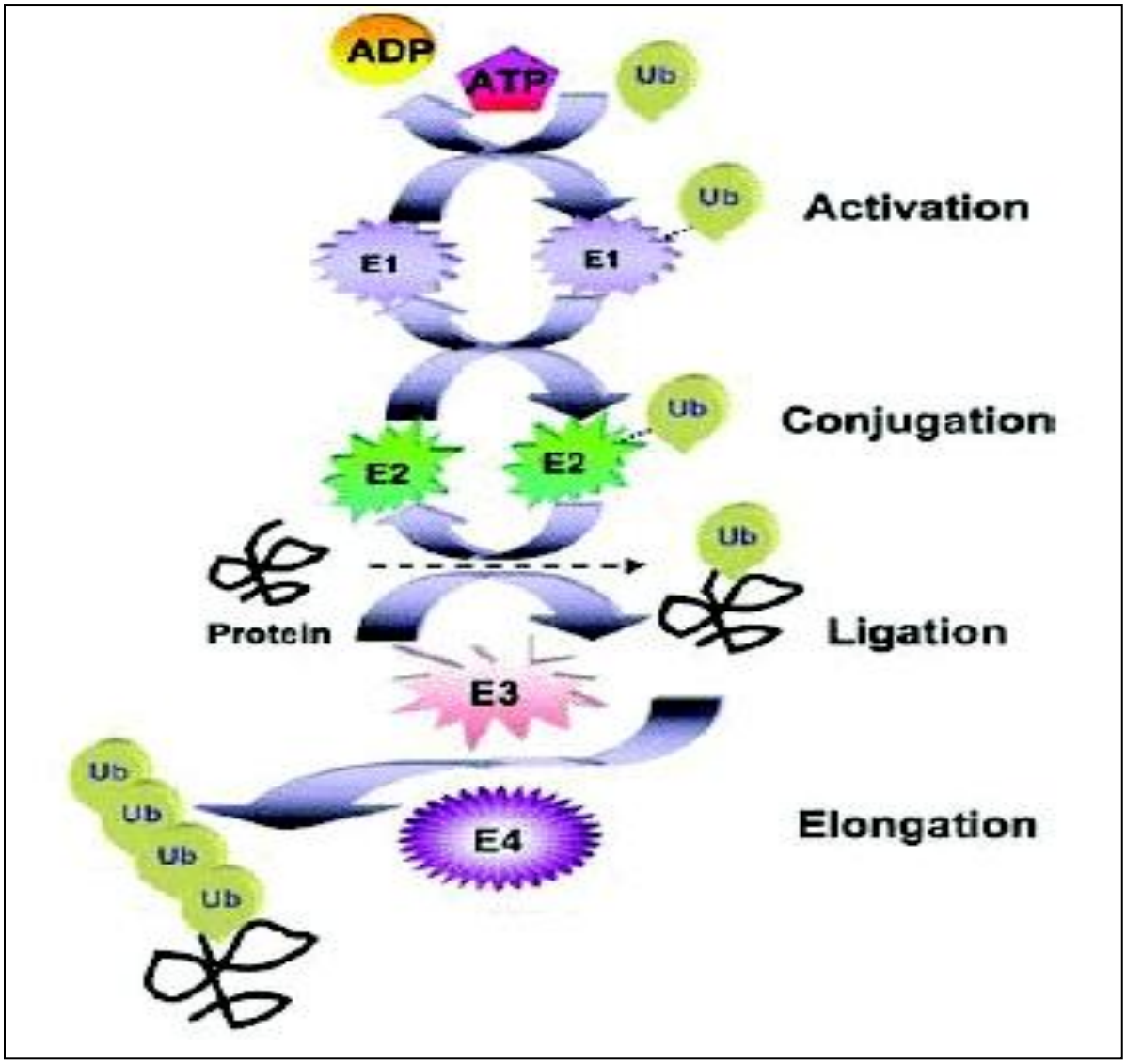


Figure 3. Protein ubiquitination. First, a high-energy thioester bond is formed between ubiquitin (Ub) and an ubiquitin-activating enzyme (E1). This reaction requires ATP hydrolysis. Secondly, the activated ubiquitin is transferred to an ubiquitin conjugating enzyme (E2). Thirdly, the activated ubiquitin is ligated to the protein substrate by an ubiquitin ligase (E3). Lastly, the ubiquitin chain is elongated by an ubiquitin-chain elongating factor (E4), which drives the assembly of the polyubiquitin chain

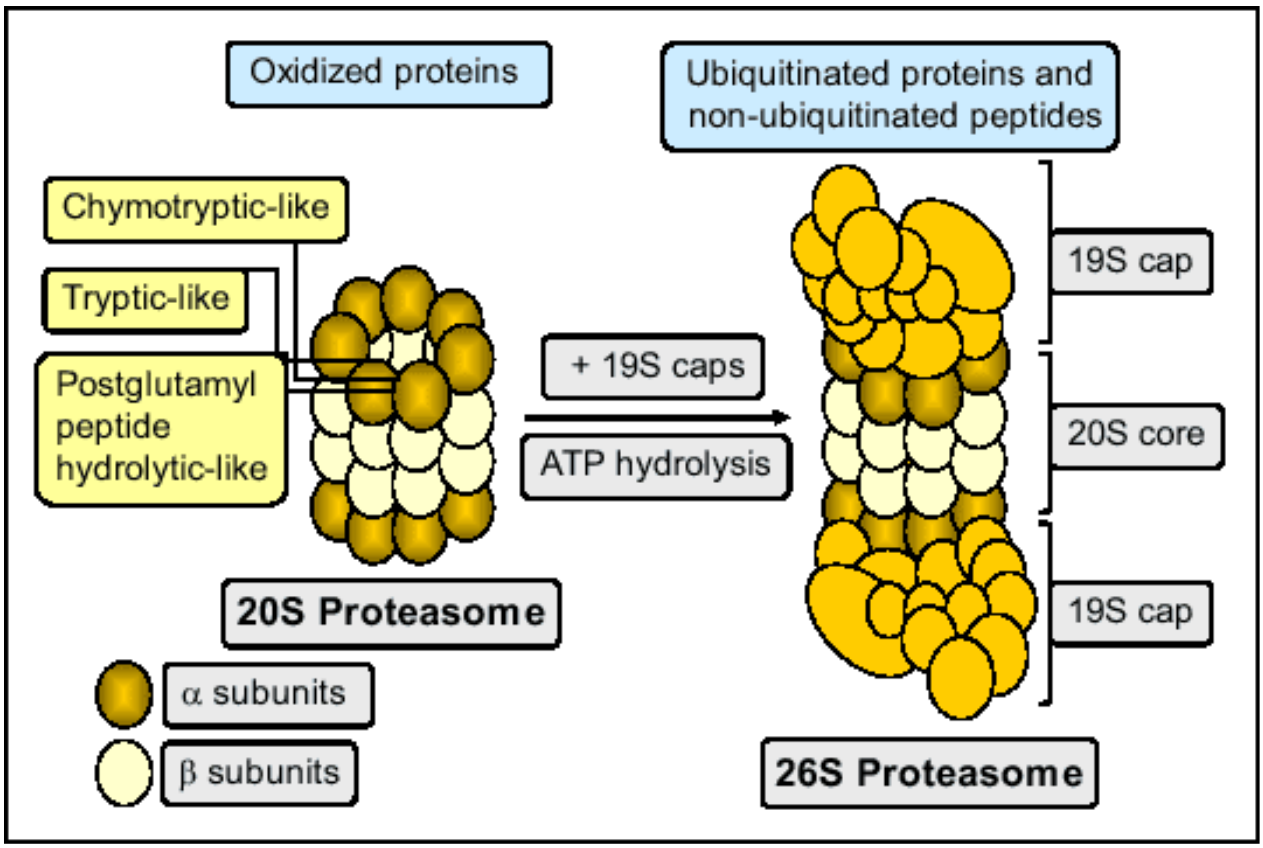


Figure 3.1 Schematic presentation of the 20S and the 26S proteasome. Proteasome peptidase activities, chymotryptic-like, tryptic-like and postglutamyl peptide hydrolytic-like are associated with ß5, ß2, and ß1 subunits of the 20S core. The 26 S proteasome consists of 20S core caped with two 19S regulatory caps under ATP hydrolysis. The 19S regulatory caps recognize ubiquitinated substrates. Substrates of the 20S proteasome are oxidized proteins. Ubiquitinated proteins and non-ubiquitinated peptides are substrates of the 26S proteasome. (277)

3.2 RESULTS

3.2.1 Basic FGF Suppression by Green Tea Catechins: Two out of four human colorectal cancer cell lines (HCT-116 and LoVo) expressed bFGF. LoVo cells highly expressed bFGF protein, whereas expression levels were marginal in HCT-116 cells. As shown in **Figure 3.2A**, bFGF protein was detected as two bands (18 and 24 kDa), and EGCG treatment reduced both bands. VEGF was expressed in all four cell lines and was also suppressed by 50 μM EGCG. LoVo cells having the highest expression of bFGF were selected for initial bFGF studies. The cells were treated with different doses of two green tea catechins, EGCG and ECG, in order to determine the effect of these compounds on the expression of bFGF. As shown in **Figure 3.2B,** left panel, RTPCR was carried out and there was no change in the expression of *bFGF* mRNA by 50 μM of EGCG or ECG. Next, we treated LoVo cells with different doses of EGCG and ECG. Both EGCG and ECG at 50 μM completely suppressed bFGF protein expression as shown in **Figure 3.2B, right panel**. Further, EGCG suppressed bFGF expression in LoVo cells in a time dependent manner, starting at 5 min (**Figure 3.2C**). To examine the effects of catechins on bFGF protein expression in HCT-116 cells, where bFGF levels were inherently lower, the ELISA assay was performed. As shown in **Figure 3.2D**, both ECG and EGCG at 50 μM also significantly suppressed intracellular bFGF ($p<0.05$). Finally, we examined non-colorectal cancer cells to see if EGCG had similar effects in other cancer cell types and found that bFGF expression was reduced in all but one (MCF-7, breast cancer) cell line as shown in **Figure 3.2E**.

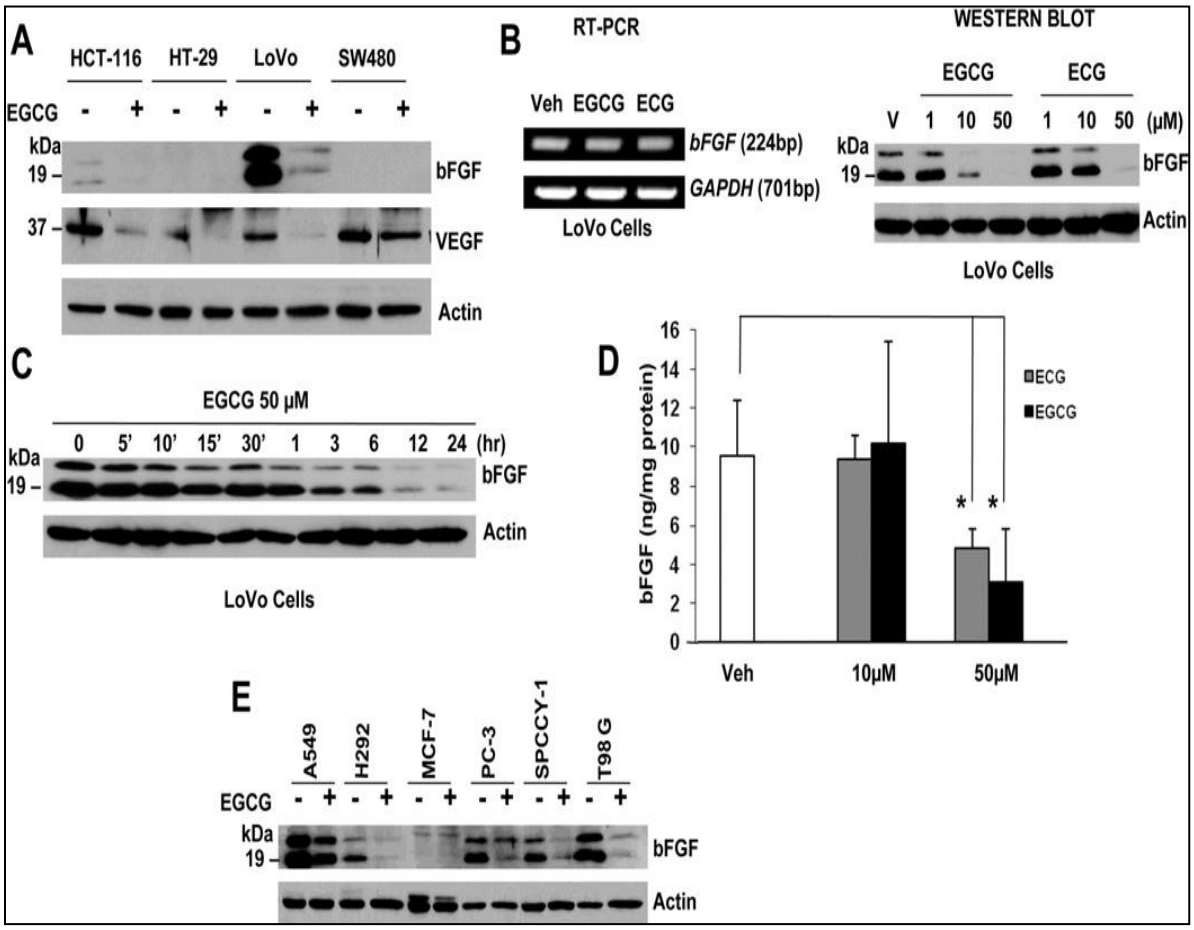


Figure 3.2. Green tea catechins suppress bFGF expression in different cell lines. (*A*) Western blot analysis of bFGF using 4 different colon cancer cell lines. The cells were treated with 50 μmol/L of EGCG for 24 hours. Equal amounts of total proteins (30 μg) were separated by SDS-PAGE and transferred to nitrocellulose membranes, and bands corresponding to bFGF were detected by enhanced chemiluminescence. (*B*) *Left panel:* RT-PCR for LoVo cells treated with 50 μM of ECG and EGCG. Number of cycles used was 29 for *bFGF* and 25 cycles for *GAPDH*. Reaction products were analyzed on 1.2% agarose gel. *Right panel:* Western blot analysis of bFGF using LoVo cell lysates treated with indicated doses of EGCG and ECG for 24 hours. (*C*) LoVo cells were treated with 50 μmol/L of EGCG, and samples were harvested at different time points. Cell lysates were then subjected to Western blot analysis for bFGF and Actin. (*D*) HCT-116 cells were treated with DMSO, ECG, or EGCG (10 and 50 μmol/L) for 24 hours. The bFGF levels were determined by ELISA and expressed as nanograms of bFGF per milligram of total cell protein content. Data represent the mean ± SD of 3 independent experiments. *$P < 0.05$, compared with control (*Veh*). (*E*) Western blot analysis of bFGF for 2 lung (A549 and H292), breast (MCF-7), prostate (PC-3), head and neck (SPCCY-1), and brain (T98G) cancer cell lines. Cells were treated with 50 μmol/L of EGCG for 24 hours and subjected to Western analysis.

3.2.2 Proteasome Inhibitor Restores the Suppression of bFGF by EGCG: To investigate whether the suppression of bFGF is related to oxidative stress after EGCG treatment in a cell culture system, LoVo cells were pre-treated with glutathione (GSH), a major intracellular anti-oxidant. As shown in **Figure 3.3A**, there is partial recovery of bFGF expression in the presence of EGCG and GSH, suggesting that EGCG-generated oxidative stress (278) may contribute to the suppression of bFGF, but that effects are largely unrelated to any pro-oxidative property of EGCG. The bFGF protein levels began to decrease as early as 5 min of EGCG treatment as seen in the time dependent experiment (**Figure 3.3C**). This pointed out to the strong possibility of either one single or multiple signaling cascade(s) being involved. Different important signaling pathways were considered like MAPK (either ERK, or JNK), NF-κB, PI3K etc and inhibitor study was carried out. Different inhibitors were used and as shown in **Figure 3.3B**, there was no restoration of bFGF expression in the presence of U0126 (ERK inhibitor), SP600125 (JNK inhibitor), MG132 (NF-κB inhibitor), or LY294002 (PI3K inhibitor). Other signaling pathway inhibitors used were for EGFR, PKC, GSK-3β, casein kinase II, mTOR, and p38MAPK, but none of these altered the expression of bFGF (data not shown). Interestingly, only 5 μM lactacystin reversed the suppression of bFGF by EGCG, suggesting that the proteasomal-dependent degradation pathway might play a role in EGCG-induced bFGF suppression. Lactacystin is a proteasomal inhibitor so finally other proteasome inhibitors, epoxomicin and AW9155 were used to see their effect, and it was found that they did not reverse EGCG-mediated bFGF suppression (**Figure 3.3B, right panel**). Proteasomal degradation is a type of degradation pathway for proteins. So we checked whether protein degradation of bFGF was involved in the EGCG-induced bFGF suppression. LoVo cells were pre-treated with DMSO (vehicle) or EGCG 50 μM (treatment) for 1 hr, followed by treatment with cycloheximide which

is a protein synthesis inhibitor (10 μM) for 1, 3, 6, 12, and 24 hr. As hypothesized, EGCG caused rapid degradation of bFGF protein in the presence of cycloheximide, first observed less than an hour after treatment (**Figure 3.3C, left panel**) in comparison to vehicle-treated samples. To confirm the efficacy of cycloheximide, other known proteins having protein stability affected were used like expression levels of cyclin D1 were checked and it was seen that it was degraded as early as 1 hr following treatment with cycloheximide in both EGCG-treated and untreated samples (**Figure 3.3C, left panel**), consistent with a previous report (279). Treatment with cycloheximide for longer periods of time (48 and 72 hr) showed similar results (**Figure 3.3C, right panel**), indicating that bFGF is very stable under cycloheximide treatment but is readily degradable in the presence of EGCG in LoVo cells. In contrast to bFGF, β-catenin protein was not degraded within 24 hr of EGCG treatment, but was slightly reduced with longer incubation periods (**Figure 3.3C, right panel**).

3.2.3 Mechanism of bFGF Protein Degradation by EGCG: Ubiquitination targets proteins involved in a large number of different biological processes for proteasome degradation (280). Once ubiquitin molecules bind on the substrate of interest, they are recognized by the 19S proteasome regulatory unit and degraded by the 26S proteasomal machinery into smaller peptides through the catalytic activities of 20S proteasome. Since Lactacystin, a proteasome inhibitor, was the only specific inhibitor we tested that could restore the suppression of bFGF by EGCG, we hypothesized that the ubiquitin-proteasome pathway plays an important role in suppression of bFGF by EGCG. Hence, ubiquitin-enriched samples of LoVo cell lysates were incubated with different doses of EGCG (1, 10, and 50 μM).

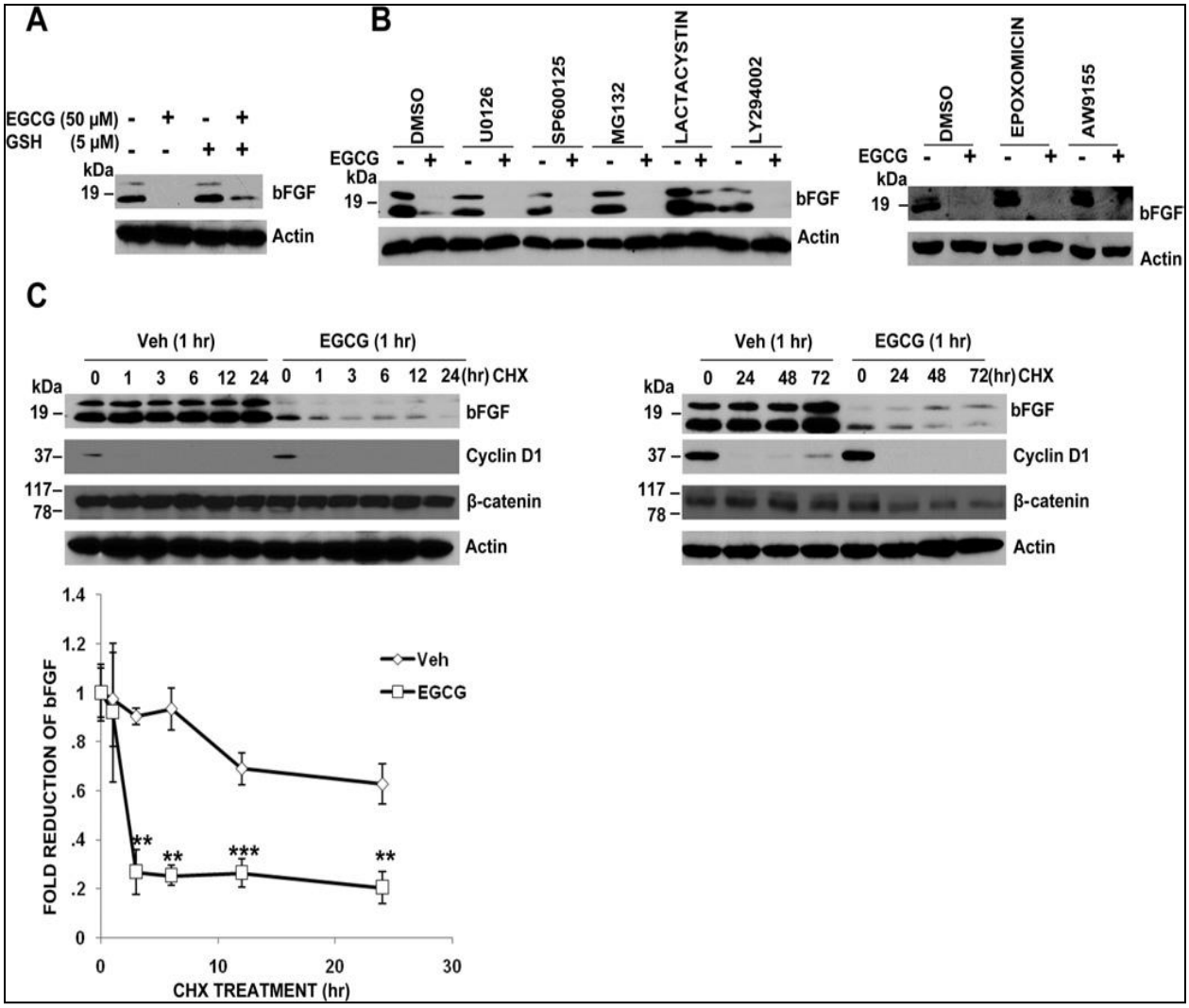


Figure 3.3. EGCG effects of signaling pathway inhibition on bFGF expression. (*A*) Prooxidative effect of EGCG. LoVo cell lysates were preincubated with GSH (5 μmol/L) for 1 hour then treated with EGCG (50 μmol/L) for 24 hours and subjected to Western analysis. (*B*) The cells were serum starved, and the following inhibitors were added 30 minutes before EGCG treatment: extracellular signal-regulated kinase inhibitor U0126 (6 μmol/L), c-Jun N-terminal kinase inhibitor SP600125 (50 μmol/L), nuclear factor-_B inhibitor MG132 (25 μmol/L), lactacystin (5 μmol/L), phosphoinositide-3 kinase inhibitor LY294002 (10 μmol/L), Epoxomicin (1 μmol/L), and AW9155 (1 μmol/L). The lysates were obtained after 24-hour treatment and subjected to immunoblot. An equal amount of loading proteins was estimated by actin probing. (*C*) Effect of EGCG on protein degradation of bFGF. LoVo cells were treated with either vehicle (*Veh*) or EGCG for 1 hour, followed by cycloheximide (10 μmol/L) treatment, and cell lysates were isolated at different time points. Immunoblot was carried out for bFGF, cyclin D1, β-catenin, and actin. The *bottom panel* shows a quantitative analysis of 3 independent experiments. Asterisks (and ***) were used to show *P* <0.01 and *P* <0 .001, respectively, using Student *t* test.**

When ubiquitin enriched samples were examined by Western blot with anti-ubiquitin antibody, the samples treated with 10 and 50 μM EGCG contained increased amounts of ubiquitination in ladder form seen in **Figure 3.4A**. Subsequently, when the same samples were subjected to Western blot using bFGF antibody, bFGF was suppressed in a dose dependent manner (**Figure 3.4A**). Next step was to examine the effect of EGCG on the 20S proteasome activity as described in *Materials and Methods*. The catalytic core of the 20S proteasome possesses three different proteolytic activities: chymotrypsin-like, trypsin-like and caspase-like (281) (**Figure 3.1**). As shown in **Figure 3.4B**, EGCG significantly decreased chymotrypsin-like and caspase-like activities, but increased the trypsin-like activity of 20S proteasome, as measured by a specific substrate. This result is consistent with previous data, showing that only lactacystin, containing property to inhibit trypsin-like activity of 20S proteasome, affects bFGF suppression by EGCG (**Figure 3.4B**).

3.2.4 Exogenous bFGF Proteins Responding to Cycloheximide: The expression vector containing the full-length of *bFGF* or *LacZ* (control) was cloned and transfected into the LoVo cells as described in *Materials and Methods*. The LoVo cells were allowed to grow for 24 hr and then subjected to cycloheximide treatment. The LacZ protein was not degraded in the presence of vehicle; however, it began to degrade 6 hr after EGCG treatment (**Figure 3.5A, top panel**). In contrast, there was drastic degradation of bFGF protein within 1 hr of EGCG treatment (**Figure 3.5A, bottom panel**), whereas bFGF levels in vehicle-treated samples only started to decrease between 1-6 hr after treatment, indicating that EGCG facilitates the degradation of recombinant bFGF protein. We also carried out the same experiment in HCT-116 cells and the results obtained were similar to those in LoVo cells (data not shown).

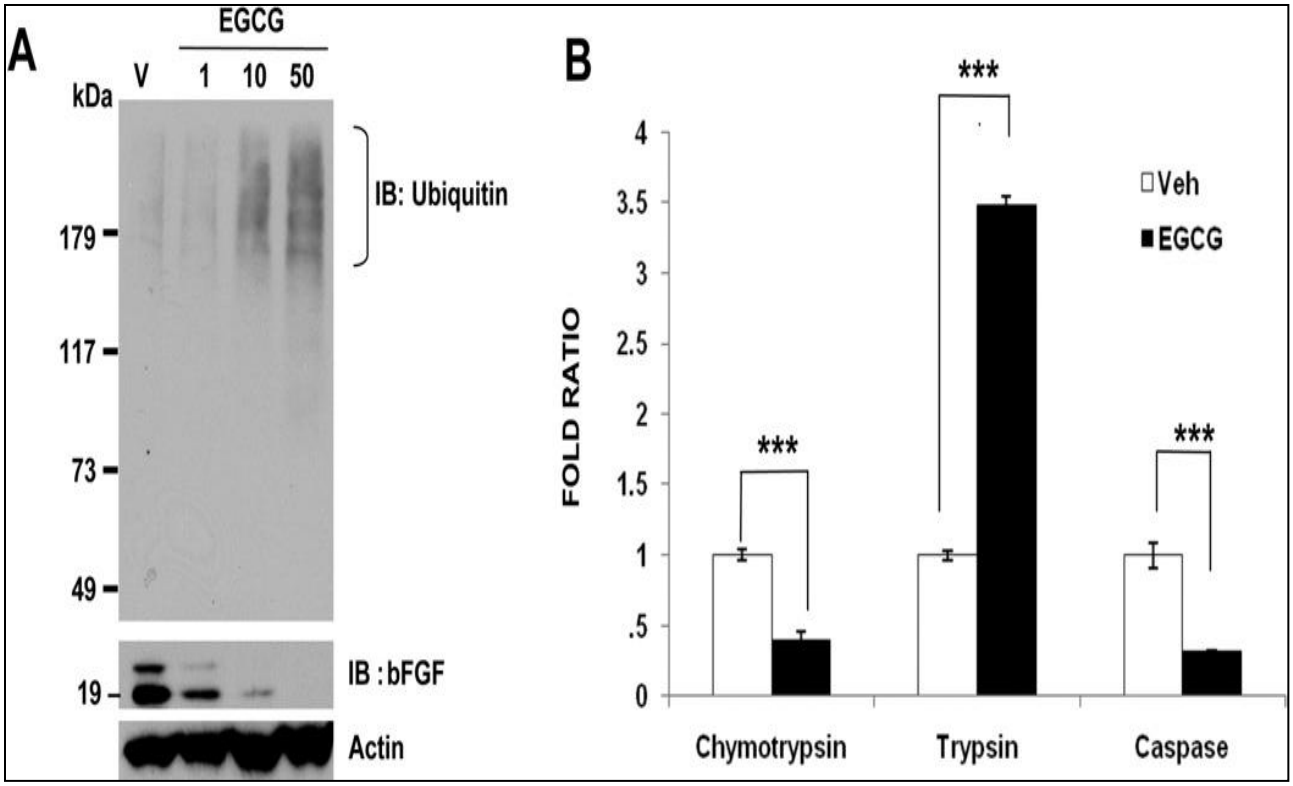


Figure 3.4. EGCG enhances ubiquitination and increases trypsin-like activity of the 20S proteasome. (*A*) Ubiquitinated enriched cell lysates treated with EGCG 1, 10, and 50 µmol/L were obtained using an ubiquitin enrichment kit and subjected to Western blot using an antiubiquitin antibody. The same cell lysates were also subjected to bFGF Western blot analysis, and data were normalized using actin. (*B*) 20S proteasome activity. LoVo cells were plated in triplicate and treated with EGCG (50 µmol/L) and vehicle (DMSO). The triplicate cell lysates were measured for chymotrypsin-like, trypsin-like, or caspase-like activity of the 20S proteasome. The luminescence was recorded on a microplate reader, and a graph was plotted using mean readings of 3 independent values. *Error bars* are SD. ***$P < 0$ .001. The y-axis was presented as the relative luminescence unit (RLU), as a vehicle-treated sample is 1.

To further elucidate whether bFGF has the ability to specifically interact with ubiquitin, we used histidine tag pull-down assays followed by Western blot analysis after transient transfections of LacZ (control) and bFGF expression vectors. Cell lysates were pulled down using nickel resin, and co-purified proteins were analyzed by immunoblot using specific antibodies for ubiquitin and V5. As shown in **Figure 3.5B**, V5-His-tagged bFGF and LacZ were efficiently pulled down by nickel resin, and the resin-purified bFGF complex contained strong ubiquitin-protein binding compared to LacZ. The purified proteins were confirmed by V5 Western blot, shown at the bottom of **Figure 3.5B**.

3.2.5 Effects of bFGF on Intestinal Tumorigenesis in APC$^{Min/+}$ Mice: Finally, we evaluated the effects of green tea catechins on tumor formation in APC multiple intestinal neoplasia (APC$^{Min/+}$) mice comparing three groups: untreated/ controls (Veh) and mice treated with either EGCG or ECG, both at a dose of 0.01% in their drinking water. The gross morphology of the small intestinal tumors revealed a striking reduction in the size and number of tumors in EGCG-treated APC$^{Min/+}$ mice, compared to the untreated group (**Figure 3.6A**). The EGCG-treated group also showed a statistically significant reduction in the total number of polyps and tumor load (p<0.05) (**Figure 3.6B and C**) compared to controls. Although ECG-treated mice did not show significant reductions, we saw a trend suggesting that ECG may also reduce polyp numbers. Adenomas were examined histologically in the small intestine (**Figure 3.6D, top**).

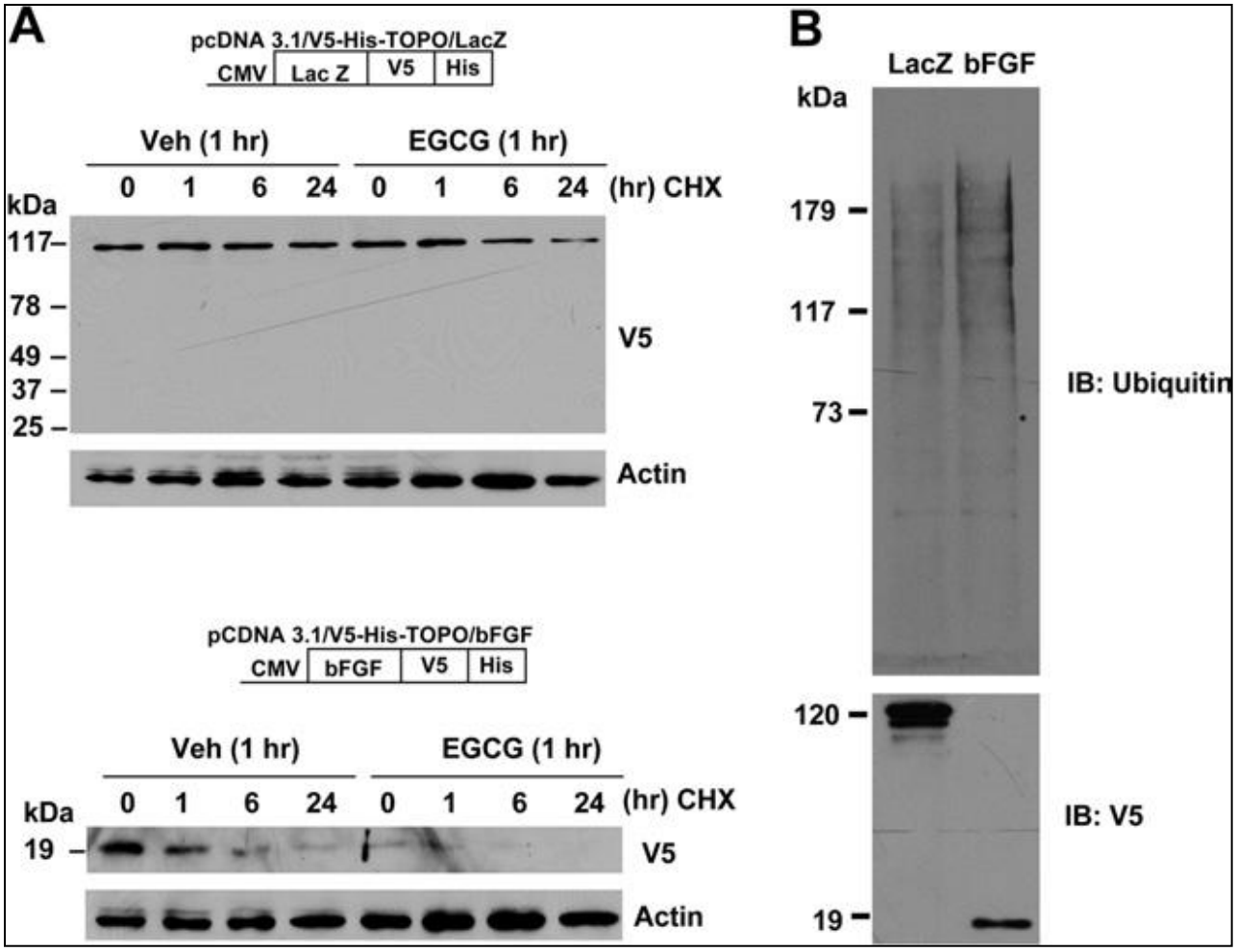


Figure 3.5. EGCG causes rapid degradation of bFGF. (*A*) LoVo cells were transfected with LacZ (*top panel*) and bFGF (*bottom panel*) expression vectors tagged with histidine and V5, using Lipofectamin 2000, and were allowed to stabilize overnight. They were subsequently treated with either vehicle (*Veh*) or EGCG (50 µmol/L) for 1 hour followed by cycloheximide (*CHX*, 10 µmol/L) treatment. The cell lysates were harvested after 0-, 1-, 6- and 24-hour treatment, and Western blot analysis was carried out for V5 and actin. (*B*) LoVo cells were transfected with LacZ and bFGF expression vectors using Lipofectamine 2000. The LacZ and bFGF (containing 6 histidine residues at their C-termini) were purified using ProBond nickel-chelating resin. The Western blot analysis was performed to measure ubiquitin and V5-Histagged recombinants from co purified proteins.

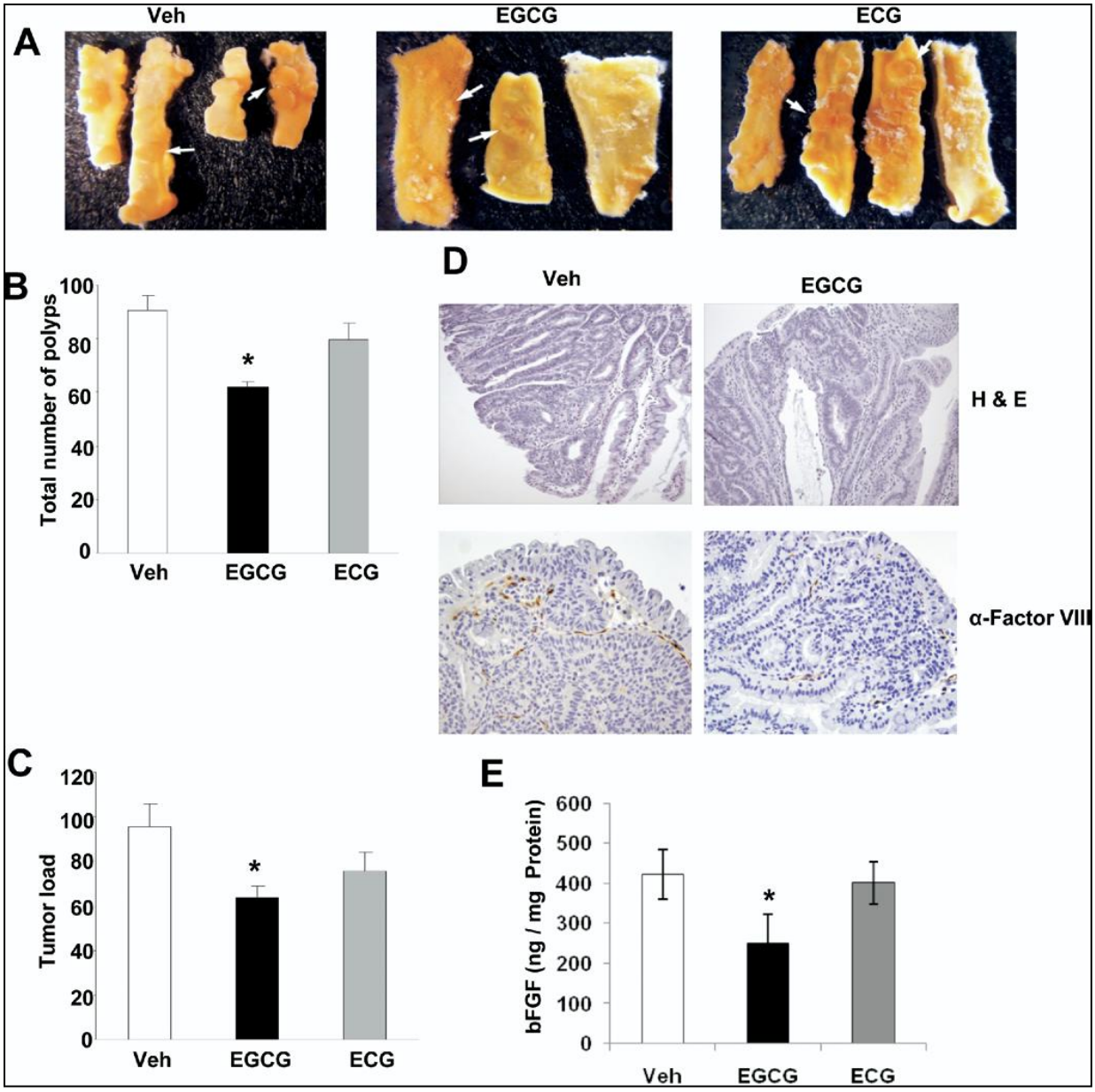


Figure 3.6. In vivo study. (*A*) Gross morphology of small intestinal tumors (*arrows*) from Veh-, EGCG-, and ECG-treated groups. (*B* and *C*) Effects of oral administration of EGCG or ECG (0.01%) on tumor number and tumor load (mean± SE) in comparison with Veh (n= 9/group). Tumor load was calculated by number of tumors ×average diameter. The *asterisk* denotes *P* value <0.05 using the Dunnett test to compare treated with control groups. (*D*) *Top: Histopathologic* representation of small intestinal tumors from control and EGCG-treated group (original magnification, ×100). *Bottom:* Immunohistochemical staining for Factor VIII (*brown*) on control and EGCG treated small intestinal tumors as an indication of vascular density and, therefore, angiogenesis (original magnification, ×200 magnification). (*E*) Mean levels of bFGF (nanogram/milligram total protein by ELISA assay) in lysates of small intestinal tumors from control (*Veh*) and *EGCG* and *ECG*-treated mice. An *asterisk* indicates a statistically significant difference compared with vehicle-treated sample. *P*<0.05 using the Dunnett test.

Factor VIII was used a marker for endothelial cells to evaluate angiogenesis (282) and bFGF is a pro-angiogenic factor in adenomas as well as adenocarcinomas (283). Immunostaining for Factor VIII on control and EGCG-treated small intestinal tumors suggested that endothelial-lined capillaries were more prevalent in the control group, compared to EGCG-treated tumors (**Figure 3.6D, bottom**). Finally, ELISA assay was performed using the small intestinal tissue samples from the respective three groups, and results showed significant suppression of bFGF by EGCG ($p<0.05$) compared to controls using the Dunnett's test (**Figure 3.6E**).

3.3 DISCUSSION

Green tea acts as an anti-cancer agent, especially in colon cancer. Although the molecular mechanism of this anti-cancer effect is still not entirely understood, most green tea effects are attributed to EGCG (284, 285). EGCG acts on several cellular and molecular targets in signal transduction pathways associated with cell death and cell survival (286). In addition to its chemo-preventive activity, EGCG is known to possess anti-angiogenic properties through inhibition of pro-angiogenic factors including VEGF and bFGF (287, 288). Our results show that EGCG suppresses the protein levels of bFGF and VEGF in colorectal cancer cells, which could account for reduced angiogenesis and hence hamper tumor growth and metastasis. The effect of EGCG on VEGF has been previously studied (289) so we investigated the molecular mechanism involved in the suppression of bFGF by EGCG. We found that bFGF was highly expressed in LoVo and HCT-116, human colorectal cancer cells and not in SW480 and HT29 colorectal cancer cells. This could be due the fact that VEGF is predominantly expressed in these cells or in the distinct the status of APC, B-catenin, P53, and MMR genes. The differences of genetic characteristic are shown in **Table 5.**

Table 5: Differences in the gene status in human colorectal cancer cells (290)

CELLS	P53	APC	β-CATENIN	MMR	COX-2	bFGF	VEGF
HCT-116	wild type	wild type	Mutant	deficient	absent	present	Present
HT-29	mutant	Mutant	wild type	proficient	present	absent	Present
LoVo	wild type	Mutant	wild type	deficient	present	present	Present
SW480	mutant	Mutant	wild type	proficient	absent	absent	Present

The examination of the transcriptional regulation of bFGF by EGCG indicated no changes in mRNA level (**Figure 3.2B, left panel**) or mRNA stability in the presence of EGCG in LoVo cells (data not shown). However the bFGF protein was reduced as early as 5 mins in a time course experiment after treatment with EGCG 50 μM. Thus, post-translational regulation of bFGF by EGCG may fully account for the EGCG-induced bFGF suppression. Also ELISA assay was performed in HCT-116 cell lysates. Although HCT-116 exhibited a very low expression of bFGF, ELISA was able to detect proteins in nanograms concentration. ELISA also showed that bFGF protein was decreased by both EGCG and ECG 50 μM (**Figure 3.2D**).

EGCG possesses a strong anti-oxidant activity (291) as well as pro-oxidant activity in cell culture systems, producing H_2O_2 in the media (292). Therefore, we investigated whether the pro-oxidant activity may cause bFGF suppression in our cell culture. Glutathione (GSH) is one of the major intracellular antioxidants. When GSH and EGCG are added to LoVo cells, bFGF protein is only partially recovered, which indicates that oxidation, presumably H_2O_2 generation by EGCG, contributes to EGCG-induced bFGF suppression but is not the only responsible factor (**Figure 3.3A**). To further elucidate the molecular effects of EGCG in signaling pathways, we tested for

involvement of different pathways using inhibitors. We found that lactacystin inhibited EGCG-induced bFGF suppression (**Figure 3.3B**). Lactacystin is a proteasomal inhibitor, which inhibits two of three proteolytic activities, chymotrypsin-like and trypsin-like. The suppression of bFGF by EGCG was not restored by other proteasomal inhibitors, MG132, Epoxomicin and AW9155 which are inhibitors of chymotrypsin-like and caspase-like activities. So this effect seemed to be specific for Lactacystin. When we carried out protein degradation assay using protein synthesis inhibitor, cycloheximide in presence and absence of EGCG, it was seen that 50 μM of EGCG causes rapid degradation of bFGF compared to controls pointing strongly towards the protein degradation pathway (**Figure 3.3C**). These results were also confirmed using the recombinant bFGF proteins (**Figure 3.5**). For proteins to be recognized and degraded by proteasome, most have to be linked to ubiquitin and hence we suspected that ubiquitination of bFGF might be occuring. This appears somewhat unique to bFGF, as EGCG had no affect on protein levels of LacZ or β-catenin, despite the fact that the latter is known to be regulated by ubiquitin-proteasomal degradation (293-295) (**Figure 3.3C, left panel**). One consideration is that LoVo cells contain a mutant APC tumor suppressor protein that would not bind to β-catenin and thereby mediate ubiquitination. However, after 24 hr, β-catenin seemed to be degraded, indicating that other pathways may be involved in β-catenin degradation in the presence of EGCG.

To check the effect of EGCG on ubiquitin, ubiquitin-enriched fractions were obtained from EGCG-treated samples, and we found that EGCG increased the ubiquitin activity in a dose-dependent manner (**Figure 3.4A**). Polyubiquitination is a chain of activated ubiquitin molecules ready to bind the substrate and hence it appears as a ladder when blotted on Western Blots (**Figure 3.4A**). When the same enriched samples were subjected to bFGF western blot, we saw a

dose dependent decrease indicating that EGCG increases ubiquitination and polyubiquitination of bFGF leads it towards the proteasome. This result was also confirmed by immunoprecipitation of bFGF and LacZ with ubiquitin and V5 antibodies as the vectors were tagged with V5 (**Figure 3.4B**). Ubiquitin is one of the most conserved eukaryotic proteins, and it conjugates other proteins through a well-defined enzymatic pathway (296). The 20S proteasome assay with specific substrates for the three catalytic activities showed an increase in trypsin-activity of 20S proteasome by EGCG. The ubiquitin-proteasome pathway is now being recognized as an important regulatory system in cancer pathways and, in fact, in many cellular processes (297). Our data support this mechanism of degradation, showing that EGCG facilitates ubiquitination of bFGF and specifically triggers trypsin-like activity of the 20S proteasome, leading to degradation of bFGF. In contrast, chymotrypsin-like activity of the proteasome is associated with tumor cell survival, and EGCG has been known to decrease chymotrypsin-like activity of the 20S proteasome (298). It is therefore surprising that EGCG increases only trypsin-like activity of the 20S proteasome in our experiments. However, our data strongly support the hypothesis that EGCG increase trypsin-like activity of 20S proteasome and in our experiments, proteasome inhibitors (Epoxomicin and AW9155) other than lactacystin did not show any effect on EGCG-induced bFGF suppression **(Figure 3.3B**). Another explanation of lactacystin specificity in EGCG-mediated bFGF degradation is that, unlike other proteasome inhibitors, lactacystin has reactive hydroxyl groups that might compete with EGCG for activation of 20S proteasomal activities. To our knowledge, this is the first report that EGCG specifically enhances trypsin-like activity of the 20S proteasome. Other proteins like BAD have been known to be targeted for proteasomal degradation by EGCG and future studies can be carried out to see whether EGCG

targets trypsin like activity of 20S proteasome in case of BAD protein degradation or if this mechanism is specific for bFGF.

An *in vivo* study using $APC^{Min/+}$ mice treated with EGCG or ECG indicated that intestinal tumor load and polyp numbers were significantly reduced only in the EGCG-treated mice. Although it is possible that the concentration and solubility of ECG reduced its effectiveness, EGCG was clearly shown to suppress bFGF to a greater extent (**Figure 3.6B**). EGCG-mediated reductions in bFGF levels in $APC^{Min/+}$ mouse tumors, which correlated with apparently fewer Factor VIII-labeled blood vessels in the tumors, suggesting an inhibitory effect on angiogenesis. The concentration of green tea catechins in human plasma has been reported to reach no higher than 1 μM even with consumption of large amounts of the beverage (174). However, higher levels are expected to be present in the lumen of the gastrointestinal tract. Although the 50 μM EGCG dose in cell culture reflects a higher range of plasma concentration, it is possible that this concentration can be reached in the intestinal tract. The exact minimum effective concentration remains to be determined, however, the bioavailability and degradation, as well as metabolic effects of catechins in the cell culture should be considered.

Our study raised many intriguing and important questions that require clarification in the future. In particular, the molecular feature of EGCG that serves as a signal trigger for ubiquitination, and the sites of ubiquitination of bFGF in the presence of EGCG should be examined. In addition, the specific enhancing activity of EGCG on trypsin-like activity of the 20S proteasome remains to be elucidated. Over expression of bFGF is very common in human colorectal cancer cells and in adenomas derived from mouse models, and these findings provide a molecular mechanism that details regulation of bFGF by EGCG. $APC^{Min/+}$ mice is a model for FAP. Other mouse models for colorectal cancer such as models with mutated MSH2, and MLH1

having adenomas and adenocarcinomas and Msh6 and Msh2 mouse developing microsatellite instability should be used in future to see the effect of EGCG on colorectal carcinogenesis.

Effect of EGCG on regulation of NUDT6 in human colorectal cancer cells

CHAPTER 4

Part of the results (Figure 4- 4.3D) are published in Journal of Nutritional Biochemistry, 2009 (*In Press*) titled "**A potential proliferative gene, *NUDT6,* is down-regulated by green tea catechins at the posttranscriptional level".**
Authors: Sukhthankar M, Choi CK, English A, Kim JS, and Baek SJ*

4.1 INTRODUCTION

Nudix (nucleoside diphosphate linked moiety X)-type motif 6 (NUDT6) also known as (Antisense basic fibroblast growth factor B, Nudix motif 6, GFG, ASGFG-2, and FGF-AS) is a member of nudix hydrolases superfamily found in all classes of organisms like bacteria, archaea, viruses and eukaryotes and consists mainly of pyrophosphohydrolases that act upon substrates of general structure nucleoside diphosphate linked to another moiety, X to yield NMP plus P-X (299). This gene caught our attention as it is an antisense product of the bidirectional transcribed gene locus, 4q26, whose sense product is *bFGF* or *FGF-2* which we have already shown to be suppressed by EGCG (300). This gene locus of 4q26 is a region of frequent gain or loss in human esophageal cancer (301). Zhang et al have shown that *FGF-AS* regulates the expression of *FGF-2* in mammalian cells and the increased risks for tumor recurrence and reduced survival after surgical resection of esophageal cancers with FGF-2 over expression was ameliorated in tumors co-expressing the endogenous *FGF* antisense RNA (302). The *FGF-AS* RNA is complementary, over a span of 464 nucleotides, to the 3' untranslated region (3'UTR) of the *FGF-2* transcripts, encompassing the proximal and distal polyadenylation sites (303). However it's been also shown that NUDT6 may have a role in modulating proliferation in response to growth signals through sense-antisense RNA interactions and, independently of FGF, via the protein itself (304).

In the view of the role of NUDT6 as both a regulator of bFGF and as cell proliferator, an understanding of the exact biological activity would provide important insights into the role of NUDT6 in colorectal carcinogenesis and to elucidate the mechanism by which green tea catechins modulate its function. In the present study, we decided to focus on effect of green tea catechins on NUDT6 and to characterize its biological function. We found that EGCG 50 μM

had stronger effect compared to ECG 50 µM in suppressing the *NUDT6* mRNA transcript. The cell proliferation assay, soft agar assay, caspase assay, and invasion assay pointed out towards NUDT6 being the novel cell proliferator blocking apoptosis.

The tumor suppressor p53 is vital in maintaining cellular genomic integrity and controlled cell growth (305-307). Loss or gain of p53 function results in the aberrant growth of cells. p53 protein has a very short half life and thus is usually present at extremely low levels within cells. In response to stress, DNA damaging agents, and chronic mitogenic stimulation, p53 is transiently stabilized and activated. Depending on cell type, cell environment and oncogenic alterations, p53 activation leads to inhibition of cell cycle progression, induction of senescence, differentiation, or apoptosis (41). Under stress, p53 can directly activate components of the apoptotic machinery by translocating to the mitochondria and leading to cytochrome C release, caspase activation, and apoptosis induction (308-310). Mitochondrial translocation of p53 precedes loss of mitochondrial membrane potential and caspase activation, suggesting that p53 may trigger mechanisms at the mitochondria that ultimately lead to induction of apoptosis (311).

4.2 RESULTS

4.2.1 Green tea catechins alter gene expression in human colorectal cancer cells: To elucidate the gene profiles affected by green tea catechins in human colorectal cancer cells, we performed microarray analysis using HCT-116 cells treated with epicatechin gallate (ECG). Among those genes altered by ECG, *NUDT6* was one of several down regulated genes (**Table 7**). We selected ten up regulated and ten down regulated genes, however we focused on the three up regulated and three down regulated genes *ATF3, NAG-1,* and *NR4A1* and *NUDT6, ID2, and*

ID1, which were already been studied in the lab or which were of particular interest respectively (**Table 6 and 7**).

HCT-116 cells were treated with ECG (50 µM) for 24 h and RNA was isolated; cDNA was made and subjected to RT-PCR. We found that ECG (50 µM) increased the expression of *ATF3, NAG-1* and *NR4A1* mRNA and decreased the expression of *NUDT6, ID2* and *ID1* mRNA (**Figure 4A, left panel**). The gel densitometry data from three independent experiments are shown in **Figure 4A, right panel**. Since we have recently reported that a major green tea catechin EGCG suppresses bFGF at the post translational level (212) and that the *NUDT6* gene is an antisense of *bFGF mRNA* (300), we decided to further investigate NUDT6 in this study. We carried out RT-PCR for *NUDT6* in other colorectal cancer cells including HT-29, SW480 and LoVo. As shown in **Figure 4B**, ECG down regulated NUDT6 in these other colorectal cancer cells. We further investigated the dose- and time-dependence. HCT-116 cells were treated with ECG (50 µM) for 0, 6, 12 and 24 h (**Figure 4C, top panel**) and ECG (10, 50 and 100 µM) for 24 h (**Figure 4C, bottom panel**). As shown **in Figure 4C**, *NUDT6* mRNA expression was suppressed at 6 h and at a dose of 10 µM, with almost complete suppression at 100 µM. Finally, we examined whether other catechins suppressed *NUDT6* by carrying out RT-PCR at a dose of 50 µM each. We found that of the major catechin of green tea, EGCG had a stronger effect on suppressing *NUDT6* mRNA expression, compared to other catechins (**Figure 4D**). Therefore, we decided to use EGCG for further experiments.

TABLE 6: Up regulated genes. Ten up regulated genes selected from Microarray analysis of human colorectal cancer cells, HCT-116 treated with green tea catechin, ECG 50µM.

GENE NAME	ACCESSION	FOLD CHANGE
GAD 2(Glutamate decarboxylase)	I_934177	16.73
ATF3(Activating transcription factor 3)	I_929428	9.59
CTGF(Connective tissue growth factor)	I_966895	6.68
CYR61(Cysteine -rich angiogenic inducer 61)	I_931923	5.55
LAMB3(Laminin beta 3)	I_939265	5.48
NAG-1/PLAB(Macrophage Inhibitory Cytokine 1)	I_966585	4.51
NR4A1/NUR-77(Nuclear Receptor Subfamily 4 Group A Member 1)	I_932430	4.38
PLAU(Urokinase Plasminogen activator)	I_1110216	4.13
BCAR3(Breast cancer anti-estrogen resistance 3)	I_936333	4.12
CNK(Cytokine inducible kinase)	I_964814	3.63

TABLE 7: Down regulated genes. Ten down regulated genes selected from Microarray analysis of human colorectal cancer cells, HCT-116 treated with green tea catechin, ECG 50µM.

ESR1(Estrogen receptor 1)	I_957799	-7.75
FGF23(Fibroblast growth factor 23)	I_945210	-6.43
CD22(CD22 Antigen)	I_966382	-6.39
FABP2(Fatty acid binding protein 2)	I_957744	-4.68
NUDT6 (Nudix Type Motif 6/Anti sense Basic Fibroblast Growth Factor)	I_958190	-4.44
ENC1 (Ectodermal neural cortex 1)	I_957125	-4.44
SOX 4(SRY box 4)	I_957058	-3.85
ID2(Member of Id helix-loop-helix family of protein)	I_943701	-3.80
ID1(Inhibitory of DNA binding 1)	I_960970	-3.47
ADD3(Adducin 3 gamma)	I_963028	-3.45

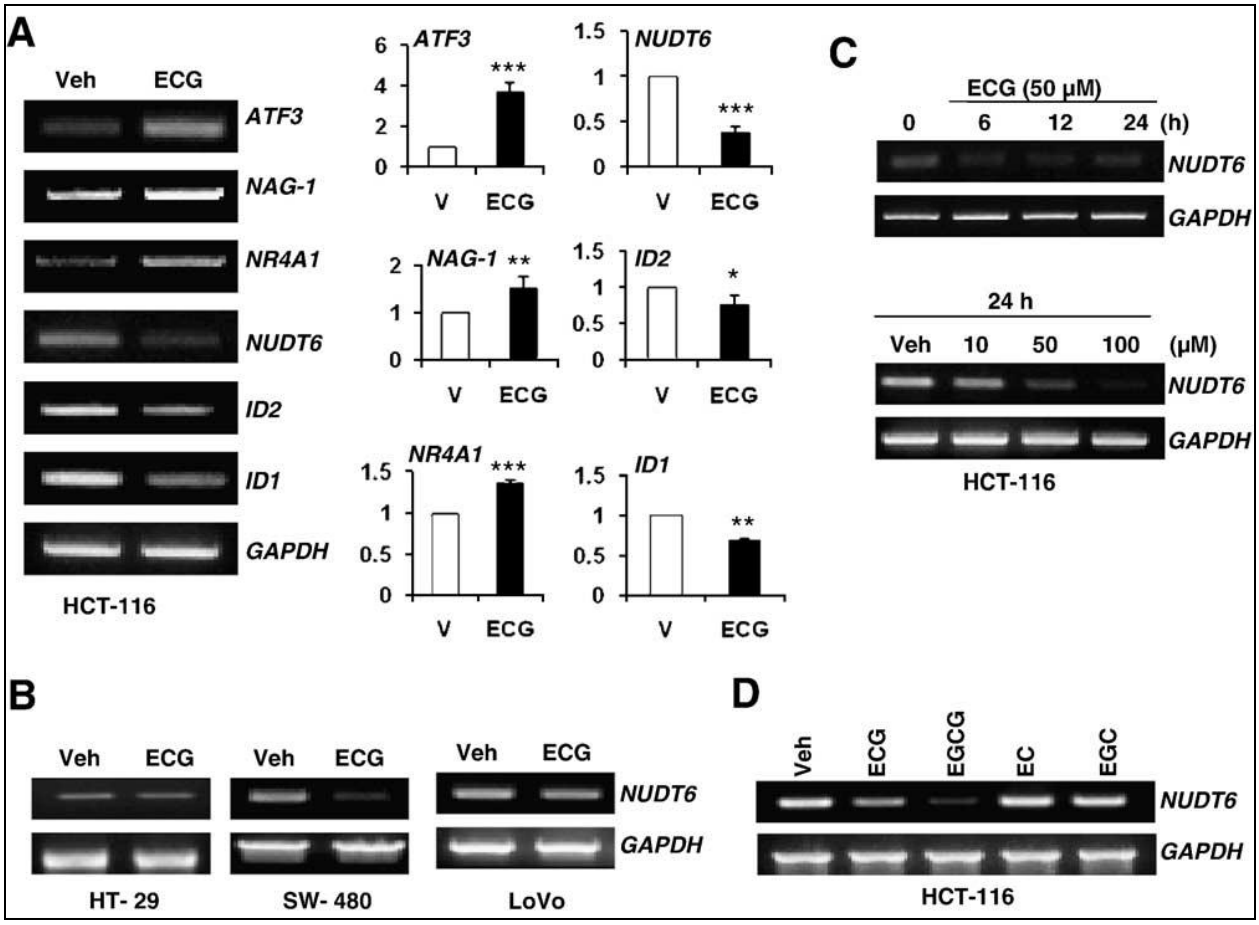


Fig. 4 NUDT6 is suppressed in the presence of catechins. (A) Left: HCT-116 cells were treated with 50 μM ECG for 24 h, and RT-PCR was performed for *NUDT6*. *ATF3*, activating transcription factor 3; *NAG-1*, NSAID-activated gene-1; *NR4A1*, nuclear receptor subfamily 4, group A, member 1; *ID2*, inhibitor of DNA binding 2; *ID1*, inhibitor of DNA binding 1. Right: Gel densitometry results from three independent experiments for the respective genes from the left panel. Significance was set at *P=.05, **P=.01 and ***P=.001. (B) Different human colorectal cancer cells were treated with ECG (50 μM) for 24 h, and *NUDT6* RNA transcripts were measured by RT-PCR. (C) HCT-116 cells were treated with ECG at different time points and at different doses. (D) HCT-116 cells were treated with different catechins (50 μM each), and *NUDT6* gene expression was determined by RT-PCR.

4.2.2 Posttranscriptional regulation of NUDT6 by EGCG: To gain insight into the mechanisms responsible for inhibition of *NUDT6* mRNA by the green tea catechin EGCG, we examined post-transcriptional regulation of *NUDT6*. Posttranscriptional control in eukaryotic gene expression can also provide a regulatory process by determining the abundance of a particular protein (312). To determine the effects of EGCG on *NUDT6* mRNA stability, the half-life of the mRNAs were first determined by adding EGCG (50 µM) to HCT-116 cells for 1 h and then treating them with actinomycin D at different time points. After treatment, mRNA was isolated at 0, 1, 2, 4 and 8 h and subjected to RT-PCR (**Figure 4.1A, top panel**). As a result, the half-life of *NUDT6* mRNA from vehicle and EGCG-treated cells was estimated to be around 7 and 2 h, respectively (**Figure 4.1A, bottom panel)**.

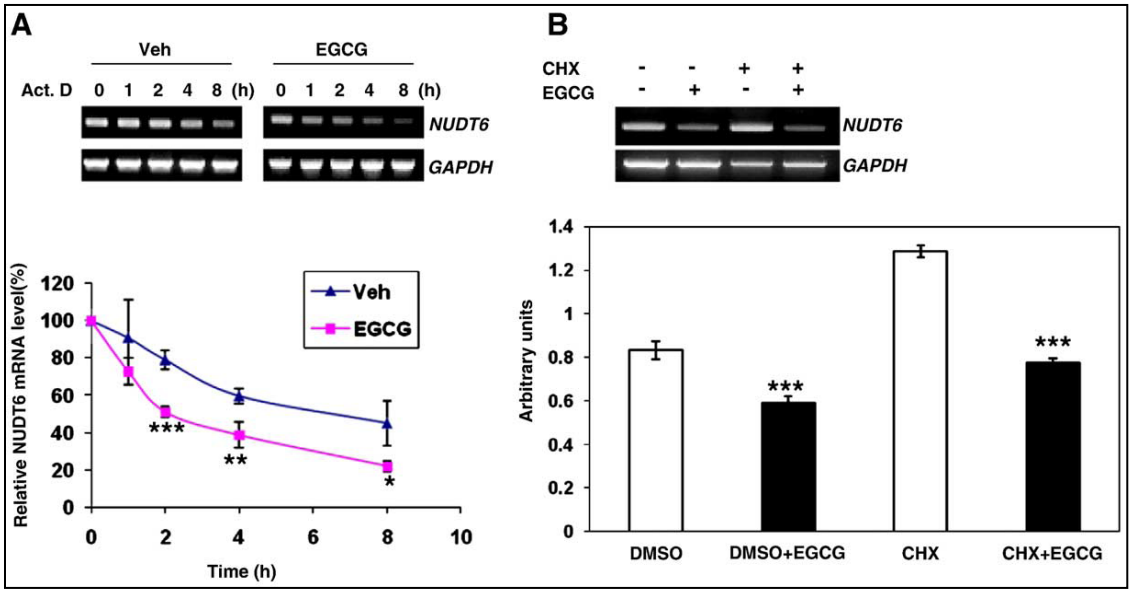


Figure 4.1. Posttranscriptional regulation of *NUDT6*. (A) EGCG facilitates *NUDT6* mRNA degradation. HCT-116 cells were incubated for 24 h with vehicle or EGCG at a concentration of 50 µM and subsequently treated with actinomycin D (5 µM). At the indicated times, total RNA was isolated, and *NUDT6* transcripts were measured by RT-PCR. The relative level of *NUDT6* mRNA was calculated, and the results were plotted as the percentage of the mRNA level present at Time 0 of actinomycin D treatment (bottom). (B) HCT-116 cells were pretreated with 10 µg/ml cycloheximide and DMSO for 1 h in serum-free media, followed by treatment with EGCG (50 µM) for 24 h. The data are representative of three independent experiments. ***P<0.001.

These results suggest that EGCG may affect *NUDT6* mRNA at the post-transcriptional level. Next, HCT-116 cells were pretreated with or without 10 µg/ ml cycloheximide and vehicle (DMSO) for 1 h, followed by treatment with EGCG (50 µM) (**Figure 4.1B, top panel**). The decreased mRNA expression of *NUDT6* was found to be insensitive to cycloheximide, suggesting that the mechanism of stabilization by EGCG is not involved in *de novo* protein synthesis (**Figure 4.1B, bottom panel**).

4.2.3. Involvement of the 3'UTR sequence of NUDT6 in mRNA stability and MAPK pathways: The stability of mRNA is determined in many cases by interactions between specific RNA-binding proteins and *cis*-acting sequences located in the 3'UTR of the mRNA (312). The AU-rich element (ARE), which targets mRNA for rapid degradation, is well known and characterized as a *cis*-acting sequence (313-315). We found one ARE consensus sequence and seven ARE-like sequences (316) in the 3'UTR of *NUDT6*. The 3'UTR of the *NUDT6* gene was cloned into a pGL3-promoter vector, downstream from the luciferase reporter gene (pSV40-LUC-*NUDT6* 3'UTR; **Figure 4.2A**). After transfection, the cells were treated with different doses of EGCG (10, 50 and 100 µM), and luciferase activity was measured. As shown in **Figure 4.2B**, the luciferase activity was decreased in a dose-dependent manner. This result clearly shows that the 3'UTR of *NUDT6* plays an important role in maintaining the stability of *NUDT6* and that EGCG affects the 3'UTR region in a dose-dependent manner.

We also checked the effect of different catechin treatments on the 3'UTR of *NUDT6* (**Figure 4.2C**), and the result was similar to RT-PCR data shown in **Figure 4D**. Although EC and EGC decreased the luciferase activity compared to vehicle, the effect was not statistically significant.

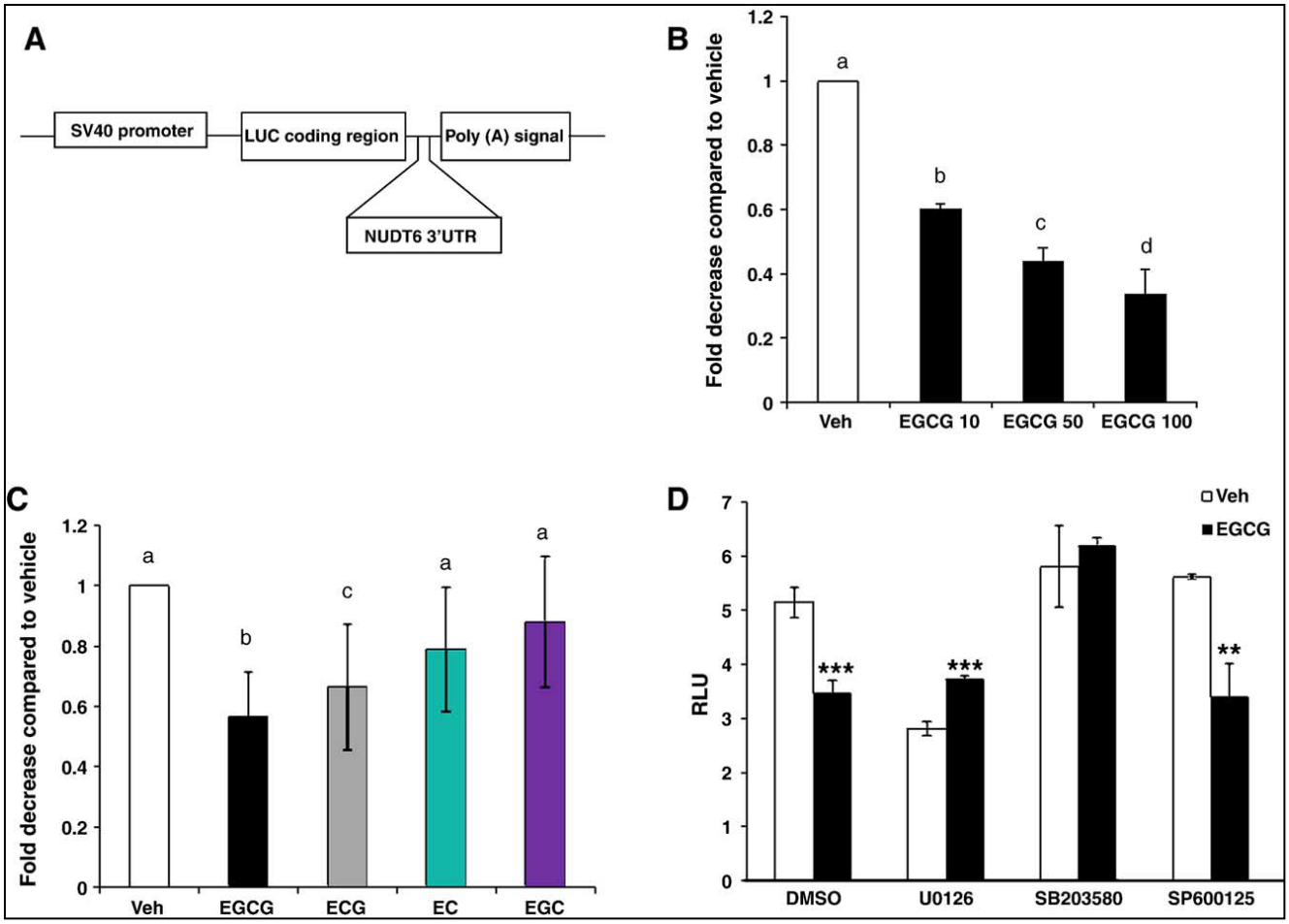


Figure 4.2. Involvement of 3′UTR sequences in EGCG-induced *NUDT6* mRNA suppression. (A) Schematic diagram of the pSV40-LUC-NUDT6 3′UTR construct. (B) The construct was transfected (0.5 µg) along with pRL-null vector (0.05 µg) into HCT-116 cells, and the cells were treated with different doses of EGCG (10, 50 and 100 µM). After 24-h treatment, luciferase activity was measured. All other values were normalized to the control (Veh) value set as 1.0. Tukey's multiple comparison test was used to express the results as the mean±SD of three independent transfections, and different letters indicate significant difference (Pb.05). (C) After transfection, HCT-116 cells were treated with four different catechins (EGCG, ECG, EC and EGC, at a dose of 50 µM), and luciferase activity was measured. The values obtained from vehicle-treated samples (Veh) were defined as 1.0, and data were analyzed using Tukey's multiple comparison test. (D) HCT-116 cells were plated in a 12-well plate; transfected with the construct mentioned in (A); pretreated with inhibitors for the ERK (U0126; 5 µM), p38MAPK (SB203580; 15 µM) and JNK pathways (SP600125; 30 µM) for 30 min; and then treated with EGCG (50 µM) for 24 h. The y-axis represents luciferase activity measured by relative luciferase unit (RLU). **P<0.01 and ***P<0.001, based on Student's t test. All experiments were performed in triplicate, with columns representing means and with bars representing S.D.

Next, we examined whether kinase pathways might be involved in EGCG-induced NUDT6 suppression. ERK, p38MAPK and JNK have been shown to be involved in ARE-driven mRNA turnover (317).

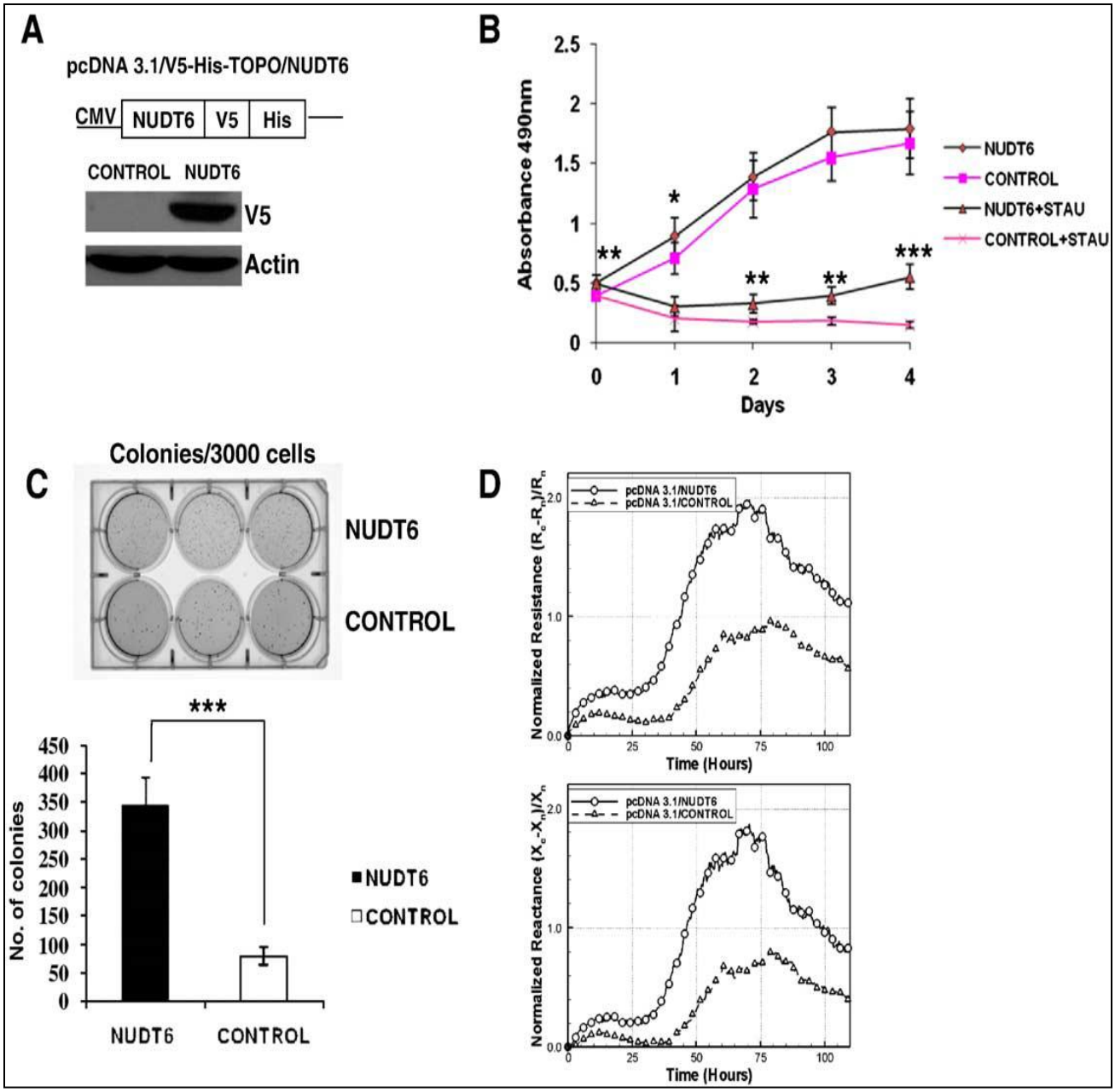
Thus, we examined the effects of MAPK pathway inhibitors on EGCG-affected *NUDT6* mRNA stability. HCT-116 cells were transfected with pSV40-LUC-*NUDT6* 3'UTR construct for 24 h, pretreated with U0126 (ERK inhibitor; 5 μM), SB203580 (p38 inhibitor; 15 μM) and SP600125 (JNK inhibitor; 30 μM) for 30 min and treated with EGCG (50 μM) for 24 h in serum-free media.

The treatment of U0126 and SB203580 with EGCG did not decrease luciferase activity compared to vehicle treated samples (**Figure 4.2D**), suggesting the potential role of ERK and, to some extent, p38MAPK in the post transcriptional regulation of *NUDT6* by EGCG.

4.2.4 Biological activity of NUDT6: Biological activity of NUDT6 has not been elucidated in detail, although there are some reports indicating that NUDT6 functions as a tumor suppressor and a tumor promoter (302, 304). Commercially available NUDT6 antibody did not work properly in Western analysis (data not shown). Therefore, we amplified the full-length *NUDT6* gene and were successful in getting the forward (NUDT6 correct orientation) and reverse (NUDT6 reverse orientation) clones of *NUDT6* (reverse clones did not have any bFGF activity, data not shown) in expression vector pCDN3.1/V5-His-TOPO (**Figure 4.3A**). We then made stable cell lines for NUDT6 in both forward and reverse orientations. The stable cell lines, pcDNA 3.1/NUDT6 and pcDNA 3.1/CONTROL were plated in 6-cm plates and were harvested and subjected to Western analysis. First, NUDT6 expression was confirmed using antibody against V5 tag (**Figure 4.3A**). Cell proliferation was carried out to determine whether NUDT6

expression affects cell growth. Staurosporine was also added to induce apoptosis, and absorbance was measured on days 0, 1, 2, 3 and 4. NUDT6-overexpressing cells increased cell proliferation in the presence of staurosporine significantly at 2, 3 and 4 days; however, a significant increase was also seen at 0 (0 day of staurosporine treatment, but 24 h after cells were plated) and 1 day without staurosporine (**Figure 4.3B**). The pro-tumorigenic activity of NUDT6 was evaluated by determining whether NUDT6 expression would affect cell growth in agarose because the ability to form colonies in soft agar is reflective of tumorigenesis (228). The NUDT6 stable cell line resulted in a dramatic increase of the clonogenic capacity of the cells (**Figure 4.3C**). To further confirm the biological activity of NUDT6, an electrical impedance assay was carried out, and the resistance and reactance were recorded as described in the Materials and Methods section. The pcDNA 3.1/NUDT6 cells showed increased resistance and reactance compared to pcDNA 3.1/CONTROL cells (**Figure 4.3D, top and bottom panel, respectively**). These data also suggest that NUDT6 may have oncogenic activity.

Zhang et al reported for the first time that NUDT6 had a mitochondrial targeting sequence in the N-terminal which is necessary and sufficient for mitochondrial localization (300). We wanted to confirm the localization of NUDT6 in the stable cell lines. The cytoplasmic and mitochondrial fractions were obtained using mitochondrial isolation kit and as shown in **Figure 4.3E**, NUDT6 is only expressed in mitochondria but not in the cytoplasm. To further confirm this result, we carried out immunostaining as mentioned in chapter 2 and NUDT6 was localized in the mitochondria whereas no expression of V5 was detected in CONTROL cells (**Figure 4.3F**). NUDT6 exhibited a role of a cell proliferator as seen in **Figures 4.3B, C, and D**. Further we wanted to check whether NUDT6 causes cell proliferation by blocking apoptosis.


A
pcDNA 3.1/V5-His-TOPO/NUDT6
CMV
NUDT6
V5
His
CONTROL
NUDT6
V5
Actin
B
Absorbance 490nm
Days
NUDT6
CONTROL
NUDT6+STAU
CONTROL+STAU
C
Colonies/3000 cells
NUDT6
CONTROL
No. of colonies
NUDT6
CONTROL
D
pcDNA 3.1/NUDT6
pcDNA 3.1/CONTROL
Normalized Resistance
Time (Hours)
Normalized Reactance
Time (Hours)

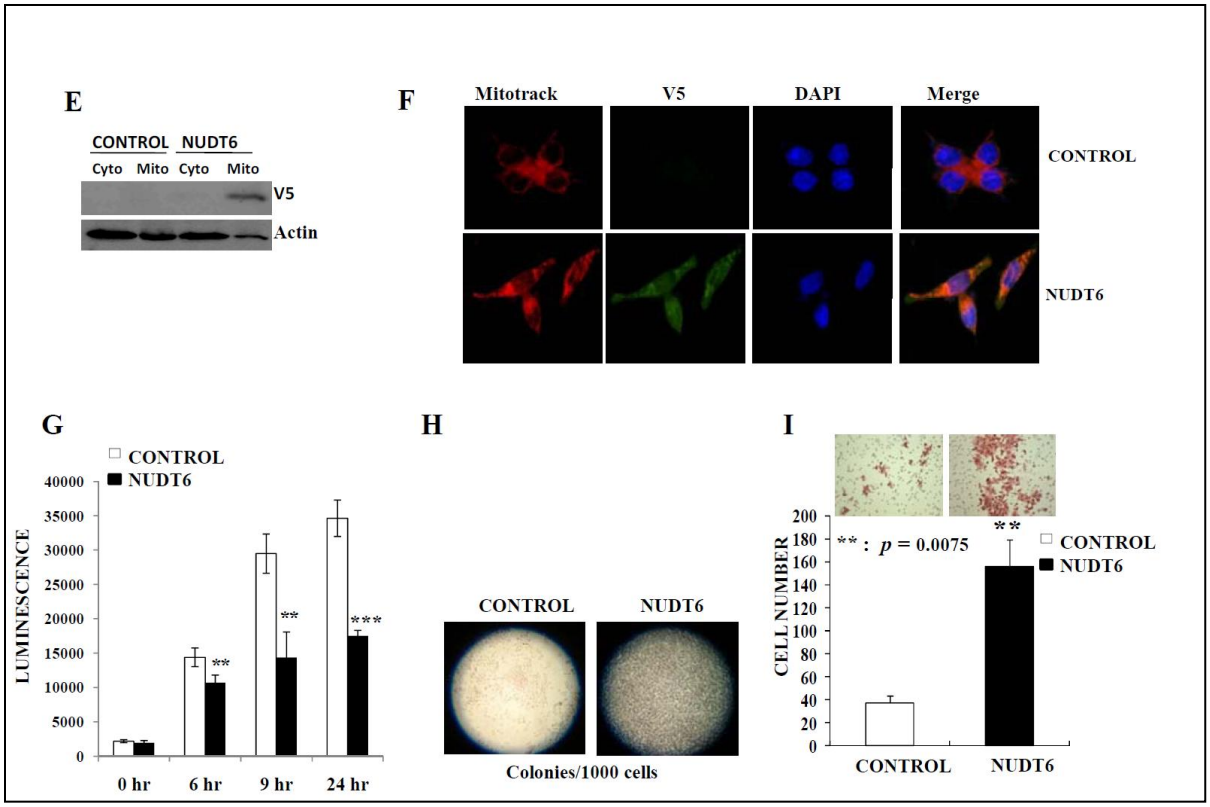


Figure 4.3. Characterization of the biological activity of NUDT6 in human colorectal cancer cells. (A) The stable cell lines pcDNA3.1/NUDT6 and pcDNA3.1/CONTROL were made as described in Materials and Methods. These clones contained tag proteins, V5 and histidine. Western blot analysis was carried out using anti-V5 antibody to confirm the integration of plasmid. Actin served as loading control. (B) CONTROL and NUDT6 cells were plated onto a 96-well plate (1 day before treatment), and either vehicle or 5 µM staurosporine was added at 0 day. Cell growth was measured using the CellTiter96 Aqueous One Solution Cell Proliferation Assay. Values are expressed as the mean±S.D. of six replicates. *P<.05, **P<0.01 and ***P<0.001 versus control cells. (C) The stable cell lines were grown for 2–3 weeks in 0.4% soft agar and stained with piodonitrotetrazolium violet solution. The results are representative of three different experiments. The data represent mean±SD. (D) Micro-impedance measurement of HCT-116 cells with stable cell lines. The corresponding normalized resistance (R) and reactance (X) were measured in HCT-116 cells over a 3.5-day period. The subscripts "c" and "n" indicate cell-covered and naked scans, respectively. For the sake of clarity, symbols represent 20 data intervals. The starting cell concentration used here was 2.4×104 cells/ml. (E) Localization of NUDT6 was studied by blotting mitochondrial fraction (mito) and cytoplasmic fraction (cyto) for V5. Actin was used as loading control. (F) The CONTROL and NUDT6 cells were stained with specific mitochondrial staining kit, Mitotrack and also with V5 and DAPI to confirm the mitochondrial localization of NUDT6. (G) The CONTROL and NUDT6 cells were treated with apoptotic inducer, staurosporine (5 µM) for 6, 9, and 24 h and caspase3/7 glo assay was used to detect luminescence. (H) Colony formation assay was carried out by plating 1000 cells/ plate of CONTROL and NUDT6 cell line and allowed to grow for around a week. (I) 1×10^6 cells were placed on matrigel and allowed to invade for 24 h

Caspases, or *c*ysteine-*asp*artic prote*ases*, are a family of cysteine proteases, which play essential roles in apoptosis (programmed cell death), necrosis and inflammation (318) among many other functions. We carried out caspase 3/7 glo assay as discussed in chapter 2 and found that NUDT6 blocks caspase activity in presence of apoptotic inducer, staurosporine used for 6, 9, and 24 h (**Figure 4.3G**). NUDT6 also increased the colony formation (**Figure 4.3H**).

Proteins involved in cell proliferation are frequently involved in cell invasion and metastasis (319). We wanted to check whether NUDT6 causes cell invasion. So CONTROL and NUDT6 cells were cultured in matrigel and allowed to grow and invade in the transwell chambers and as shown in **Figure 4.3I**, NUDT6 cells invaded the matrigel with much more efficacy compared to CONTROL cells. All these data point out towards a very potent cell proliferator role of NUDT6.

4.2.5 NUDT6 suppresses p53 expression: The tumor suppressor protein p53 mutation is associated with cell invasion and cell proliferation (320). Hence, we checked whether NUDT6 has any effect on p53 protein status and found that NUDT6 cell line had decreased p53 expression compared to CONTROL cell line shown in **Figure 4.4A, left panel**. NAG-1 is downstream target of p53 (321, 322) and its expression was decreased in NUDT6 cells. BCL2 family also includes anti-apoptotic agents like Bcl-2 (323, 324) which many a times, inhibit the activity of pro-apoptotic agents. We checked the expression level of BCL2 in NUDT6 stable cell lines and found it to be increased but there was no change in cyclin D1 in NUDT6 cells (**Figure 4.4A**). The p53 protein expression was reduced in NUDT6 cell line, so we wanted to check if *p53* gene transcription is affected by NUDT6. Hence we carried out RT-PCR but there was no change between the mRNA transcripts of *p53* in CONTROL or NUDT6 cell line as shown in **Figure 4.4A, right panel.** Once p53 is activated due to stress, it causes transcription initiation of

its downstream targets like MDM2, which in turn acts as E3 ligase and causes ubiquitination and degradation of p53 by proteasomal pathway (325, 326). We used MDM2 promoter having two p53 binding sites and transfected into the stable cell lines and as shown in **Figure 4.4B** as a reporter for activation of promoter by p53. NUDT6 cells showed decreased activity of p53 compared to CONTROL. We blocked NUDT6 using siRNA but could not restore p53 expression (data not shown). Since p53 is known to undergo protein degradation, we examined whether NUDT6 affects p53 at translational level. The use of puromycin, which is protein synthesis inhibitor, showed that NUDT6 causes degradation of p53 starting at around 1 to 3 h compared to degradation at 6 h in CONTROL cell line (**Figure 4.4C**). Since NUDT6 causes degradation of p53, we speculated that it binds to p53. Hence, we carried out immunoprecipitation. Pull down of V5-His tagged CONTROL and NUDT6 by Nickel column and immunoblotting with p53 showed that NUDT6 binds to p53. When p53 was pulled down in these two stable cell lines and immunoblotted with V5 and p53, binding between these two proteins was also confirmed as shown in **Figure 4.4D.**

4.2.6 NUDT6 increases tumor volume in vivo: Our in vitro experiments showed that NUDT6 is a novel cell proliferator, and it affects apoptosis perhaps through binding to p53, and causing its degradation. Therefore we wanted to see its effects *in vivo*. The xenograft model was used and the 10 male nude mice were given 3% sucrose water for drinking. Each mouse was injected with CONTROL cells on right flank and NUDT6 cells on left flank. The tumor volume was calculated as mentioned in chapter 2. The tumor volume was increased in NUDT6 injected cells compared to CONTROL cells (**Figure 4.5A**).

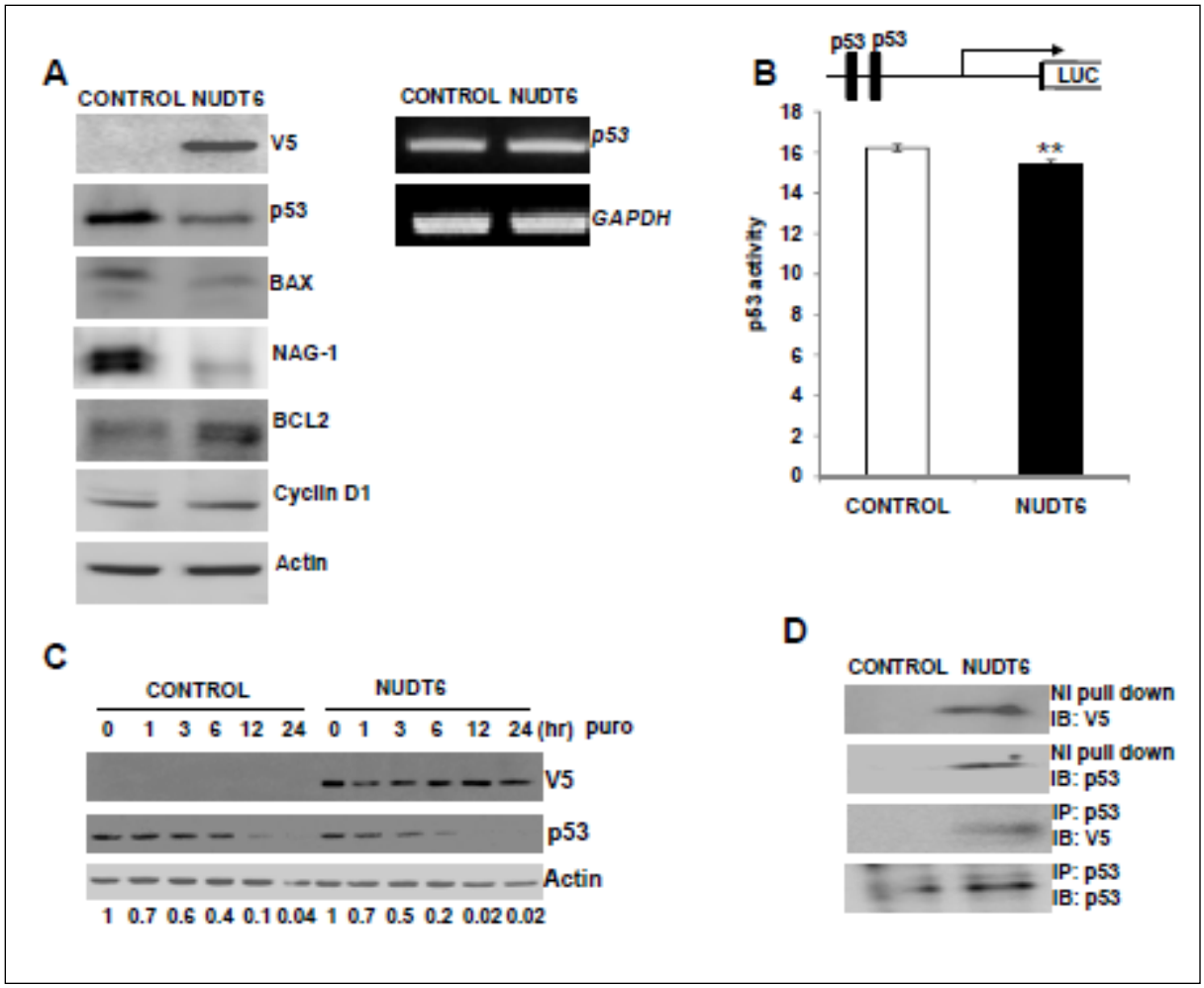


Figure 4.4 Involvement of p53. (A) Left panel: Western Blot analysis in CONTROL and NUDT6 cells for different proteins. Actin was used as a loading control. Right panel: RTPCR for *p53* and *NAG-1* in CONTROL and NUDT6 stable cells. *GAPDH* is used as a control. (B) MDM2 promoter containing two p53 binding sites transfected in CONTROL and NUDT6 cells and luciferase assay was measured. Experiment was carried out in triplicates and standard deviation was calculated. (C) The CONTROL and NUDT6 cells were grown till 60-80% confluent and were subsequently treated with puromycin (puro, 50 µmol/L) treatment. The cell lysates were harvested after 0-, 1-, 3-, 6-, 12- and 24-h treatment, and Western blot analysis was carried out for V5, p53 and actin. The numbers below the actin band indicate the fold decrease of p53 over Actin. The 0-h time point for both CONTROL and NUDT6 was adjusted to 1. (D) The CONTROL and NUDT6 stable cells were purified using ProBond nickel-chelating resin. The Western blot analysis was performed to measure V5-His tagged and p53. The proteins of CONROL and NUDT6 were pull down using p53 antibody, 5 µg were subjected to immunoblot with V5 and p53.

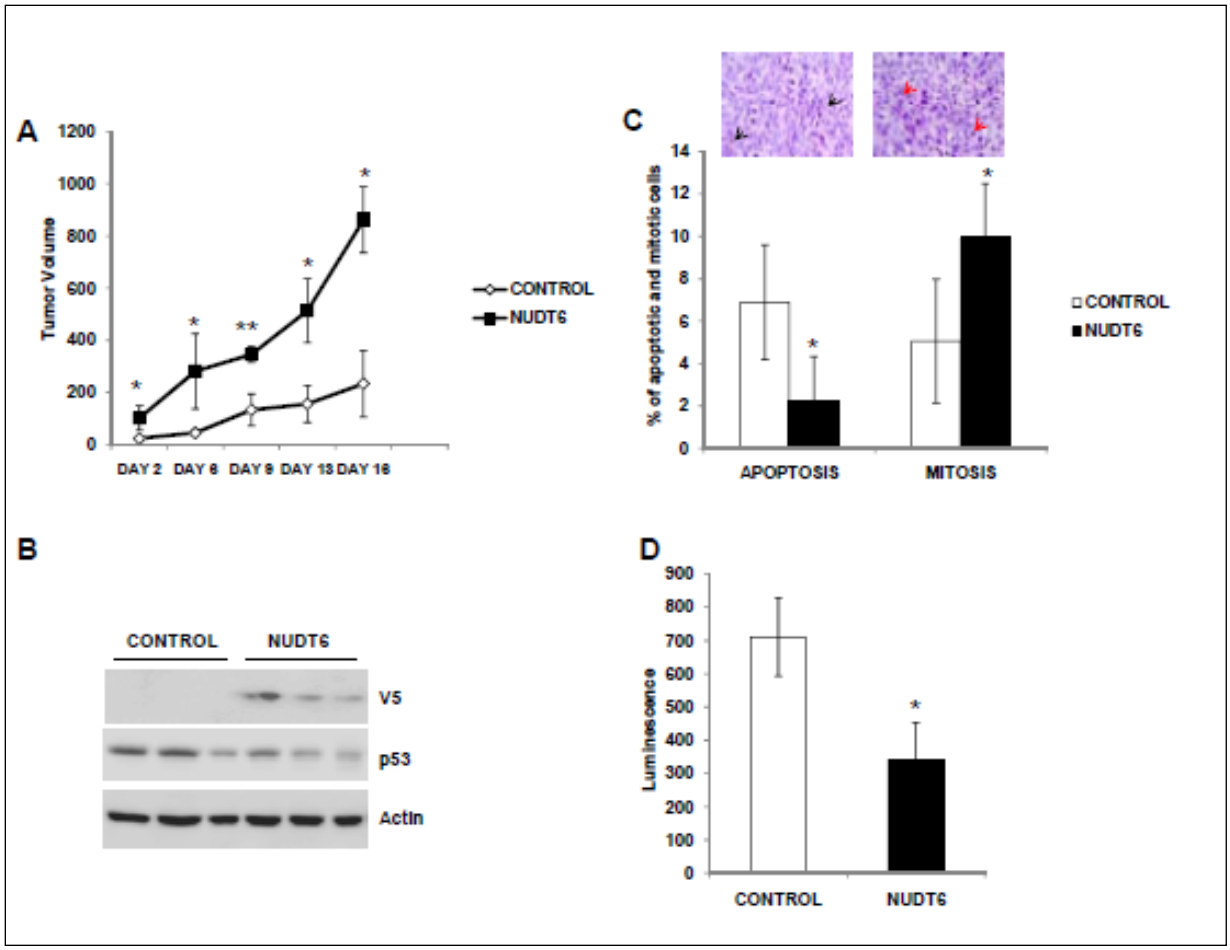


Figure 4.5. Xenograft study. (A) Comparison of tumor volume in NUDT6 injected mice tumors (mean± SD) in comparison with CONTROL. The *asterisk* denotes *P* value <0.05 using the Student t test to compare NUDT6 and CONTROL groups. (B) Western Blot using tumor tissue lysates from CONTROL and NUDT6 injected mice for V5, p53 and Actin. (C) H&E staining for CONTROL and NUDT6 tumors. Black arrows point towards apoptotic bodies, Red arrows point towards mitotic figures. The graph indicates the densitometry for % of apoptotic and mitotic cells amongst the CONTROL and NUDT6 injected mice. The one asterisk signs denote a *P* value of <0.05 using Student t test. (D) Caspase 3/7 Glo assay for tumor tissue lysates for CONTROL and NUDT6 tumors. Luminescence was measured. Samples were in triplicates and values show mean±SD. *P<0.05.

The tissue lysates made from the tumors of these mice were subjected to immunoblotting and V5 antibody could detect expression in NUDT6 cells (**Figure 4.5B**). The p53 expression was suppressed in NUDT6 cells compared to CONTROL cells (**Figure 4.5B**). In H&E staining in NUDT6 showed increased percentage of mitotic figures and decreased apoptotic figures compared to CONTROL (**Figure 4.5C**). Finally NUDT6 also decreased caspase activity in tissue lysates compared to CONTROL cells (**Figure 4.5D**). There was another group of nude mice which was given 0.1% EGCG in 3% sucrose drinking water to mask the bitter taste of EGCG (data not shown). The tumor volume was increased in NUDT6 injected mice after EGCG treatment. However the tumor volume in CONTROL injected cells was decreased by EGCG treatment. The percentage of mitotic cells was increased and caspase activity was decreased in NUDT6 injected mice treated with EGCG. The results were similar to vehicle group in NUDT6 injected mice. EGCG could not restore p53 expression in NUDT6 tumors.

4.2.7 Expression of NUDT6 in human tissues: Finally, we wanted to check the expression of NUDT6 in human colorectal normal and tumor tissues. We isolated RNA from the tissues and carried out RTPCR and found that NUDT6 is highly expressed in tumor tissues compared to normal as shown in **Figure 4.6A**.

The Figure 4.6B shows graphical comparison between normal and tumor human colorectal tissue. The β-microglobulin was used as house keeping control gene. NUDT6 expression is almost not seen in the normal tissue of human colorectal tissue samples.

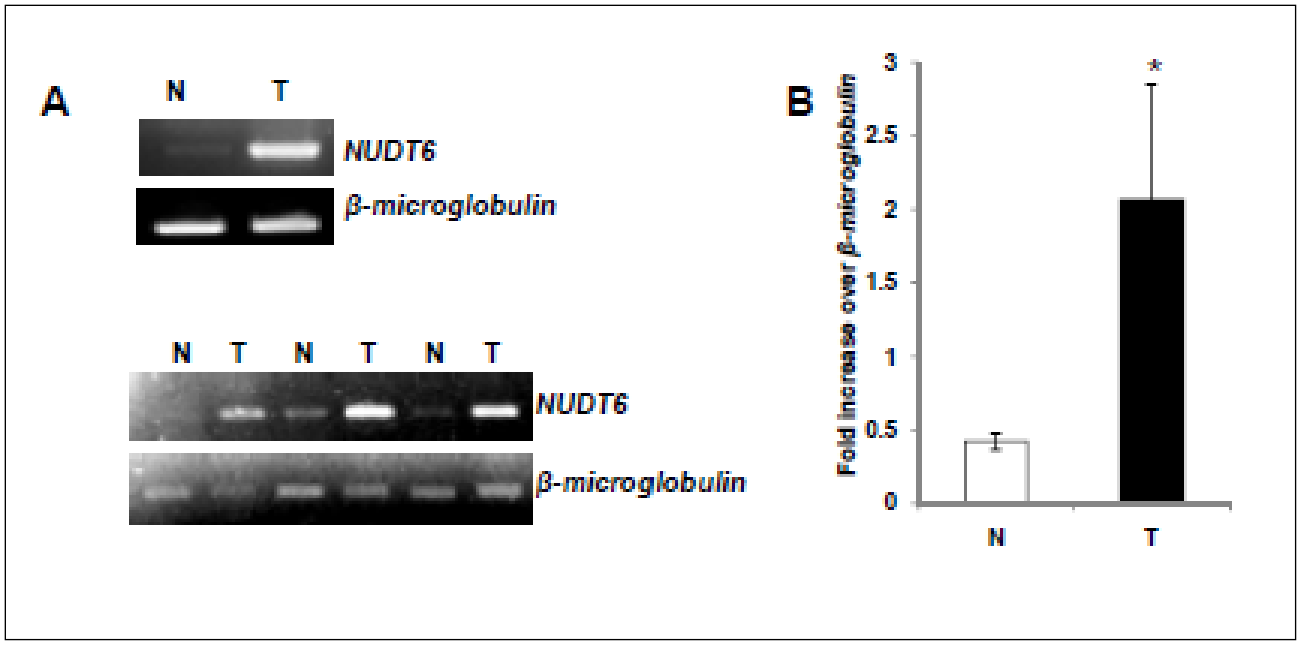


Figure 4.6. Human normal and tumor colon tissue samples. (A) RT-PCR for measuring *NUDT6* transcript in human colorectal normal (N) and tumor (T) tissue samples. *β-microglobulin* was used as control. (B) Shows a quantitative analysis of 3 independent experiments. Asterisks (*) was used to show *P* <0.05, using Student *t* test.

4.3 DISCUSSION

Dietary compounds have been known to have fewer side effects and play an important role in pathogenesis and prevention of cancer (327). Green tea catechins are among such dietary compounds, and they are well established as anti-cancer agents (328, 329). The green tea catechins are known to inhibit cell growth, angiogenesis, and metastasis and to promote apoptosis accompanied by alterations in gene expression of tumor suppressor proteins NAG-1 and EGR-1, and oncogenic proteins such as bFGF (212, 330, 331). In this chapter, we have provided a new mechanism by which EGCG/ECG acts as an anticancer agent. ECG 50 μM suppressed *NUDT6* transcript in four different human colorectal cells, HCT-116, HT-29, SW-480, and LoVo cells and it did so in dose and time dependent manner (**Figure 4A, B, C**). However EGCG showed a stronger suppression amongst four green tea catechins (**Figure 4D**). EGCG affected the post-transcriptional regulation of the cell proliferative gene *NUDT6* through ERK and p38MAPK pathways. Further we have also shown that NUDT6 may increase cell proliferative activity at least partly by binding to p53 and causing degradation of p53 protein ultimately leading to uncontrolled cell proliferation in vitro and increased tumor volume in vivo.

Regulation of gene expression by post-transcriptional modification of mRNA stability is an important mechanism used in the control of cell growth. Many but not all eukaryotic mRNAs are longer lived with average half lives of several hours for mammals (332). There are several factors regulating the rate of mRNA turnover (333-335) and one such factor is the intrinsic stability of the mRNA molecule, as expressed in the free-folding energies of its secondary and tertiary structures, which means the rate of mRNA degradation determines the extent and duration of gene expression (335). Due to this the gene expression may be regulated by a molecule in two ways: either as a positive regulator, in which mRNA stability is associated with

longer turnover rates of mRNA molecules within a cell and therefore increased translational rate, or, as a negative regulator, in which mRNA stability is associated with shorter turnover rates of mRNA molecules and therefore decreased translational rate of highly expressed genes (336, 337). The differences in decay rates in stable and unstable mRNAs are attributable to the presence or absence of specific sequence elements called AU rich elements (ARE). The sequence consensus for the ARE is loosely defined as a pentamer comprising AUUUA, repeated once or several times within the 3'UTR. It is often found within a U-rich region of the mRNA. However, recent work has suggested that a nonamer, UUAUUUA (U/A) (U/A), is more indicative of rapid destabilization (338). It is now becoming clear that it is the combination of functionally and structurally distinct sequence motifs, such as AU-pentamers, nonamers and U-rich stretches, which determines the ultimate stabilizing ability of each particular, ARE. The 3'UTR of mammalian mRNAs contain AREs, exhibiting the well characterized adenosine (A) + uridine (U)-rich elements, which regulate degradation of mRNAs and provide an effective way to control protein expression by regulation of mRNA half-life and translation (339). In particular, cellular events such as differentiation, proliferation, apoptosis, and inflammation are known to be related to modifications of the rate of RNA degradation mediated by the ARE motif (313, 339). Based on their sequences and their effects on mRNA stability, three classes of AREs have been defined (340). Class I AREs contain scattered copies of the AUUUA sequence within a U-rich region. Class II AREs contain overlapping AUUUA motifs within a U-rich region. Class III AREs contain U-rich sequences rather than the AUUUA motif. Emerging evidence suggests that there is another novel ARE that does not fit in any of the three classes. Examination of sequences revealed that the 3'UTR of *NUDT6* mRNA contains a highly conserved ARE (**Figure 4.2A**) and 7 ARE-like sequences, and these AREs should indicate the rapid degradation of mRNA. Indeed,

insertion of the 3'UTR of *NUDT6* into the reporter vector caused a dramatic reduction in luciferase activity in the presence of EGCG (**Figure 4.2B**). In addition, EGCG affected the mRNA stability of *NUDT6* and degraded the mRNA as quickly as 2 h compared to 7 h in vehicle (DMSO) (**Figure 4.1A, top and bottom panel**). There was however no involvement of any *de novo* protein synthesis as depicted in the cycloheximide study (**Figure 4.1B, top and bottom panel**), indicating an involvement of signaling pathways that do not require new protein synthesis.

The MAPK pathway could play a role in the post-transcriptional regulation of genes because a number of ARE-binding proteins require phosphorylation by MAPK for activation (341). We have shown that treatment with JNK inhibitor and EGCG did not affect luciferase activity of the pSV40-LUC-*NUDT6* 3'UTR construct **(Figure 4.2D**); however, luciferase activity was even increased and restored when U0126 and SB203580 were added, respectively. Interestingly, U0126 treatment with EGCG increased luciferase activity (**Figure 4.2D**), indicating that the ERK pathway may affect not only the 3'UTR of *NUDT6* but also SV40 promoter activity located in the promoter of this reporter vector. Luciferase activity was decreased in the presence of U0126, compared to the vehicle-treated samples, clearly suggesting that the ERK pathway is involved in stabilizing mRNA of *NUDT6.* Overall, our data suggest that EGCG modulates the ERK and p38MAPK pathways and leads to degradation of *NUDT6* mRNA. Further studies are required to determine the ERK and p38MAPK pathway-related ARE-binding proteins.

NUDT6 belongs to the Nudix hydrolase family and is hypothesized to have both pro- and anti-proliferative effects (299). There was a 4 to 5 fold decrease in the *NUDT6* gene expression in our microarray data treated with ECG. To confirm the microarray data, we selected three up regulated genes, *ATF3*, *NAG-1* and *NR4A1*, and three down regulated genes, *NUDT6*, *ID2* and

ID1. We previously reported that *NAG-1* and *ATF3* are induced by green tea catechins, and *NR4A1, ID1, ID2* are known to play a role in tumorigenesis (342-344). In contrast, the biological function of NUDT6 and its role in tumorigenesis have not been well elucidated (300). We generated stable cell lines with V5/His tags as shown in **Figure 4.3A**. The cell proliferation assay showed that the NUDT6 cells had an increased rate of cell growth compared to control at the early time points (**Figure 4.3B, 0 and 1 day**); however, effects on cell proliferation was not seen at the later time points (**Figure 4.3B, 2, 3, and 4 days**) when an apoptosis inducer, staurosporine, was added to the cells. This suggests that NUDT6 may contribute to the initiation of cell proliferation and that its expression makes cells less sensitive to the apoptosis inducer. Cell proliferative properties of NUDT6 are more evident by soft agar cloning and impedance assays. The NUDT6 stable cell line had more clonogenic properties compared to CONTROL cells, as shown in **Figure 4.3C**. The impedance technique has been applied to a number of biological studies that deal with cellular barrier function, attachment, spreading, and adhesion (345). In addition, frequency-dependent electrical impedance measurements have been used to evaluate the model parameters associated with cell-cell and cell-matrix junction formation (346). In the present study, micro-impedance measurements were used to quantitatively examine proliferation and morphological changes such as cell-cell adhesion and cell-substrate adhesion under NUDT6 expression. The electrical impedance assay confirmed that NUDT6 expression resulted in cell proliferation with enhanced cell motility (**Figure 4.3D top and bottom panels**). Caspases are essential in cells for apoptosis, or programmed cell death, in development and most other stages of adult life, and have been termed "executioner" proteins for their roles in the cell. To further complement the role of NUDT6 as cell proliferator, we checked whether NUDT6 could block caspase activity in the presence of apoptotic inducer staurosporine, confirmed that

NUDT6 can indeed cause cell proliferation by blocking apoptosis in dividing cells (**Figure 4.3G**). NUDT6 also caused increase in colony formation (**Figure 4.3H**). The two defining characteristics of cancer cells are uncontrolled cell proliferation and aggressive cell invasion (347). NUDT6 cells showed more cell invasion activity as shown in **Figure 4.3I**, stating that it can not only initiate primary tumor formation but also allow cancer cells to possibly metastasize to secondary sites.

Zhang et al showed that NUDT6 has a mitochondrial peptide sequence and hence is located in mitochondria (300). We found similar results with the stable cell lines which showed NUDT6 expression only in the mitochondrial fraction. Mitotrack is a cell permeable chemical stain containing thiol-reactive chloromethyl moiety for labeling mitochondria. Mitotrack passively diffuses through plasma membrane and stains active mitochondria. Mitotrack staining was seen co localizing with NUDT6 stain when merged. However there was no expression of either V5 or mitotrack in CONTROL (**Figure 4.3E and F**).

The roles of p53 as a tumor suppressor have been extensively studied mostly for its functions in the regulation of cell cycle progression and apoptosis. Accumulating data in recent years have strongly implicated a new role for p53 in the remodeling of the actin-based cytoskeleton, playing a role in cell invasion (348, 349). In response to genotoxic stress, including DNA damage, hypoxia, and activated oncogenes, p53 rapidly translocates to the mitochondria (311, 350-352). We hypothesized that there might be some relationship between NUDT6 over expression and p53 where in under stress, p53 moves to mitochondria, where mitochondrial protein, NUDT6 binds it and targets it for degradation. Our Western Blot data showed that p53 expression was decreased in the NUDT6 over expressing cell line (**Figure 4.4A, left panel**). Post translational modification of p53 is very common mechanism (353). The downstream targets of p53, BAX

and NAG-1 were also decreased and the anti-apoptotic protein BCL2 was increased which could be due the disturbance in balance of pro-apoptotic and anti-apoptotic proteins (**Figure 4.4A**). NUDT6 did not have any effect on the transcription of *p53* gene indicating the significant changes occurring at translational or post-translational level (**Figure 4.4A, right panel**). The half life of p53 protein is around 20minutes, however when compared between CONTROL and NUDT6, NUDT6 caused degradation of p53 protein at an earlier time point in the presence of protein synthesis inhibitor puromycin (**Figure 4.4C**) suggesting NUDT6 could have a potential role in degradation of p53 acting as E3 ligase like MDM2 (354) but further detailed studies are required. Ubiquitination targets proteins involved in a large number of different biological processes for proteasome degradation (355) so we checked if ubiquitination of p53 occurs in NUDT6 cell line but there was no ubiquitination seen suggesting degradation of p53 by NUDT6 might follow an ubiquitin-independent pathway (data not shown). It will be worthwhile to look at ubiquitin independent pathway.

The in vivo experiments complemented the in vitro experiments showing NUDT6 is a cell proliferation promoter and causes increase in tumor volume (**Figure 4.5A**). Western Blot analysis showed decreased p53 expression in tumor lysates in vehicle treated groups (**Figure 4.5B**). NUDT6 decreased the caspase activity in tissue lysates similar to the in vitro experiment (**Figure 4.5D**). The mitotic bodies were more in NUDT6 tumors as shown in **Figure 4.5C** indicating the NUDT6 tumor cells undergo more proliferation compared to CONTROL. NUDT6 also decreased the apoptotic bodies complementing the caspase activity and in vitro studies and is an anti-apoptotic agent. The results in EGCG treated nude mice injected with NUDT6 were similar to vehicle.

There could be several explanations; 1) the 0.1% EGCG dose was not enough to have an effect, 2) nude mice are immunodeficient mice and EGCG might require an active immune system to exhibit the beneficial effects, 3) EGCG leads to p53-dependent apoptosis, or 4) NUDT6 desensitizes the effect of EGCG. Multidrug resistance is very common problem in successful chemotherapy (356).

NUDT6 increased the mRNA transcripts of multidrug resistance genes, *ABCC1* and *ABCB1* (data not shown). Further studies are required to gain deep insight into the mechanism by which NUDT6 blocked EGCG effects.

NUDT6 expression was also more common in human colorectal tumor samples compared to normal colon samples indicating increased NUDT6 expression in tumors (**Figure 4.6A and B**). All these data show that NUDT6 is a novel and potent tumor proliferator, exhibiting its effect perhaps by binding p53 in mitochondria and causing its degradation (may be in cytoplasm after translocation), decreasing pro-apoptotic effects of EGCG. Hence the potential significance of NUDT6 as a biomarker for colon carcinogenesis needs to be established in future studies.

Conclusion: EGCG is a chemopreventive agent

CHAPTER 5

Colorectal cancer is a third leading cause of cancer related deaths in the Western World. It has a natural history of transition from adenoma to adenocarcinoma that spans, on average, 15-20 years, providing a window of opportunity for effective interventions and prevention (357). The chemotherapy regimen used against colorectal cancer is stressful because it has undesirable side effects that can diminish the quality of life. Also recent data have suggested that non-steroidal anti-inflammatory agents like aspirin, celecoxib, and sulindac sulfide, all have the potential to reduce both adenomas and colorectal adenocarcinomas (358). However such agents have issues of safety and therapeutic indices for example, aspirin shows effects only at a weekly total dose of 1000 mg or greater and has serious side effects like cerebrovascular and gastrointestinal adverse effects associated with long-term use (359, 360). Many dietary compounds like green tea, resveratrol, genistein, apigenin, exhibit beneficial effects to health including prevention of diseases like cancer. The ultimate goal today is prevention of cancer. Green tea is considered one of the most promising dietary agents for the prevention and possibly early treatment of many cancers including colorectal cancer worldwide. The green tea catechin, EGCG, possesses cancer–preventive, antioxidant, anti-mutagenic, apoptotic, and anti-inflammatory properties. Our group and others demonstrated the beneficial properties of EGCG in colorectal cancer using different molecular targets like induction of proapoptotic agents like NAG-1, EGR-1 and ATF3 (208). EGCG was found to inhibit growth and activation of the epidermal growth factor receptor and human epidermal growth factor receptor-2 signaling pathways in human colon cancer cells (213). EGCG also caused inhibition of inflammation (214), translocation of different genes (215), cell cycle regulation (216), inhibiting activation of IGF-1 (217) and many more functions. In this thesis, two new molecular targets of EGCG, bFGF and NUDT6, have been described.

The pro-angiogenic factor, bFGF causes angiogenesis leading to growth and nourishment of tumors and eventually metastasis. Suppression of bFGF would deprive tumor of nourishment. My data shows that EGCG inhibits bFGF expression at the post-translational level by activating ubiquitin proteasomal degradation which is a novel mechanism of EGCG action and a first report showing involvement of ubiquitin proteasome pathway. EGCG increased ubiquitination of bFGF as well as the trypsin like activity of 20S proteasome. The polyubiquitinated bFGF was recognized by 20S proteasome and increased trypsin-like catalytic activity caused rapid degradation of bFGF protein probably into peptides. EGCG also reduced adenoma formation in $APC^{Min/+}$ mice and bFGF downregulation by EGCG plays at least a partial role in EGCG-induced anti-tumorigenesis activity. EGCG is a potent anti-angiogenic agent as well as a chemopreventive dietary agent inhibiting growth and development of polyps. In the future it will be interesting to see whether EGCG targets other pro-angiogenic agents through ubiquitin proteasomal pathway or if the effect is specific for bFGF. $APC^{Min/+}$ mice are a model for FAP in humans and have intestinal adenomas but seldom colonic adenomas; therefore other colorectal cancer models like mouse models with mismatch repair gene mutations which have colonic adenomas should be explored for EGCG effects on colorectal cancer.

NUDT6 is the anti-sense gene of *bFGF*. The biological activity of NUDT6 is not known. However there are contradicting reports, one report says NUDT6 regulates bFGF activity (300) whereas other report states that NUDT6 causes cell proliferation (361). My work characterizes the biological activity of NUDT6 as a novel potential cell proliferation factor for the first time. I was successful in making stable cell lines for NUDT6 and CONTROL as there are no antibodies available for it and stable cells are very useful especially in characterization of biological activities. For a gene to be characterized as an oncogene, four properties are required: 1) It

should be able to cause cell proliferation, 2) it should evade apoptosis, 3) it should be able to increase invasion of cells, and 4) should be able to induce growth of tumors in xenograft models. The data suggested that NUDT6 increased cell proliferation, increased number of colonies in soft agar, increased electrical resistance and reactance between cell to cell and cell to substrate suggesting cells are tightly bound and frequently resistant to treatment and decreased caspase activity in presence of an apoptotic inducer. NUDT6 made cells resistant to induction of apoptosis. Increased invasion potential was seen in NUDT6 cells compared to CONTROL and using in vivo study we found that NUDT6 over expression cells formed tumors which showed an increased volume compared to CONTROL cells when injected into nude mice. NUDT6 is highly expressed in human colorectal tumors and less or no expression is seen in paired normal human colorectal tissue.

NUDT6 is a mitochondrial protein. I have also found that NUDT6 can bind p53 protein and this binding may lead to p53 degradation. Whether the binding occurs in mitochondria with p53 translocation to the cytoplasm for degradation is yet to be determined. It will also be very interesting to see whether NUDT6 can degrade p53 through the proteasomal pathway. However p53 is not ubiquitinated in presence of NUDT6. There is still a possibility that the degradation occurs in an ubiquitin independent manner. In the future, studies can also be carried out to elucidate in detail the mechanisms of degradation of p53 by NUDT6.

EGCG suppressed *NUDT6* at a post-transcriptional level probably through ERK and p38 MAPK pathways. EGCG reduced the half life of *NUDT6* mRNA by targeting the AU-rich elements in its 3’ UTR causing rapid degradation. However EGCG could not suppress NUDT6 expression in vivo and NUDT6 increased tumor volume and decreased caspase activity in EGCG treated nude mice. The failure of EGCG action in controlling tumors in NUDT6 over expression cells injected

mice could be due to desensitization of EGCG by NUDT6. I also found that NUDT6 increases the mRNA transcript of multidrug resistant genes, *ABCC1* and *ABCB1* (data not shown). Multidrug resistance is the biggest problem of chemotherapy failure (362). Future studies elucidating the role of NUDT6 in multidrug resistance can lead to finding of a novel marker in colon carcinogenesis. If it is proved that NUDT6 is indeed causing cancer cells to become resistant to EGCG, it can be used as a marker to determine the prognosis of cancer. Screening the effects of chemotherapeutic drugs like 5-FU, cisplatin etc in NUDT6 expressing cells is another promising future study. New arrays can be designed to detect NUDT6 expression in cancerous tissues.

Another aspect is deciding the most relevant and effective concentration of dietary agents, which is always a problem for a number of reasons. First, adding a compound directly to a cell culture will deliver a much higher local dose than occurs in the body following ingestion. Second, some if not all of the compounds may undergo metabolism *in vivo* to other more or less active derivatives and such metabolism may not be replicated in culture. Third, higher *in vivo* doses than those so far reported may be achievable by administration of a pure compound rather than as a dietary source, or an optimized formulation of a pure compound. Fourth, some target tissues may receive a higher (or more prolonged) dose than the reported peak levels in plasma. Fifth, some target tissues, such as skin, oral cavity, gastrointestinal tract, and bladder, may receive higher doses because they are not dependent on circulating levels. The colon, for example, can be exposed to significant amounts of (unabsorbed) material in the lumen. Finally, where the diet is concerned, any one compound may be poorly bioavailable, but with dozens, even hundreds, of active molecules being ingested together, the cumulative dose of similar acting compounds may be significantly higher. It should not be forgotten though; some natural products have exhibited

toxicity *in vivo* when given in high doses, so there is also an argument for the use of higher concentrations *in vitro* to indicate the full range of activity of these molecules. However the only toxicity observed with green tea is insomnia due to caffeine and it mostly occurs in people sensitive to caffeine. But decaffeinated green tea is also available.

Since EGCG beneficial health effects are being increasingly proved, it seems to be even more advisable to encourage the regular consumption of green tea as a widely available, palatable and inexpensive beverage. Taking all this into account, it would be advisable to consider the regular consumption of green tea in Western diets. Drinking four to five cups of freshly brewed green tea at a temperature below boiling point (140 to 185 ^{0} F), keeping the green tea solution in mouth for one minute, increases salivary concentration of catechins and helps exposing the human body to beneficial effects of green tea.

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VITA

Mugdha Girish Sukhthankar was born in Mumbai, India on November 8th, 1980. Her father's job made her travel to different States of India and she became fluent in different Indian languages. Due to her mother's chronic ulcerative colitis problem, she moved back to Mumbai where she earned her B.V.Sc (DVM) from Bombay Veterinary College. She was ranked second all over the State. After graduation, she totally dedicated her life to treatment and welfare of animals in the clinics of the Bai Sakarbai Dinshaw Petit Hospital for Animals. She was an active member of IDA (In defence of animals), PETA (People for Ethical Treatment of Animals) and BSPCA (The Bombay Society for the Prevention of Cruelty to Animals).

Mugdha joined the Graduate School of Veterinary Medicine at the University of Tennessee, Knoxville in August 2005 to pursue her NIH funded doctorate in Comparative and Experimental Medicine. She focused on dietary compound, green tea catechins and its molecular targets in prevention of colorectal carcinogenesis. During the course of her studies, Mugdha received two International Conference travel awards from The American Society for Biochemistry and Molecular Biology (ASBMB) and The American Institute for Cancer Research (AICR) where she presented her work. She was also successful in getting the University of Tennessee, Knoxville Research Symposium Award for best presentation for all three years since the Symposium was introduced. She was also able to achieve a strong publication record with her first paper being published in Gastroenterology journal with an impact factor of 12.5. Later together with first author and co-author publications, she has total of 6 papers. During this period, she got married to her childhood best friend, Bhupesh in December 2008.

Following the completion of her PhD in Comparative and Experimental Medicine in December 2009, Mugdha is starting as a postdoctoral research associate in St.Jude's Children Research

Hospital in Memphis, Tennessee. Her research project will focus on the ABC transporters playing a key role in Cancer.