Effects of 5-fluorouracil Drug Treatment on the Expression Profile of MicroRNAs in MCF7 Breast Cancer Cells

by

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Abstract

Breast cancer is one of the leading causes of deaths in women worldwide. 5-flourouracil (5-FU) is a classic chemotherapeutic drug that has been widely used in the treatment of breast cancer patients. In this study, using several biochemical techniques, we studied the global effects of 5-FU treatment on MCF7 breast cancer cells. The dose-response curve obtained after the treatment of MCF7 cells with 23 different 5-FU concentrations for 48 hours showed an atypical bimodal or biphasic curve, thus indicating a plausible dual mechanism of action for 5-FU. After 48 hours of treatment with 5-FU, the cells were found to be apoptotic, with a distinct reduction in the cell size, compromised anchorage ability but no significant alteration in the cell cycle progression. These findings provided evidence of the global inhibitory effects of 5-FU on human breast cancer cells *in vitro* and warranted further evaluation to study the molecular basis of aberrant expression of protein-coding genes previously reported after 5-FU treatment. We hypothesized that microRNAs (miRNAs), the newly identified class of small regulatory RNAs, might play a mediator role in inducing the cytotoxicity of 5-FU, by regulating the expression of

its target genes. Using a combined advanced microarray and quantitative real time PCR (qRT-PCR) technology, we found for the first time that 5-FU significantly altered the global expression profile of miRNAs in MCF7 breast cancer cells. After 48 hours of treatment with a low dose (0.01μM), 42 miRNAs were differentially expressed in MCF7 cells (23 up-regulated, 19 down-regulated). A majority of these miRNAs have been previously associated with cancer development, and were predicted to potentially target many oncogenes and tumor suppressor genes. To further understand the connection between miRNA dysregulation and 5-FU therapy, we investigated the dose- and time-dependent modification in the miRNA expression levels after 5-FU treatment. Eleven miRNAs (let-7g, miR-10b, miR-15a, miR-16, miR-21, miR-27a, miR-365, miR-374b, miR-483-5p, miR-574-3p and miR-575) previously identified in the microarray to be differentially expressed after treatment were selected to analyze their responsiveness to eight different 5-FU dosages of 0.001, 0.005, 0.01, 0.1, 0.7, 1, 5 and 10μM. Of these, miR-10b, miR-21, miR-365 and miR-483-5p were shown to be significantly regulated in a beneficial way. Time-response data was also generated for miR-10b, miR-21, miR-483-5p, miR-574-3p and miR-575 following 12, 24, 36, 48, 60 and 72 hours treatment with 0.1, 0.7 and 10μM 5-FU. The data obtained suggested that miRNA expression in MCF7 cells is sensitive to 5-FU therapy at low doses and shorter treatment durations. The down-regulation of an important oncomir, miR-21; and alteration in the expression of three new miRNAs with no previous breast cancer association, miR-483-5p, miR-574-3p and miR-575 indicates that miRNA might play an important role in 5-FU therapy. In conclusion, miRNAs were shown to play an important regulatory role in 5-FU induced cytotoxicity and fit in perfectly in the intricate network of 5-FU activity.

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5-FU	5-fluorouracil
µM	Micromolar
ANOVA	Analysis of Variance
AO	Acridine Orange
BCL2	B-cell CLL/lymphoma 2
BRCA1	Breast cancer type 1 susceptibility protein
BRCA2	Breast cancer type 2 susceptibility protein
Cdk	Cyclin Dependent Kinase
cDNA	Complementary DNA
CLL	Chronic lymphocytic leukemia
Cy3/5	Cyanine dyes
DA	Dead Apoptotic
DN	Dead Normal
DNA	Deoxy Ribonucleic acid
DLBCL	Diffuse large B cell lymphoma
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
dNTP	Deoxyribonucleotide triphosphate
dsRDB	Double stranded RNA binding domain protein
dTMP	Deoxythymidine monophosphate
dTTP	Deoxythymidine triphosphate

dUMP	Deoxyuridine monophosphate
dUTP	Deoxyuridine triphosphate
EB	Ethidium Bromide
EDTA	Ethylenediaminetetraacetic Acid
eIF	Eukaryotic initiation factors
ER	Estrogen Receptor
FUMP	Fluorouridine monophosphate
FUTP	Fluorouridine triphosphate
FdUMP	Fluorodeoxyuridine monophosphate
FdUTP	Fluorodeoxyuridine triphosphate
GO	Gene Ontology
G1	Gap 1 of Cell Cycle
G2	Gap 2 of Cell Cycle
HCC	Hepatocellular carcinoma
HER2/neu	Human Epidermal Growth Factor Receptor 2
IC	Inhibitory Concentration
IV	Intravenous
kDa	Kilo Daltons
Kg	Kilograms
LA	Live Apoptotic
LN	Live Normal
LOWESS	Locally Weighted Scatterplot Smoothing
MCF7	Human Breast Adenocarcinoma cell line

M	Molar concentration
m	Meters
ml	Milliliters
mg	Milligrams
miR	MicroRNA
miRNA	MicroRNA
miRNA*	MicroRNA complementary sequence
mRNA	Messenger RNA
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
nM	nanomolar
OD	Optical density
OSCC	Oral Squamous cell carcinoma
p53	Tumor protein 53
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PR	Progesterone Receptor
Pre-miRNA	Precursor miRNA
Pri-miRNA	Primary microRNA
PTEN	Phosphatase and Tensin homolog
qRT-PCR	Quantitative Real Time Polymerase Chain Reaction
Rb	Retinoblastoma tumor suppressor protein
RISC	RNA-induced Silencing Complex
RNA	Ribonucleic acid

RNP	Ribonucleoprotein complex
RNU48	Endogenous reference gene
RPMI	Cell Culture Media
RT	Reverse Transcriptase
S phase	Synthesis phase of Cell Cycle
SEM	Standard error of the mean
TS	Thymidylate synthase
TNF	Tumor necrosis factor
UTR	Untranslated Region

Chapter 1: Review of Breast Cancer, Chemotherapy and MicroRNAs

Breast cancer: An Overview

Breast cancer is one of the leading causes of deaths worldwide. It is the most common cause of death in women between the ages of 45 and 55. The American Cancer Society reported 465,000 deaths worldwide due to breast cancer alone in 2007. Breast cancer incidence in women in the United States is about 1 in 8 (~13%). Although it is a common form of cancer in women, male breast cancer cases have also been reported and it accounts for about 1% of all cancer deaths in men (American Cancer Society). Breast cancer is the cancerous growth of the tissue in the breasts, which can be benign or malignant. Benign breast tumors result in fibrocystic deposition in the breast, causing lumpiness of the breast. Malignant breast tumors can spread (metastasize) to different tissues or organs by the blood stream or lymph system; the most common sites are the bones, liver, lungs, and brain. According to the American Cancer Society, an estimated 192,370 new cases of breast cancer were diagnosed and approximately 40,170 women died from breast cancer in the year 2009.

There are several risk factors associated with breast cancer. Genetic mutation and heredity is one of the major reason causing breast cancer; for example, genetic mutations in the BRCA1 and BRCA2 genes. However, only about 5-10% of breast cancers are hereditary; other causes include age, alcohol consumption, obesity, hormone replacement therapy, and birth control pills consumption. Early breast cancer does not show any significant symptoms. Gradual development of the cancer results in formation of a lump in the breasts, change in shape, size, color or texture of breast or discharge from nipple. Breast cancer can be diagnosed by careful physical examination, mammography, ultrasonography, Magnetic Imaging Resonance (MRI) and breast biopsy. With recent technological advances, gene expression profiling is also used to

detect early breast cancer and predict their prognostic outcomes. Three such gene expression–based prognostic breast cancer tests have been licensed for use: Oncotype DX (Genomic Health, Redwood City, California), MammaPrint (Agendia BV, Amsterdam, the Netherlands), and H/I (AvariaDX, Carlsbad, California).

Types of breast cancer

There are several types of breast cancer, but the most common types are ductal carcinoma and lobular carcinoma. The other types of breast cancers are relatively rare. Some of these cancers are in situ carcinomas, which mean that the cancer cells remain confined to ducts or lobules and do not grow into deeper tissues in the breast or spread to other organs in the body. These are sometimes referred to as non-invasive or pre-invasive breast cancers. The more aggressive breast cancers are the invasive or infiltrating carcinomas which have spread to the other breast tissues and other organs of the body.

The following list entails the different types of breast cancers: (American cancer Society, Inc.; National Breast Cancer Foundation, Inc.; Breastcancer.org)

Ductal carcinoma: This is the cancer of the ducts, the passageway which carries milk from the milk-producing lobules to the nipple. It can be non-invasive (in situ) or invasive (infiltrative). It is the most common type of cancer, accounting for about 85 – 90% of breast cancer cases.

Lobular carcinoma: It arises in the lobules, the milk-producing gland of the breast. It is the second most common type of breast cancer, occurring in about 8% of the cases.

Inflammatory breast cancer: This is an uncommon type of invasive breast cancer, in which the cancer cells block the lymph vessels around the breast, making the breast look red,

warm and with a pitted appearance. There is usually no lump or tumor found, and it accounts for about 1-3% of all breast cancer cases.

Medullary carcinoma: This is a rare type of invasive ductal carcinoma in which the cancer cells are morphologically different and bigger than the normal breast cells, and there is a well defined boundary observed between the cancerous cells and normal cells.

Metaplastic carcinoma: This type of breast cancer includes a range of cancers of mixed epithelial (cells lining the breast) and mesenchymal (connective tissue if the breast) cells, which then change into squamous (nonglandular) cells. This cancer does not have estrogen receptors (ERs), progesterone receptors (PRs), or HER2/neu protein.

Mucinous (colloid) carcinoma: It is the cancer of the mucus-producing cells. They are usually estrogen receptor positive and HER2/neu negative.

Tubular carcinoma: This is a rare type of invasive ductal carcinoma in which the cancer cells have a tubular microscopic appearance. Tumors are generally small, estrogen receptor positive and HER2/neu negative.

Papillary carcinoma: It is a type of rare ductal carcinoma in situ in which the cells are arranged in small, finger-like projections (papilla) when viewed under the microscope. These are rarely invasive and are more prominent in older women.

Adenoid cystic (adenocystic) carcinoma: These cancers have both glandular (adenoid) and cylinder-like (cystic) features under the microscope. They make up less than 1% of breast cancers and are rarely invasive.

Phyllodes (phylloids) tumor or cystosarcoma phyllodes: It is a rare sarcoma, cancer of the stroma (connective tissue), which is usually benign.

Angiosarcoma breast tumor: A breast angiosarcoma is a rare type of breast cancer which starts in cells that line the blood vessels within the breasts.

Molecular genetics of breast cancer

Carcinogenesis is a multistep process which usually involves genetic alterations that influence important cellular pathways. About 10% of the breast cancer cases are shown to be hereditary, which results mainly due to genetic defects or mutations in the genome inherited from the parent. Women with these mutations have up to 80% risk of developing breast cancer during their lifetime. About 90% of breast cancers are due not to heredity, but mainly sporadic cancers, resulting from acquired somatic mutations or genetic abnormalities that happen as a result of aging process and life in general. More recently, using advanced technologies such as comparative genome hybridization (CGH), fluorescence *in situ* hybridization (FISH), chromosome painting and microsatellite marker analysis, the genome of the breast cancer cells have been shown to be highly unstable (Hedley, Rugg et al. 1987; Tirkkonen, Johannsson et al. 1997; Ingvarsson, Geirsdottir et al. 1998; Lingle, Barrett et al. 2002). These are due to mutations or other alterations in the tumor suppressor genes and amplification of the oncogenes. Alterations in the tumor suppressor genes would cause loss-of-function effects, while oncogenes cause gain-of-function effect that contribute to the malignant breast cancer phenotype. Table 1.1 gives the list of different oncogenes and tumor suppressor genes which usually undergo alterations in breast cancer.

Strategies for breast cancer treatment

There are several treatment options available for the management of breast cancer. It can be treated either locally or systemically. Local treatment includes surgery (mastectomy or lumpectomy) and radiation therapy; while systemic treatment includes chemotherapy and hormone replacement therapy. There are several chemotherapeutic drugs used for the treatment of breast cancer (Table 1.2) that are usually used as an adjuvant therapy along with surgery. In many cases, a combination of two or more medicines will be used as chemotherapy treatment for breast cancer (Table 1.3). However there are several side-effects associated with treatment with chemotherapeutic drugs, like anemia, hair loss, nausea, vomiting, diarrhea, etc . Hormonal therapy is also widely used for the management of breast cancers that are sensitive to hormones. These are usually estrogen and progesterone-receptors positive breast cancers. Table 1.4 gives the list of drugs used in hormone therapy.

Recent advances in research and technology has seen the development of targeted drug therapies such as bevacizumab (Avastin – anti-angiogenesis drug designed to inhibit the signaling of cancer cells to new blood vessels); lapatinib (Tykerb – targets the protein HER2) and trastuzumab (Herceptin – targets the protein HER2), which are more specific for breast cancer treatment and are less likely to harm normal, healthy cells. More work is needed to improve chemotherapy for breast cancer and to develop new therapies to alleviate breast cancer and improve the general quality of living.

Chemotherapy and 5- Fluorouracil

Several chemotherapeutic drugs are used for the treatment of breast cancer. 5-fluorouracil (5-fluoro-1*H*-pyrimidine-2,4-dione or 5-FU) is an important chemotherapeutic drug which is widely used for the treatment of different cancers, mainly breast (Fumoleau, Bonneterre et al. 2003), colorectal (Wils, O'Dwyer et al. 2001) and head and neck cancers (Posner, Colevas et al. 2000). It was first synthesized in 1957 by (Heidelberger, Chaudhuri et al. 1957). It has been in use for about 50 years and is available in market under different trade names – Adrucil®, Efudex®, Fluoroplex®, and Carac™. The drug can be administered as IV (intravenous) infusion or bolus, or applied topically, depending on the type of cancer treated. The patients are usually subjected to a continuous drug infusion at the constant rate of 450 – 966 mg/m^2/day, causing 5-FU plasma concentration to reach values of order of magnitude of 5µM or more (Petit, Milano et al. 1988). However, the daily dose of 5-FU is not to exceed 800 mg.

5-FU is an antimetabolite drug. These drugs are cell-cycle specific, which affects cells only when they are in the S-phase or getting divided. Since the cancerous cells divide more rapidly compared to the normal cells, they take up these antimetabolites more rapidly than the normal cells, and hence are more toxic to them. 5-FU is a pyrimidine antagonist. It is an analogue of uracil, in which the hydrogen at C-5 position is substituted with a fluorine atom (Figure 1.1), hence called a Fluoropyrimidine. Its cytotoxicity has been ascribed to its inhibition of the thymidylate synthase (TS) and misincorporation into RNA and DNA, finally inducing cell-cycle arrest and apoptosis.

Mechanism of action

Following administration, 5-FU enters the cell through the same facilitated transport mechanism as used by uracil (Wohlhueter, McIvor et al. 1980). It is then converted intracellularly into 3 different active metabolites: fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP) and fluorouridine triphosphate (FUTP) (Figure 1.2).

The first step in the activation on 5-FU is its conversion to fluorouridine monophosphate (FUMP). This conversion can occur in two ways: a) Direct conversion: by action of orotate phosphoribosyltransferase (OPRT) with co-factor phosphoribosyl pyrophosphate (PRPP); and b) Indirect conversion: through sequential conversion from 5-FU to fluorouridine (FUR) and then to FUMP by the action of enzymes uridine phosphorylase (UP) and uridine kinase (UK) respectively (Daher, Harris et al. 1990). FUMP can then be phosphorylated to fluorouridine diphosphate (FUDP), which can be converted to the active compound fluorouridine triphosphate (FUTP), causing the cytotoxic effects. FUDP can also be converted to fluorodeoxyuridine diphosphate (FdUDP) by ribonucleotide reductase (RR). FdUDP can then be phosphorylated or dephosphorylated to give the active compounds fluorodeoxyuridine triphosphate (FdUTP) or fluorodeoxyuridine monophosphate (FdUMP) respectively. Alternatively, 5-FU can also be converted to FdUMP by thymidine phosphorylase (TP) catalysed conversion of 5-FU to fluorodeoxyuridine (FUDR), which is then phosphorylated to FdUMP by thymidine kinase (TK). The three active metabolites, FdUMP, FdUTP and FUTP elicit the cytotoxicity of 5-FU in cancerous cells.

Inhibition of thymidylate synthase (TS)

TS is a 36kDa dimeric protein which contains a nucleotide – binding site and a folate – binding site. It catalyses the conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP). dUMP undergoes reductive methylation to dTMP with N^5,N^{10}-methylenetetrahydrofolate (CH_2THF) serving as the methyl donor. This reaction is a part of the de novo synthesis of thymidine and is the only source of thymidine, which is required for DNA replication and repair.

FdUMP actively binds to the nucleotide-binding site of TS, which results in the formation of a stable ternary complex of TS, FdUMP and CH_2THF (Figure 1.3). Binding of FdUMP to TS prevents the binding of the normal substrate dUMP to TS, resulting in inhibition of dTMP synthesis (Santi, McHenry et al. 1974; Sommer and Santi 1974). Reduction in the level of dTMP causes succesive reduction in dTTP levels, which in turn induces imbalances in the deoxynucleotide pool (dATP, dCTP and dGTP) and particularly dATP/dTTP ratio (Yoshioka, Tanaka et al. 1987; Houghton, Tillman et al. 1995). These imbalances result in inhibition of DNA synthesis and repair, and thus cause DNA damage.

Misincorporation into DNA and RNA

FdUTP gets misincorporated into the DNA strands in place of dTTP during DNA replication. Additionally, accumulation of dUMP results in allevated levels of dUTP inside the cells (Mitrovski, Pressacco et al. 1994; Aherne, Hardcastle et al. 1996). This causes misincorporation of dUTP in DNA strands. This misincorporation cannot be corrected / repaired by nucleotide excision due to the high dUTP/dTTP ratios, and this ultimately results in DNA strand breaks and cell death.

Further, FUTP is extensively misincorporated in RNA strands, which disrupts the normal RNA processing. This results in disruption of the further synthesis and processing of mRNAs (Doong and Dolnick 1988; Patton 1993), tRNAs (Randerath, Tseng et al. 1983; Santi and Hardy 1987) and rRNAs (Kanamaru, Kakuta et al. 1986; Ghoshal and Jacob 1994) and in inhibition of post-transcriptional conversion of uridine to pseudouridine in these RNAs (Samuelsson 1991). This results in RNA toxicity and imbalances, causing variability in cellular metabolism and functioning.

Inactivation of 5-FU

5-FU is primarily catabolized in the liver, where the enzyme dihydropyrimidine dehydrogenase (DPD) is present in abundance. DPD catalyses the conversion of 5-FU to inactive compound dihydrofluorouracil (Diasio and Harris 1989) (DHFU, Figue 2).

Dosage and clinical pharmacokinetics

5-FU is generally administered as an intravenous injection. The actual doses are dependent on the weight of the patient. Usually, 12 mg/kg of 5-FU are given intravenously once daily for 4 successive days. The daily dose should not exceed 800 mg. If no toxicity is observed, six mg/ml are given on the 6^{th}, 8^{th}, 10^{th} and 12^{th} days. Therapy is then discontinued at the end of 12^{th} day (Fluorouracil Injection, USP; Gensia Sicor Pharmaceuticals, Inc., Irvine, CA 92618).

Following intravenous injection, 5-FU gets distributed mainly into the tumors, bone marrow, liver, intestinal mucosa and other tissues in the body. It also diffuses readily across the blood brain barrier and gets distributed into the CSF (cerebrospinal fluid). Seven to 20% of the parent drug is excreted unchanged in the urine in about 6 hours (Diasio and Harris 1989), while 60-90% of the administered dose is excreted in urine within 24 hours, primarily as α-fluoro-β-

alanine (Heggie, Sommadossi et al. 1987). The mean half life of elimination from plasma is dose-dependent and in the range eight to 20 min (Heggie, Sommadossi et al. 1987; Diasio and Harris 1989). No intact drug can be detected in the plasma after three hours of intravenous injection (Heggie, Sommadossi et al. 1987; Diasio and Harris 1989). However, 5-FU active metabolites have prolonged elimination half lives (Heggie, Sommadossi et al. 1987).

5-FU modulations / Improvements

5-FU drug treatment is associated with varied side effects, ranging from mild to severe. Common side effects include nausea, vomiting, diarrhea, weakness, mouth sores, poor appetite, discoloration of the vein through which the medication is given, sensitivity to light (photophobia), reduced white and red blood cell and platelet counts which can cause increased risk of infections. More rare side effects include skin reactions like hyperpigmentation (darkening of skin), dryness or cracking of skin; discoloration of nails; thinning of hair and hand and foot syndrome. Several strategies have been adopted to improve the 5-FU therapy (Figure 1.4). These include adjunct therapy of 5-FU with other chemotherapeutic drugs like Leucovorin, Methotrexate or Irinotecan. Oxaliplatin, 5-FU and Leucovorin adjunct therapy have also been used for advanced colorectal cancer. Various 5-FU pro-drugs have been synthesized, like Capecitabine and tegafur, which improves the targeted delivery of 5-FU and significantly reduces the associated side effects.

Effect of 5-FU on gene expression

5-FU has been shown to modify the expression of protein-coding genes. 5-FU may alter the gene expression levels of its target enzyme, TS and its metabolic enzymes, DPD, OPRT, TP and UP (Inokuchi, Uetake et al. 2004; Mauritz, van Groeningen et al. 2007). In a previous study,

five 5-FU-inducible transcriptional targets have been identified: SSAT (spermine / spermidine acetyl transferase), annexin II, thymosin β-10, chaperonin-10 and MAT-8 (Maxwell, Longley et al. 2003). Further, p53 have been shown to be a potential target of 5-FU (Hernandez-Vargas, Ballestar et al. 2006). 5-FU also upregulates FAS expression (Tillman, Petak et al. 1999). Another study reported differential expression of a set of genes between 5-FU sensitive and 5-FU resistant colon carcinoma cell line, including ornithine decarboxylase, spermine/spermidine synthases, spermine/spermidine acetyltransferase, p21/WAF1, mdm2, Fas, mic-1, EphA2, and ferredoxin reductase (Zhang, Ramdas et al. 2003). Most importantly, they reported role of p53 and tumor necrosis factor (TNF) regulation in 5-FU treatment (Zhang, Ramdas et al. 2003). A study performed global gene expression pattern following 5-FU treatment on MCF7 cells (Hernandez-Vargas, Ballestar et al. 2006). A total of 300 genes were shown to be regulated at any time point following treatment at 10μM and 500μM of 5-FU (Hernandez-Vargas, Ballestar et al. 2006). Many of these genes were found to be related with the p53 transcription factor. A dose- and time-dependent pattern was observed in the gene expression, with gene expression changes correlated with cell cycle and cell death parameters (Hernandez-Vargas, Ballestar et al. 2006). Thus, 5-FU modifies the expression levels of several protein–coding genes. However, the molecular mechanism of this effect is still unknown.

MicroRNAs

MicroRNAs (miRNAs) are an important class of endogenous, non- protein-coding RNA molecules which play a regulatory role in gene expression. They are small RNA molecules, about19-25 nucleotides in length, which negatively regulate gene expression by binding to the

3’-Untranslated region (3’-UTR) of the target mRNAs, and causing mRNA degradation or translational repression. Most of the miRNAs are highly conserved interspecies. It has been predicted that miRNAs constitute more than 1% of the total protein coding gene, while they target more than 30% protein coding genes (Lewis, Shih et al. 2003).

The first miRNA, lineage-deficient-4 (*lin-4*), was discovered by Victor Ambros’s group in Caenorhabditis elegans in 1993 (Lee, Feinbaum et al. 1993). The *lin-4* RNA showed near perfect antisense complementarity with the 3’ – UTR of mRNA of the *lin-14* gene, which is important for regulation of developmental timing in C. elegans. Since its discovery, thousands of miRNAs have been discovered in animals, plants and several viruses. These miRNAs play an important role in multiple biological processes, including developmental timings, embryogenesis, cell differentiation, organogenesis, metabolism, apoptosis and various diseases, including cancers.

Biogenesis of microRNAs

MiRNAs are transcribed from miRNA genes, which can be transcribed as autonomous transcription units, or as clusters from a polycistronic transcription unit (Bartel 2004; Kim and Nam 2006). miRNA genes can be divided into three groups based on their genomic location: first, genes located in the exonic region of non-coding transcriptions units; second, genes located in the intronic regions of non-coding transcription units; and third, genes located in the intronic region of protein-coding transcription units (Kim 2005; Kim and Nam 2006). A majority of them are, however, located in the intronic region of a protein-coding gene. miRNA genes are generally transcribed by RNA polymerase II (pol II) (Cai, Hagedorn et al. 2004; Lee, Kim et al. 2004), or sometimes by RNA polymerase III (pol III) (Borchert, Lanier et al. 2006); to give primary

miRNA transcript, called pri-miRNA (Figure 1.5). These pri-miRNAs are usually several thousand bases long and consists of a local hairpin structure. They may also contain a 5'-cap and 3'-poly(-A) tail (Cai, Hagedorn et al. 2004; Lee, Kim et al. 2004). This stem-loop structure is then cleaved by a Microprocessor complex to give precursors of microRNA, called pre-miRNAs (Denli, Tops et al. 2004; Gregory, Yan et al. 2004). The Microprocessor complex is composed of nuclear RNase III Drosha and double-stranded RNA binding domain protein DGCR8 (DiGeorge syndrome critical region 8). Drosha is a 160kDa protein consisting of two RNase III domains (RIIIDs) and a catalytic double-stranded RNA binding domain (dsRBD); whereas DGCR8 is a 120kDa human protein which consists of two dsRBDs (Han, Lee et al. 2004). Drosha binds to the pri-miRNA and cleaves it at approximately two helical turns (about 22 nt) from the terminal loop, to give the pre-miRNAs (Zeng, Yi et al. 2005). The flanking regions are degraded in the nucleus. DGCR8 is supposed to aid substrate recognition by Drosha, by assisting in binding of microprocessor complex to RNA or orientating the complex to RNA (Denli, Tops et al. 2004; Gregory, Yan et al. 2004; Landthaler, Yalcin et al. 2004). These pre-miRNAs have a typical stem-loop secondary structure, comprising of a about 22bp stem, a terminal loop and a 3'-overhang of around 2 nt (Lee, Ahn et al. 2003). The pre-miRNAs thus processed are then exported to the cytoplasm by the nuclear transport receptor, Exportin-5 (Yi, Qin et al. 2003; Lund, Guttinger et al. 2004).

Exportin-5 binds cooperatively with the pre-miRNAs and its cofactor GTP-bound-Ran in the nucleus (Bohnsack, Czaplinski et al. 2004). This complex then transports the pre-miRNAs across the nuclear membrane through the nuclear pore complexes. Following export, the GTP is hydrolyzed to GDP and in the process, pre-miRNAs are released into the cytoplasm. The secondary stem-loop structure and short 3'-overhang of the pre-miRNAs are significant

structural requirements for their transport by Exportin-5 (Zeng and Cullen 2004). Following their export from the nucleus, these pre-miRNAs are further processed to about 22 nucleotide miRNA:miRNA* duplexes by the cytoplasmic RNase III enzyme Dicer, and its dsRBD TRBP (the human immunodeficiency virus transactivating response RNA-binding protein) (Hutvagner, McLachlan et al. 2001; Chendrimada, Gregory et al. 2005). Dicer is a about 160kDa protein, consisting of two RIIIDs, a dsRBD and a long N-terminal segment that contains a Dead-Box RNA helicase domain, a DUF283 domain and a PAZ domain. It cleaves the pre-miRNAs at approximately 2 helical turns (about 22 nucleotides) from the 3'-terminus to give the miRNA:miRNA* duplex. One strand of the duplex is then selected as the mature miRNA and the other strand is degraded in the cytoplasm by unknown mechanism. Studies on siRNA duplexes have shown that the relative thermodynamic stability of the two ends of the duplex plays a role in strand selection (Khvorova, Reynolds et al. 2003; Schwarz, Hutvagner et al. 2003). The strand with relatively unstable base pairs at the 5'-end is usually selected as mature miRNA, while the other miRNA* strand is degraded. The mature miRNA then gets incorporated into the ribonucleoprotein complexes (RNPs) called microRNA-RNPs (miRNPs) or into miRNA-induced silencing complexes (miRISCs), which bring about gene regulation. The Agronaute (Ago) proteins are an important protein constituent of these complexes.

Mechanism of miRNA-mediated gene regulation

MiRNAs interact with their target mRNAs by base pairing (Figure 1.6). In plants, a majority of miRNAs base pair with near perfect complementarity with the mRNAs, but in animals there is imperfect base pairing which follows certain rules in most miRNAs, as shown by experimental and bioinformatic analysis (Brennecke, Stark et al. 2005; Lewis, Burge et al. 2005; Grimson, Farh et al. 2007) (Figure 1.7). The miRNAs bind perfectly with the mRNAs

between nucleotides 2 and 8, known as the “seed” region (Bartel 2004; Doench and Sharp 2004; Brennecke, Stark et al. 2005). The seed region is generally devoid of any mismatching base pairs or bulges. It is flanked by an A or U residue on positions 1 and 9, which may help in improving site efficiency (Lewis, Burge et al. 2005; Grimson, Farh et al. 2007). The bulges or mismatches may be present in the central region of the miRNA-mRNA duplex. Finally, the 3’-end of miRNA should bind complementarily with the mRNA to stabilize the interaction (Brennecke, Stark et al. 2005; Grimson, Farh et al. 2007). The miRNA-mRNA duplex thus formed brings about the gene regulation.

Eukaryotic translation

The process of eukaryotic translation consists of three basic steps: initiation, elongation and termination. Initiation of translation involves recognition of the mRNA 5’-terminal 7-methylguanosine (m^7G) cap by eukaryotic initiation factor 4E (eIF4E) subunit of initiation factor eIF4F. Other important initiation factors are eIF4A, eIF4G, eIF3 and eIF6. Interaction of eIF4G with eIF3 results in recruitment of 40S ribosomal subunit, which identifies the start codon. Another important role of eIF4G is its ability to interact with the polyadenylate-binding protein 1 (PABP1), which is associated with the poly(A) tail. Simultaneous interaction of eIF4G with eIF4E and PABP1 causes circularization of the mRNA, increasing the proximity of eIF4E and 5’-m^7G cap, and thus stimulating initiation of translation. Identification of start codon is followed by association of the large 60S ribosomal subunit and the start of elongation step. As translation proceeds, newly synthesized proteins (nascent polypeptides) emerge from the large ribosomal subunit. Several ribosomes can translate a single mRNA at the same time, resulting in formation of ‘Polysomes’ (ribosome clusters). Termination occurs when an elongating ribosome

encounters the stop codon; the ribosome dissociates from the mRNA, and the completed protein is released.

Presently there are four proposed mechanisms of miRNA-mediated gene repression.

mRNA cleavage

MiRNAs that bind perfectly with their target mRNAs bring about direct cleavage of a phosphodiester bond in the mRNA (Figure 1.6). In plants, most miRNAs bind to target mRNAs with near perfect complementarity, and the mRNA is cleaved endonucleolytically in the middle of the miRNA-mRNA duplex (Jones-Rhoades, Bartel et al. 2006). This cleavage occurs between the residues base paired to the 10 and 11 nucleotides of the miRNAs (counting from the 5'- end of miRNA) (Elbashir, Martinez et al. 2001). This cleavage is caused by the 'slicer' activity of the RISC. The Agronaute proteins (Ago) are an important constituent of this RISC. Ago are about 100 kDa proteins which contain the PAZ and PIWI domains (Carmell, Xuan et al. 2002). The PAZ domain contains an oligonucleotide-binding fold which binds the single-stranded 3'- end of miRNAs (Lingel and Sattler 2005), while the PIWI domain has a conserved pocket for binding the 5'-phosphate of miRNAs (Parker, Roe et al. 2005). Thus, the miRNAs is lodged between the PAZ and PIWI domains of the Ago protein. In addition, the PIWI domain also shows the presence of a catalytic site which functions similar to RNase H enzyme, capable of cleaving the RNA strand. Thus, the 'slicer' activity of the RISC complex is associated with the Ago proteins. However, only Ago-2 protein complexes are capable of inducing mRNA cleavage (Meister, Landthaler et al. 2004). In human, mRNA cleavage is not the usual method of miRNA-induced gene silencing. miR-196 is the only miRNA which binds perfectly to 3'-UTR of the Hoxb8

mRNA, causing its cleavage (Yekta, Shih et al. 2004). All others miRNA induce gene silencing by imperfect binding.

Translational repression

Another mechanism of mRNA silencing by miRNA is by interfering with their translation (Elbashir, Lendeckel et al. 2001). This was first suggested by the observation that miRNA lin-4 reduced the amount of LIN-4 protein without significantly affecting the amount of lin-4 mRNA (Lee, Feinbaum et al. 1993). Presence of multiple miRNA binding sites on target mRNAs further supports this model. Translational repression generally occurs in the absence of perfect complementarity between miRNA and the target mRNA. Currently two main models of translational inhibition by miRNAs are available: repression at initiation step or at post-initiation step. Studies performed in various labs have provided support for both the mechanisms. It is still unclear as to which mechanism is more prevalent and what factors determine as to which mechanism will be followed.

Translational repression at initiation step is proposed to be affected by two possible mechanisms. First mechanism is by suppression in recognition of 5'-m^7G cap by the eIF4E (Humphreys, Westman et al. 2005; Pillai, Bhattacharyya et al. 2005) . It has also been reported that Ago2 can bind to the ^{7}mGpppN cap via a motif resembling a cap-interacting sequence of the initiation factor eIF4E, preventing the cap–eIF4E interaction, and thus stopping the recruitment of the small ribosomal subunit to mRNA (Kiriakidou, Tan et al. 2007). Inefficient repression of mRNA containing IRES (Internal ribosome entry site) or a nonfunctional cap further supports this model. Briefly, miRNAs are supposed to prevent the synergy between the 5'-cap and the 3'-

poly(A) tail. An alternative mechanism for translational repression is by obstructing the joining of 60S subunit, which is affected by eIF6 protein (Chendrimada, Finn et al. 2007)

Translational repression has also been showed to occur post-initiation. The observation that lin-14 and lin-28 mRNAs, targets of lin-4 miRNA, remain associated with polysomes despite reduction in their protein products (Olsen and Ambros 1999; Seggerson, Tang et al. 2002) supports this model. A 'drop-off' mechanism of post-initiation repression has been proposed by many researchers, which states that miRNAs cause ribosomes to become prone to premature termination of translation (Petersen, Bordeleau et al. 2006). Another possible mechanism includes the slowing down of ribosome elongation (Mootz, Ho et al. 2004).

The research does not unequivocally state the exact mechanism of translational repression. One possible reason for the ambiguity could be adoption of different experimental techniques for the study. Another possible explanation could be that miRNA function through multiple mechanisms. The complementary binding with the target mRNA, and the Ago and GW182 proteins may play a role in deciding the mechanism of miRNA silencing. Further, there isn't enough evidence to support that initiation and post-initiation mechanisms are mutually exclusive.

Cotranslational protein degradation by Proteolysis

Another method of gene silencing by miRNA is at the protein production stage. Certain proteins can be found associated as polysomes after the binding of miRNA to its target mRNA (Olsen and Ambros 1999). These polysomes or nascent peptides are continuously synthesized from the mRNAs, but are not accumulated. They undergo rapid degradation by the proteases in

the cytosol (Nottrott, Simard et al. 2006). The association of miRISC or miRNP with the target mRNAs recruits the proteases and promotes its activity and affinity for the polysomes.

Deadenylation and Decapping

mRNA destabilization is another possible mechanism of miRNA-induced gene silencing. The destabilization is a sequential process, with the initial step being shortening of the 3'-Poly(A) tail (Giraldez, Mishima et al. 2006; Wu, Fan et al. 2006). This is followed by degradation step which can occur via two possible pathways: the mRNA can be progressively degraded in 3'-5' direction by an exosome, or it can be first 5'-decapped , followed by 5'-3' degradation by the exonuclease XRN1(Parker and Song 2004) .The degradation step usually occurs in the P bodies, cellular structure involved in mRNA catabolism and translational repression (Parker and Sheth 2007) . The GW182 protein and the Ago PIWI domain are important constituents for this mechanism.

Thus miRNA-mediated gene silencing can be brought about by many different mechanisms (Figure 1.8).

miRNAs, Breast cancer and 5-FU

Thousands of miRNAs have been detected and characterized in plants, animals and viruses (Zhang, Pan et al. 2006). miRNAs play a very important role in many biological processes, including developmental timing, organ development, stem cell maintenance and differentiation, disease, cell proliferation, apoptosis and response to different stresses (Ambros 2001; Bartel 2004; Alvarez-Garcia and Miska 2005; Cheng, Tavazoie et al. 2005). miRNA has also been reported to play a role in cancer initiation and progression (Calin and Croce 2006) and

cancer metastasis (Cheng, Byrom et al. 2005). 50% of miRNA genes are localized in cancer-associated genomic regions or in fragile sites (Calin, Sevignani et al. 2004). miRNA expression profiles have helped in classifying human cancers and development of a miRNA signature for different types of cancers (Lu, Getz et al. 2005). In most cancers, miRNAs show aberrant levels of expression. Those which are over-expressed in tumors are thought to be oncogenic in nature, called as 'Oncomirs', such as miR-17-92 cluster, which targets the E2F1 oncogenes in lymphoma, or miR-21, which targets PTEN tumor suppressors in hepatocellular carcinoma (O'Donnell, Wentzel et al. 2005; Meng, Henson et al. 2007). Those miRNAs whose expression is reduced in tumors are thought to be Tumor suppressors or 'TSmirs', such as in case of miR-15a and miR-16-1 which targets the BCL2 oncogene in Chronic Lymphocytic Leukemia (CLL) or let-7 family which target RAS oncogene in lung cancers (Johnson, Grosshans et al. 2005; Calin and Croce 2006).

miRNAs also play an important role in breast cancer. miRNA signature pattern have been shown to predict estrogen, progesterone and HER2/neu receptor status in breast cancer (Lowery, Miller et al. 2009) (Ma, Teruya-Feldstein et al. 2007) showed that miR-10b initiates breast cancer invasion and metastasis. On the other hand, breast cancer metastasis is suppressed by miRNAs such as miR-335 and miR-126 (Tavazoie, Alarcon et al. 2008). Further, miR-373 and miR-520c stimulate cancer cell migration and invasion (Huang, Gumireddy et al. 2008). miRNA gene expression levels are also aberrantly altered in breast cancer. miR-21, miR-10b are over-expressed (Ma, Teruya-Feldstein et al. 2007); whereas miR-125b and miR-145 are reduced in breast cancer (Iorio, Ferracin et al. 2005). These studies highlight the importance of miRNAs as both stimulators and inhibitors in breast cancer. Currently, a growing body of evidence has suggested the importance of miRNAs in modulating the chemosensitivity and chemoresistance

of tumor cells (Meng, Henson et al. 2006; Blower, Verducci et al. 2007; Blower, Chung et al. 2008). (Si, Zhu et al. 2007) reported that suppression of miR-21 sensitized MCF7 cells to anticancer drug topotecan. Similar studies have been reported for the drugs gemcitabine, doxorubicin and tamoxifen (Kovalchuk, Filkowski et al. 2008; Miller, Ghoshal et al. 2008; Zhao, Lin et al. 2008) illustrating the importance of miRNAs in drug sensitivity and resistance. Further, miRNAs such as miR-15b and miR-16 have been shown to modulate multidrug resistance by targeting the anti-apoptotic bcl2 gene (Xia, Zhang et al. 2008). These studies reveal the intrinsic role of miRNAs in managing the efficiency of chemotherapy in several human cancers.

Currently, no work has been reported on the effect of 5-FU on miRNAs. 5-FU has been widely used for treatments of various types of cancer, including breast cancer. However, its exact mechanism of toxicity at the molecular level is still not clearly understood. Also, the mechanism of cancer cell resistance to drug is still eluded. 5-FU has been shown to modify the expression of protein-coding genes (Longley, Harkin et al. 2003; Maxwell, Longley et al. 2003; Hernandez-Vargas, Ballestar et al. 2006; Rossi, Bonmassar et al. 2007), but the regulatory mechanism is unknown. Further, 5-FU was recently reported to modify the expression of several miRNAs in colon cancer cells (Rossi, Bonmassar et al. 2007), indicating the potential ability of 5-FU in altering miRNA expression. However, there is no report on the effect of 5-FU on miRNAs in human breast cancer. Considering the critical role of miRNAs in cancer and drug chemosensitivity, we hypothesized that the cytotoxicity of 5-FU in breast cancer may be partially elicited by regulation of miRNA expression levels. Determination of the effects of 5-FU on expression of miRNAs can help to identify the miRNAs which play a role in chemosensitivity and resistance of 5-FU in particular, and other chemotherapeutic drugs in general. It may also help to improve the efficacy of 5-FU treatment, by reducing its side-effects or by decreasing the

incidence of 5-FU resistant cancers. Finally, the data can be extrapolated to other chemotherapeutic drugs, and targeted delivery systems, which can target these specific miRNAs, can be synthesized.

Hypothesis

5-FU induces its cytotoxicity partially by altering the expression levels of microRNAs associated with breast cancer, which accounts for the modification of the gene expression levels induced by 5-FU. 5-FU also modifies the expression of target mRNAs transcripts and proteins of associated miRNAs, which could play an important role in inducing 5-FU cytotoxicity.

Research Objectives

The goal of this proposed research project is to elucidate the molecular mechanism of 5-FU and its effect on the global microRNA expression profile. A combined experimental and computational approach will be employed to achieve the following specific objectives:

Specific Aim 1: To investigate the effects of 5-FU treatment on MCF7 breast cancer cells (Chapter 2)

The sensitivity of MCF7 breast cancer cells to 5-FU treatment was analyzed using Trypan blue dye exclusion assay and MTT dye reduction assay. Modifications in the cell growth and proliferation after 5-FU exposure were studied by determining the inhibitory concentrations of 5-FU and the dose- and time-dependence of these responses. The effect on cell cycle and induction of apoptosis was also studied using Flow cytometry and Acridine Orange/Ethidium

Bromide (AO/EB) dye staining assay. This study helped us to classify the general effect of 5-FU treatment on MCF7 breast cancer cells.

Specific Aim 2: To identify the miRNAs which are differentially expressed after 5-FU treatment (Chapter 3)

In this objective, a comparative analysis of the expression pattern of miRNAs in normal and 5-FU treated MCF7 cells was performed using miRNA microarray technology. Six potential miRNAs, miR-575, miR-671-5p, miR-483-5p, miR-574-3p, miR-365 and miR-374b, which showed maximum differential expression after 5-FU treatment, were selected based on fold – change and statistical analysis. The differential expression levels of these miRNAs post – 5-FU treatment will be validated by qRT-PCR technique.

Specific Aim 3: To determine the dose-dependence and time-dependence of the miRNA expression response to 5-FU treatment (Chapter 4)

The dose and time dependence of the expression levels of the miRNAs selected in Objective 2 after 5-FU treatment were analyzed at different concentrations and for varying time periods using the qRT-PCR analysis. This study helped to identify any significant pattern in the expression levels of miRNA following 5-FU treatment.

Experimental model

The model for my experiment is the MCF7 human breast adenocarcinoma cell line. MCF7 cell line was originally isolated in 1970 from the pleural effusion of a 69-year old Caucasian woman with metastatic mammary carcinoma (Soule, Vazguez et al. 1973). These cells are positive for estrogen receptor, progesterone receptor, epidermal growth factor receptor and E-cadherin expression. They lack expression of apoptotic enzyme caspase-3 and basic

fibroblast growth factor. It is an adherent cell line which grows in monolayer. MCF7 breast cancer cells are widely used as an in vitro model of breast cancer, mainly to study the role of estrogen in breast cancer due to the presence of well-characterized estrogen receptors. They are easy to culture and maintain, and show many genetic and morphological similarities to the breast cancer cells.

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Table 1.1: Oncogenes and tumor suppressor genes and their functions in breast cancer (modified from (Osborne, Wilson et al. 2004))

Gene	Classification	Function
HER2	Oncogene	Tyrosine kinase receptor; involved in signal transduction pathways of cell growth and differentiation
Ras	Oncogene	G Protein involved in cellular signal transduction important in cell growth, differentiation and survival
PI3K	Oncogene	Kinase involved in cellular functions such as cell growth, proliferation, differentiation, motility, survival and intracellular trafficking
Akt	Oncogene	Kinase regulating cell-cycle, cell survival, metabolism and in angiogenesis
eIF-4E	Oncogene	Initiator of protein translation
Cyclin D1	Oncogene	Cell-cycle mediator required for cell cycle G1/S transition; interacts with tumor suppressor gene Rb
Cyclin E	Oncogene	Cell-cycle mediator required for cell cycle G1/S transition
c-myc	Oncogene	Transcription factor which binds with Enhancer box sequence (E-boxes) and recruits Histone acetyltransferases (HATs)
c-fos	Oncogene	Transcription factor
p53	Tumor suppressor gene	Transcription factor; response to DNA damage and stress; induces cell-cycle arrest; cell-cycle checkpoint activation; triggers/facilitates apoptosis
p27	Tumor suppressor gene	Inhibit cyclin-dependent protein kinases; arrest cell cycle in G_1 phase;
BRCA1	Tumor suppressor gene	Regulates DNA transcription; acts in DNA repair; interacts with Rad 51 protein; involved in cell cycle control and apoptosis
BRCA2	Tumor suppressor gene	Acts in DNA repair; interacts with Rad 51 protein; also involved in transcriptional regulation
CHK2	Tumor suppressor gene	Cell cycle checkpoint kinase; activates p53 after DNA damage
ATM	Tumor suppressor gene	Checkpoint kinase; acts in DNA repair; activates CHK2; induction of p53; phosphorylation of BRCA1
PTEN	Tumor suppressor gene	Phosphatase; negative regulator of Akt kinase; controls PIP3 pathway in cell growth
Rb	Tumor suppressor gene	Retinoblastoma gene, repressor of cell cycle and protein translation

Table 1.2: Common chemotherapeutic drugs used for the treatment of breast cancer

Chemical name	Brand names	Class	Mechanism
Capecitabine	Xeloda	Antimetabolite	Pro-drug for 5-fluorouracil, an antimetabolite
Carboplatin	Paraplatin	Alkylating agents	Forms DNA intrastrand crosslinks, inhibits replication
Cyclophosphamide	Cytoxan, Neosar	Alkylating agents	Forms DNA intrastrand crosslinks, inhibits replication
Daunorubicin	Cerubidine, DaunoXome	Anthracyclins	Prevents cell division by disrupting the structure of the DNA in two ways: intercalate into the base pairs in the DNA minor grooves; and cause free radical damage of the ribose in the DNA.
Docetaxel	Taxotere	Taxanes (Mitotic inhibitor)	Disrupts microtubules, arrests mitosis in metaphase
Doxorubicin	Adriamycin, Doxil, Rubex	Anthracyclins	Prevents cell division by disrupting the structure of the DNA in two ways: intercalate into the base pairs in the DNA minor grooves; and cause free radical damage of the ribose in the DNA.
Epirubicin	Ellence, Pharmorubicin	Anthracyclins	Prevents cell division by disrupting the structure of the DNA in two ways: intercalate into the base pairs in the DNA minor grooves; and cause free radical damage of the ribose in the DNA.
Fluorouracil or 5-fluorouracil	Adrucil; Efudex; Fluoroplex; Carac	Antimetabolite	Pyrimidine analog, interferes with DNA synthesis
Gemcitabine	Gemzar	Antimetabolite	Pyrimidine analog, interferes with DNA synthesis
Idarubicin	Idamycin	Anthracyclins	Prevents cell division by disrupting the structure of the DNA in two ways:

			intercalate into the base pairs in the DNA minor grooves; and cause free radical damage of the ribose in the DNA.
Ixabepilone	Ixempra	Epothilones (microtubule inhibitor)	Disrupts microtubules
Methotrexate	Amethopterin, Mexate, Folex	Antimetabolite	Inhibits folate metabolism, blocks nucleoside synthesis
Mitoxantrone	Novantrone	Type II topoisomerase inhibitor	Intercalates with DNA; disrupts DNA synthesis and DNA repair
Mitomycin	Mutamycin	Cell-cycle inhibitors	Inhibits DNA synthesis by inhibiting cell-cycle transition
Paclitaxel	Abraxane, Taxol	Taxanes (Mitotic inhibitor)	Disrupts microtubules, arrests mitosis in metaphase
Thiotepa	Thioplex	Alkylating agents	Forms DNA intrastrand crosslinks, inhibits replication
Vincristine	Oncovin, Vincasar PES, Vincrex	Mitotic inhibitor	Disrupts microtubules, arrests mitosis in metaphase
Vinorelbine	Navelbine	Mitotic inhibitor	Disrupts microtubules, arrests mitosis in metaphase

Table 1.3: Standard chemotherapy drug combination regimens used for the treatment of breast cancer (*American cancer Society, Inc.)*

- AT: Adriamycin and Taxotere
- AC ± T: Adriamycin and Cytoxan, with or without Taxol or Taxotere
- CMF: Cytoxan, methotrexate, and fluorouracil
- CEF: Cytoxan, Ellence, and fluorouracil
- FAC: fluorouracil, Adriamycin, and Cytoxan
- CAF: Cytoxan, Adriamycin, and fluorouracil

  (The FAC and CAF regimens use the same medicines but use different doses and frequencies)
- TAC: Taxotere, Adriamycin, and Cytoxan
- GET: Gemzar, Ellence, and Taxol

Table 1.4: Hormone therapy treatment used for the treatment of breast cancer

Drug class	Mechanism	Examples
Selective Estrogen-Receptor Modulators (SERMs)	Binds to the estrogen receptors in breast cancer cells, thus blocking the effects of estrogen on breast cancer cells	Nolvadex (Tamoxifen) Evista (Raloxifene) Fareston (Toremifene)
Aromatase Inhibitors	Inhibits the enzyme aromatase, which prevents the production of estrogen in adrenal glands	Aromasin (Exemestane) Femara (Letrozole) Arimidex (Anastrozole) Megace (Megestrol)
Hormone agonists	Causes an initial increase in the production of hormones by the body, which is then subsequently suppressed due to negative feedback loop mechanism of the body.	Zoladex (Goserelin) Lupron (Leuprolide)
Estrogen receptor antagonist	Binds to the ER and prevents ER dimerization, which leads to the rapid degradation of the fulvestrant-ER complex, producing the loss of cellular ER	Faslodex (Fulvestrant)

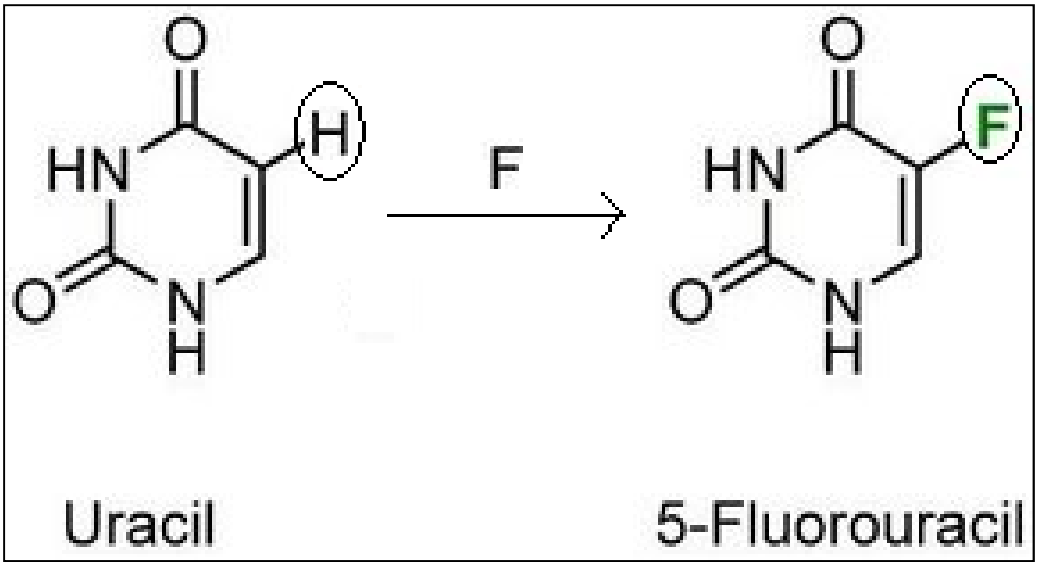

Figure 1.1: Structure of 5-fluorouracil, a fluoropyrimidine, in which the hydrogen atom at the 5th carbon is replaced by a fluorine atom.

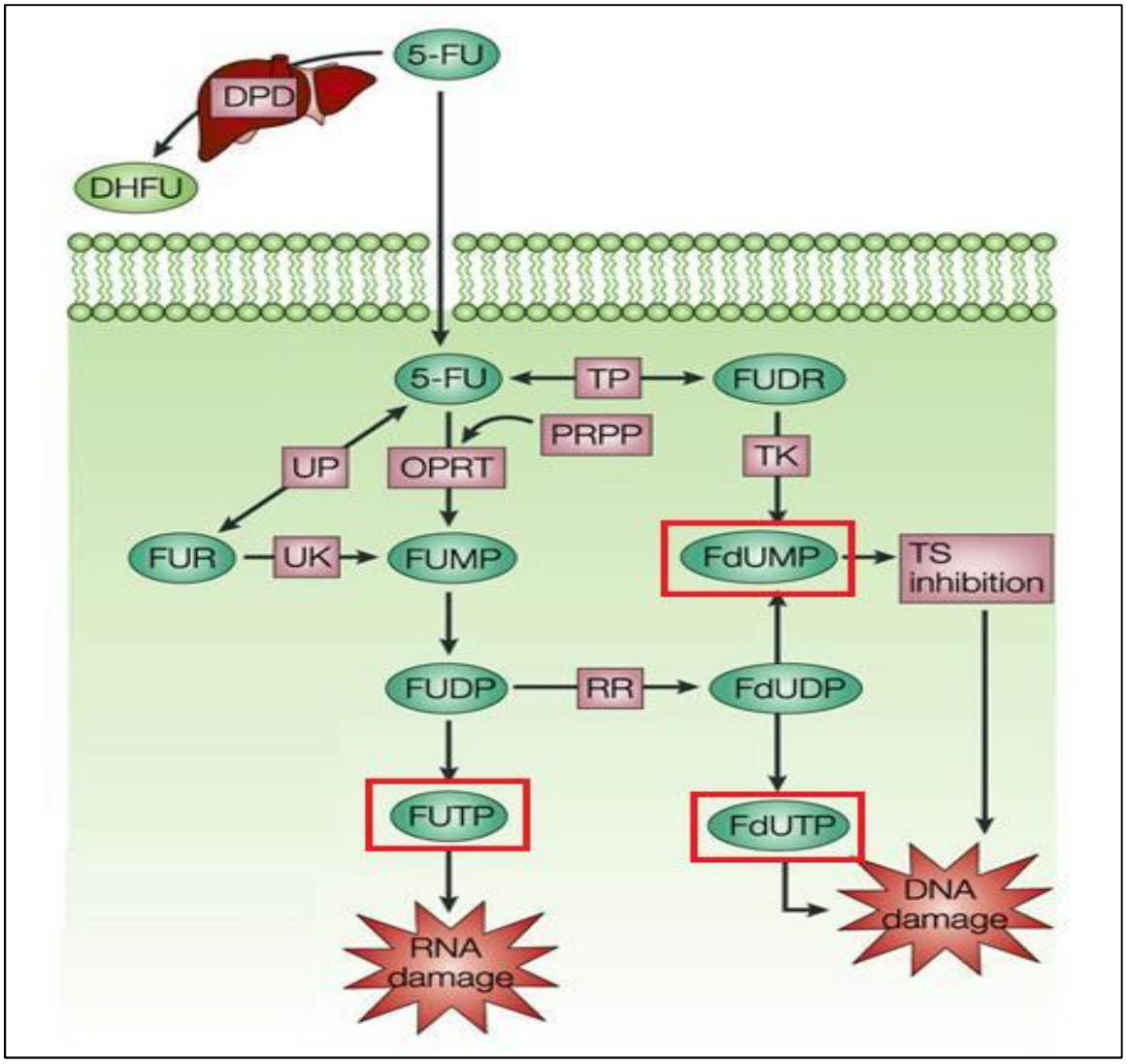

Figure 1.2: Metabolism of 5-FU to active products FdUMP, FdUTP and FUTP (Longley, Harkin et al. 2003).

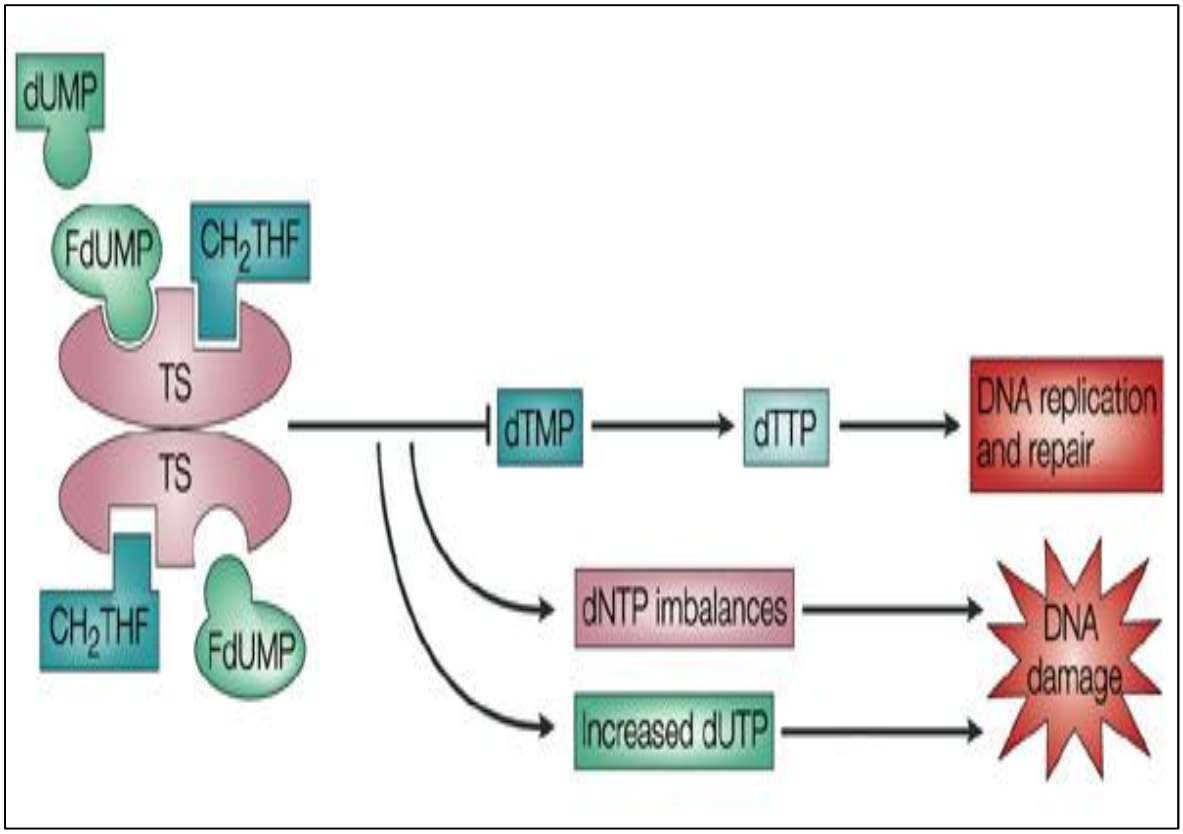

Figure 1.3: Inhibition of TS by 5-FU metabolite FdUMP (Longley, Harkin et al. 2003).

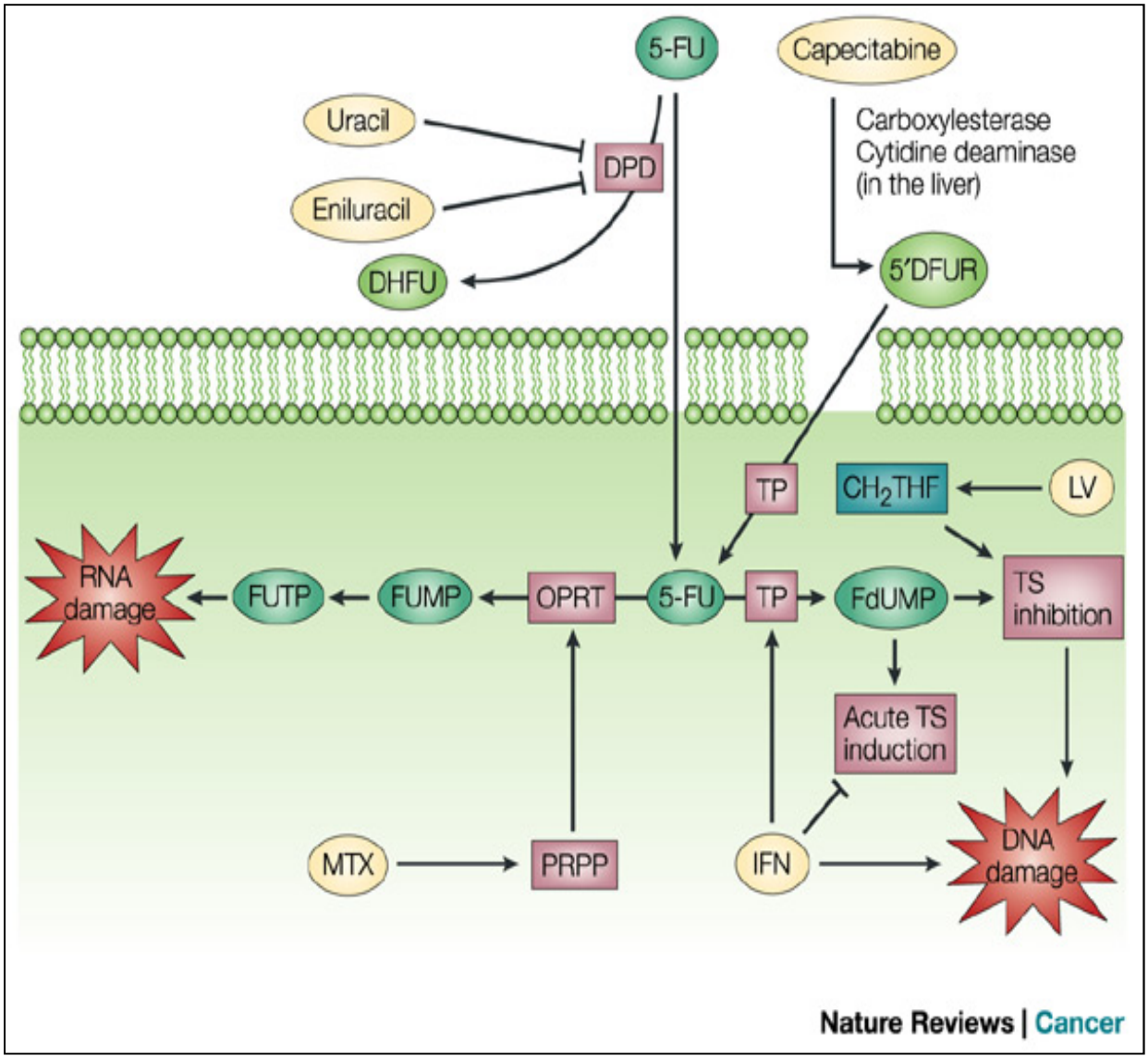

Figure 1.4: Summary of some of the strategies investigated for improving the efficiency of 5-FU treatment: Leucovorin (LV), Eniluracil, Uracil, Methotrexate (MTX), Interferones (IFNs), Capecitabine (Longley, Harkin et al. 2003).

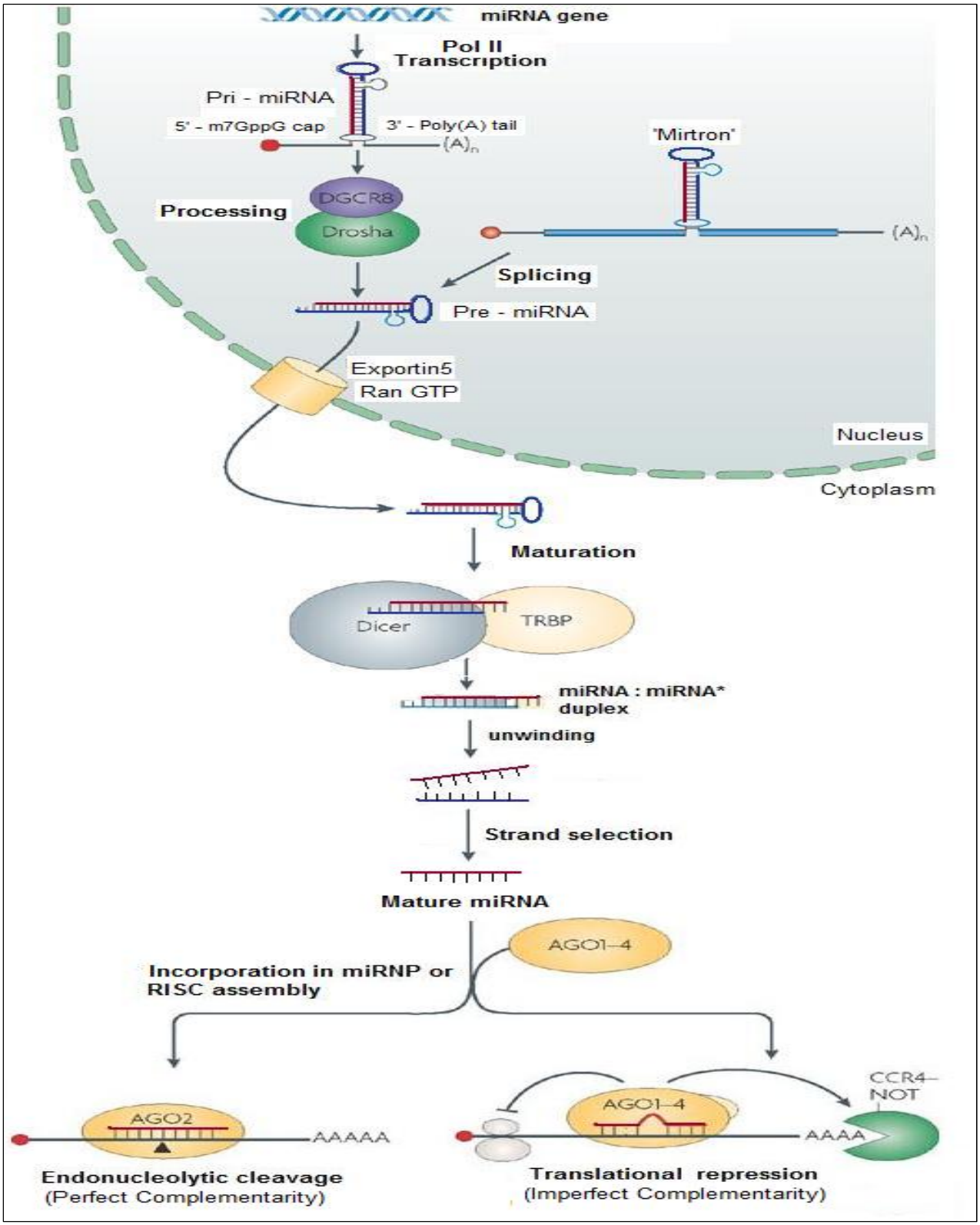

Figure 1.5: Biogenesis of miRNAs (modified from (Filipowicz, Bhattacharyya et al. 2008).

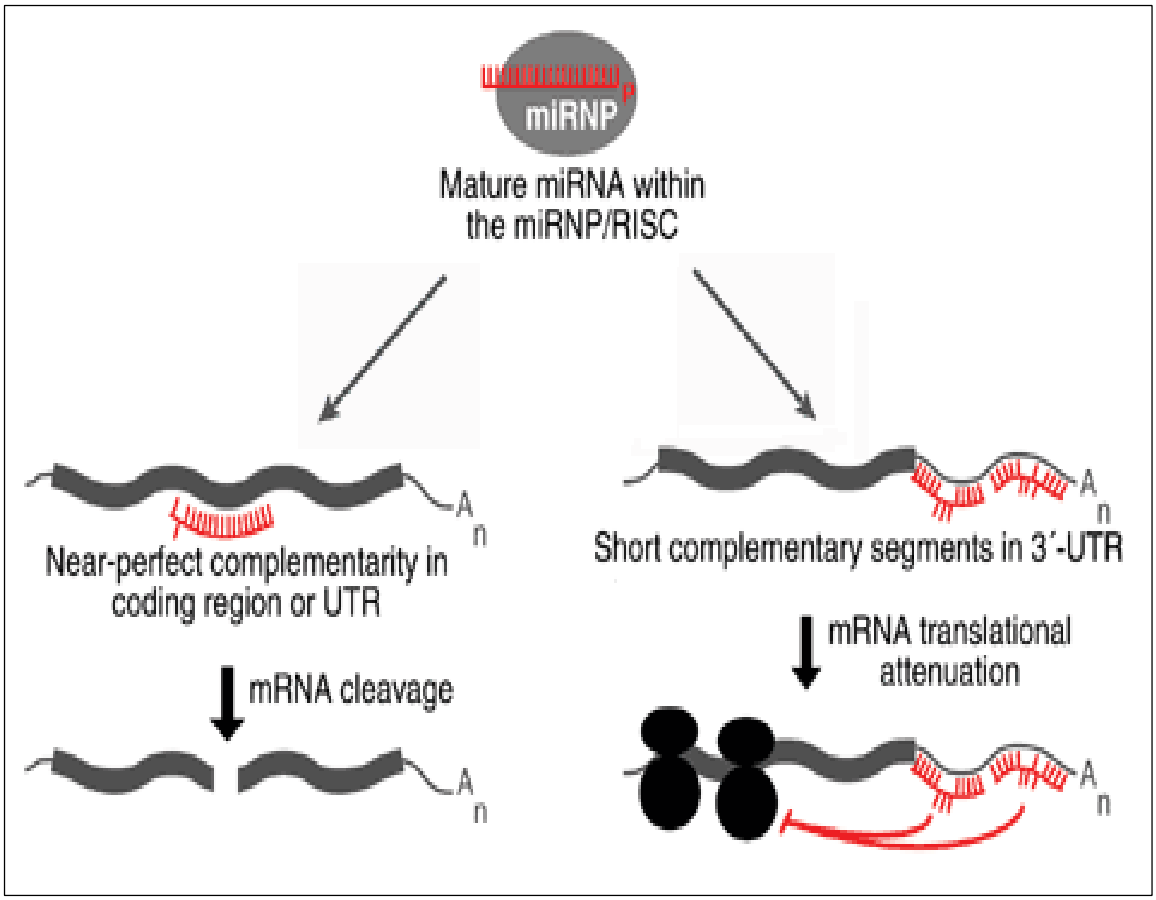

Figure 1.6: Mechanism of miRNA-induced gene silencing based on complementarity with target mRNAs (modified from (Bartel 2004).

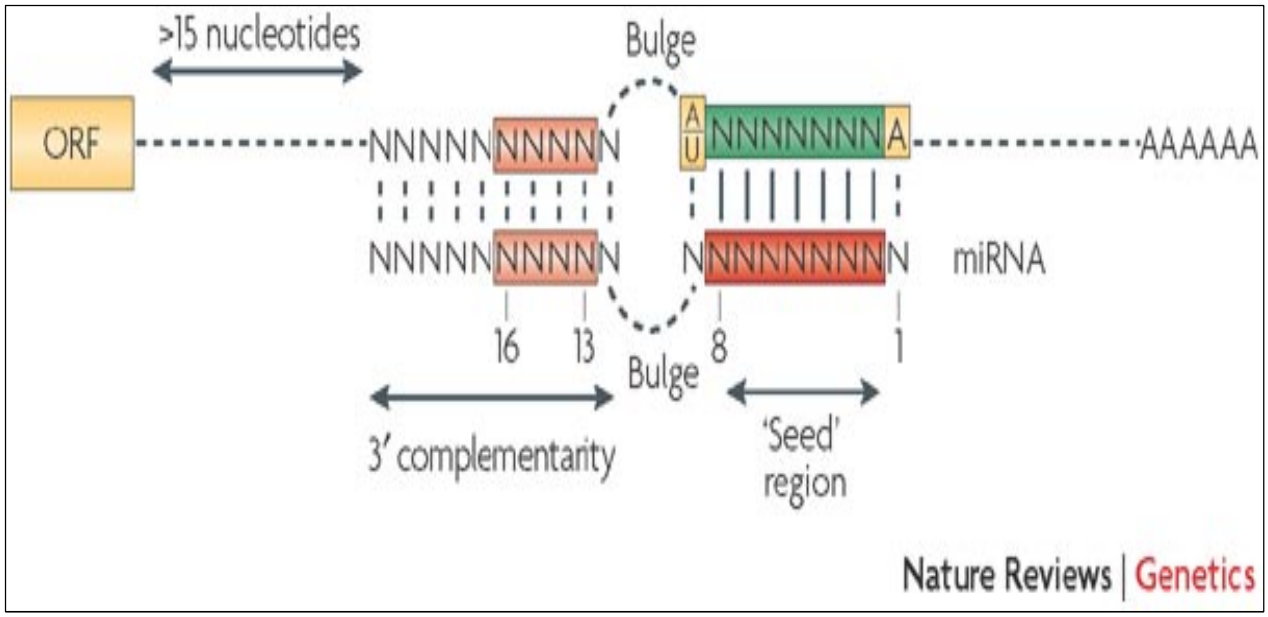

Figure 1.7: Imperfect base-pairing of miRNA and its target mRNA in animals

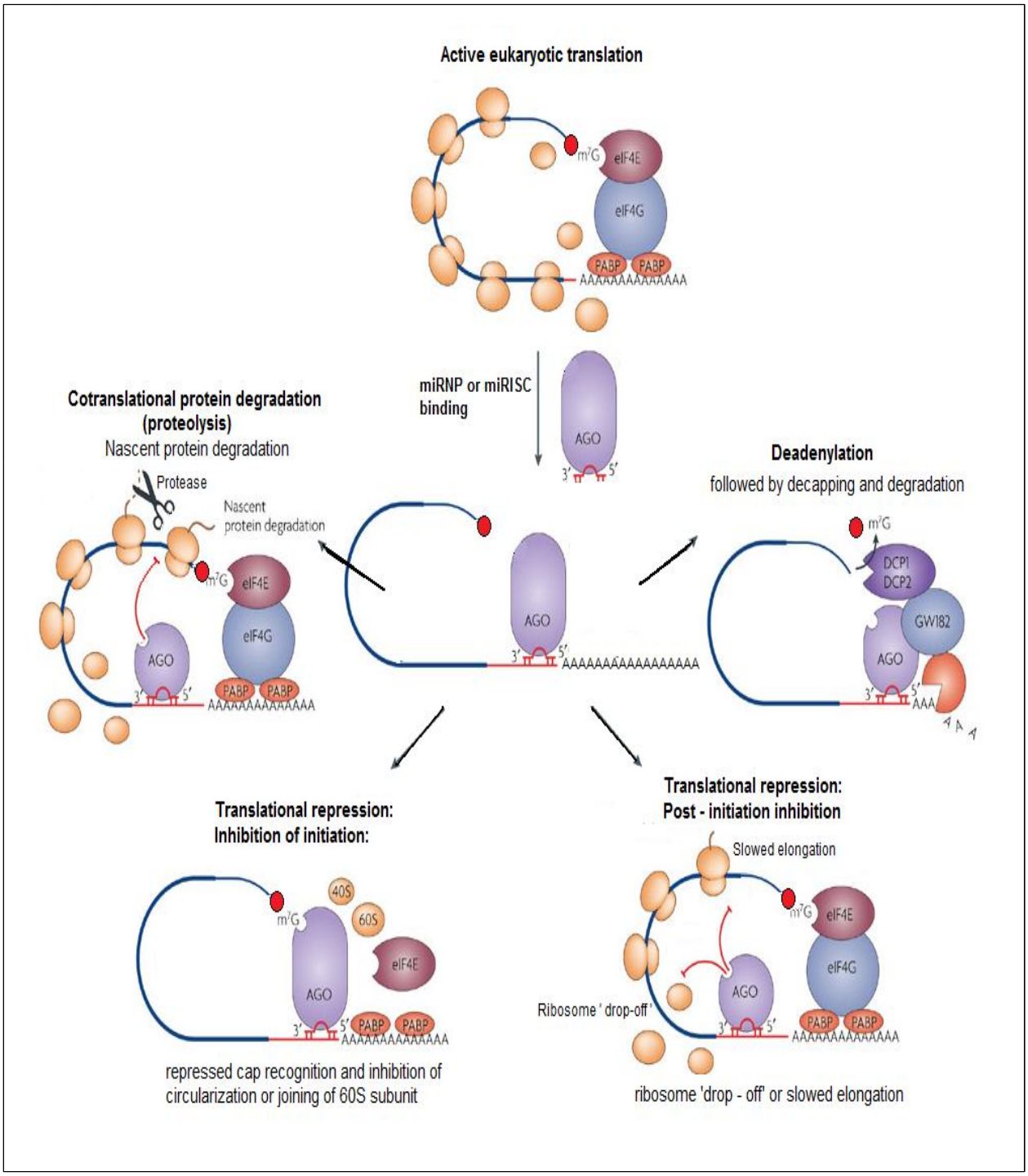

Figure 1.8: Mechanism of gene regulation by miRNA in animals (modified from (Filipowicz, Bhattacharyya et al. 2008).

Chapter 2: Effects of 5-fluorouracil on Growth, Cell Cycle Progression and Apoptosis of MCF7 Breast Cancer Cells

Abstract

Breast cancer is one of the leading causes of deaths in women worldwide. 5-flourouracil (5-FU) is an important chemotherapeutic drug used in the treatment of breast cancer patients. In this study, using several biochemical techniques, we studied the global effects of 5-FU treatment on MCF7 breast cancer cells. The dose-response curve obtained after the treatment of MCF7 cells with 23 different 5-FU concentrations for 48 hours showed a dose-dependent decrease in cell proliferation. However, an atypical bimodal or biphasic curve was obtained, with two distinct curves observed between 0.001-0.1µM and 0.7-2000µM, thus indicating that 5-FU might act via a dual mechanism. Two different IC_{50} (Inhibitory concentration 50 %) values were calculated to be 0.007 and 2.8µM. A similar bimodal inhibition of the cellular metabolism of MCF7 cells was also observed after 48 hours of treatment using the MTT assay. The acridine orange/ethidium bromide nuclei staining assay determined that the mechanism of cell death induced was mainly by apoptosis after 48 hours. The morphology of the cells also showed a distinct reduction in the cell size, with compromised anchorage ability. The cell cycle progression however remained unaffected even after treatment with high 5-FU doses after 48 hours of exposure. These findings provide evidence of the global inhibitory effects of 5-FU on human breast cancer cells *in vitro* and warrant further evaluation to study the molecular basis of its cytotoxicity.

Keywords: 5-fluorouracil, breast cancer, MCF7, bimodal, viability, apoptosis, cell cycle

Introduction

Chemotherapy with antineoplastic (or cytotoxic) drugs has been extensively used in the palliative treatment and management of breast cancer. Chemotherapeutic drugs having varying mechanisms of action are used for the treatment of breast cancer. Of the several classes of drugs, antimetabolites are one of the most effective first-line drugs. They are used as monotherapy as well as in combination regimens with other drugs. Fluoropyrimidines, mainly 5-fluorouracil (5-fluoro-2,4(1H,3H)-pyrimidinedione or 5-FU) is one such antimetabolite which is widely used mainly for the treatment of breast (Fumoleau, Bonneterre et al. 2003), colorectal (Wils, O'Dwyer et al. 2001) and head and neck cancers (Posner, Colevas et al. 2000). It is mainly used to treat more aggressive forms of breast cancer, but can be used to treat any stage of breast cancer as a part of the combination therapy of CAF (Cyclophosphamide, Adriamycin and 5-fluorouracil); CMF (Cyclophosphamide, Methotrexate and 5-fluorouracil) and CEF (Cytoxan, Ellence, and fluorouracil) (Smalley, Lefante et al. 1983; Falkson, Falkson et al. 1992). Extensive research has been performed to improve the effectiveness of these combination strategies for the treatment of breast cancer. The combination of 5-FU with other newly developed anticancer drugs such as irinotecan, tomudex (TDX), and oxaliplatin has also help improve the response rates for breast cancer (Longley, Harkin et al. 2003).

At the cellular level, 5-FU is a pyrimidine antagonist which is rapidly taken inside actively dividing cancerous cells by facilitated transport similar to uracil (Wohlhueter, McIvor et al. 1980). Once inside the cancer cells, 5-FU is converted intracellularly into three cytotoxic metabolites: fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP) and fluorouridine triphosphate (FUTP) by the sequential action of different enzymes, orotate phosphoribosyltransferase (OPRT), uridine phosphorylase (UP), uridine kinase (UK),

ribonucleotide reductase (RR) and thymidine kinase (TK) (Daher, Harris et al. 1990). These three metabolites account for all the cytotoxic effects of 5-FU. The primary mechanism of cytotoxicity of 5-FU is the inhibition of enzyme thymidylate synthase (TS) activity by FdUMP, resulting in inhibition of dTMP *de novo* synthesis (Santi, McHenry et al. 1974; Sommer and Santi 1974) and subsequent imbalances in the deoxynucleotide pool, particularly dATP/dTTP ratio (Yoshioka, Tanaka et al. 1987; Houghton, Tillman et al. 1995). This in turn inhibits DNA synthesis and repair, and thus causes DNA damage. In addition, FdUTP and FUTP gets misincorporated into the DNA strands in place of dTTP during DNA replication, resulting in allevated levels of dUTP inside the cells (Mitrovski, Pressacco et al. 1994; Aherne, Hardcastle et al. 1996). Further, FUTP is extensively misincorporated in RNA strands, which disrupts the normal RNA processing. This results in disruption of the further synthesis and processing of mRNAs (Doong and Dolnick 1988; Patton 1993), tRNAs (Randerath, Tseng et al. 1983; Santi and Hardy 1987) and rRNAs (Kanamaru, Kakuta et al. 1986; Ghoshal and Jacob 1994) and in inhibition of post-transcriptional conversion of uridine to pseudouridine in these RNAs (Samuelsson 1991). This results in RNA toxicity and imbalances, causing variability in cellular metabolism and functioning.

Cell cycle arrest and induction of apoptosis are the primary mechanisms of inhibition of cell growth by most of the anti-cancer drugs (Barry, Behnke et al. 1990; Hickman 1992; Lundberg and Weinberg 1999; Shapiro, Koestner et al. 1999; Qin and Ng 2002). Previous studies have demostrated the ability of 5-FU to arrest the cell cycle pregression of human breast cancer and colorectal cancer cells. It mainly causes a G1/S phase arrest in the cancer cells (Grem, Nguyen et al. 1999; Tokunaga, Oda et al. 2000; Mirjolet, Didelot et al. 2002; Hernandez-Vargas, Ballestar et al. 2006). Further, previous studies have attributed the cellular effects of 5-FU as

well as 5-FU in combination with other drugs to changes in p53 and p53 target molecules (Lowe, Ruley et al. 1993; Lowe, Bodis et al. 1994; Grem, Nguyen et al. 1999; Petak, Tillman et al. 2000; Tokunaga, Oda et al. 2000; Backus, Dukers et al. 2001; Yoshikawa, Kusunoki et al. 2001; Yukimoto, Nakata et al. 2001; Mirjolet, Didelot et al. 2002; Zhang, Ramdas et al. 2003; Hernandez-Vargas, Ballestar et al. 2006). Another study has reported that the induction of apoptosis by 5-FU has been linked with FAS regulation, a member of the Tumor-necrosis factor (TNF) receptor superfamily (Maxwell, Longley et al. 2003). It has also been shown to induce overexpression of several p53 target genes involved in apoptosis and cell cycle regulation, like APAF1, BCL2, BAK1; and repression of the c-Myc gene (Hernandez-Vargas, Ballestar et al. 2006).

Unfortunately, 5-FU is rarely used as a monotherapy now-a-days, and is mainly used in combination with other drug regimens. Recent research has been focussed on improving the efficacy of therapeutic drugs with fewer side effects and more specific action in cancer cells only. In this process, several prodrugs have been synthesized which have a lower hydrophobicity and more specific action in cancer cells to improve 5-FU therapy. These include Tegafur, S1, Capecitabine, Eniluracil, and several other compounds (Miwa, Ura et al. 1998; Cunningham and James 2001; O'Shaughnessy, Twelves et al. 2002; Dominguez, Marchal et al. 2003; Marchal, Rodriguez-Serrano et al. 2007). Studying the molecular effects of 5-FU on breast cancer cells will help better understand the mechanism of 5-FU cytotoxicity, which in turn would help to improve the efficacy of 5-FU treatment.

The aim of this study was to investigate the global effects of 5-FU on MCF7 breast cancer. The MCF7 breast cancer cell line has been shown to be an excellent experimental model to study breast cancer (Horwitz, Costlow et al. 1975; Engel and Young 1978; Gioanni, Le

Francois et al. 1990; Levenson and Jordan 1997; Simstein, Burow et al. 2003). Hematocytometer cell counts, microscopy, flow cytometry and staining assays were performed to determine the effect of 5-FU on cell proliferation, morphology, cell cycle progression and induction of apoptosis. The results of our study illustrated that 5-FU inhibits cell proliferation and induces apoptosis in a dose-dependent manner. However, no significant effect was observed on the cell cycle clock of the cells. Thus, the data helps to better appreciate the biological effects of 5-FU and confirms its efficiency in treatment of breast cancer.

Materials and Methods

Cell line and cell culture

All cell culture reagents were purchased from Invitrogen, Inc, Carlsbad, CA. The human breast adenocarcinoma cell line MCF7 (HTB-22™) was obtained from the American Type Culture Collection (ATCC, Rockville, MD). They were cultured in RPMI 1640 media containing L-Glutamine and 25 mM HEPES, and supplemented with 10% Fetal Bovine Serum (FBS), 10 µg/ml gentamicin and 4 µg/ml insulin and sterilized using a 0.22µM polyethersulfone filter (Corning Inc., Corning, NY). The cells were maintained at 37˚C in a humidified incubator with 5% CO_2. The media was replaced every 48 hours, and the cells were passaged once a week by trypsinization using 0.05% trypsin/0.02% EDTA (Sigma, St. Louis, MO).

Growth Curve Analysis

The growth curve and the doubling time of MCF7 cells were determined by monitoring the growth of the cells for 5 days. For this purpose, cells were seeded at $1.5x10^4$ cells/cm^2

($9.6x10^4$ cells/well) in flat-bottom 6-well plates in 2ml of RPMI 1640 complete media. After every 24 hours interval thereafter, the cells were harvested using 0.05% trypsin/0.02% EDTA and counted by the Trypan blue dye exclusion assay as described below. The media was replaced every 48 hours. The experiment was performed in triplicates on four separate occasions.

5-fluorouracil Drug treatment

5-fluorouracil (5-FU) was purchased from Sigma-Aldrich (St. Louis, MO) and stored at 4˚C, away from light and moisture. For cell treatments, a 10 mM stock solution of 5-FU was prepared in RPMI complete media and stored at 4˚C for not more than two weeks. The stock was filtered through a 0.22µM polyethersulfone filter prior to further dilution. The effect of 5-FU on the growth curve of MCF7 cells was assessed every 24 hours using the MTT dye reduction assay as described below. In the preliminary experiment, we determined that the trypan blue dye exclusion assay for cell viability correlates well with the MTT dye reduction assay. Briefly, the cells were seeded at $1.5x10^4$ cells/cm^2 (4800 cells/well) in flat-bottom 96-well plates in 100µl of complete media without phenol red; and after 24 hours, treated with four different concentrations of 5-FU (0.01µM, 1µM, 10µM and 50µM) as described below. In order to determine the cell number in each sample following the 5-FU treatments, the cells were treated with MTT (as described below) and the Optical Density (OD) was measured directly at a wavelength of 540nm using a microplate reader (Multiscan MCC/340, Fisher Scientific, Pittsburg, PA). The OD was measured before treating the cells (control), and at 24 and 48 hours. The OD of each sample was then compared with a standard curve, in which the OD was directly proportional to the actual cell numbers. The experiment was performed in triplicates in five separate trials.

Cell morphology analysis

Briefly, $5x10^5$ cells were seeded in a $25cm^2$ cell culture flask ($2.0x10^4$ cells/cm^2) in 5ml of complete media. After 24 hours, the fresh media was replaced in control flasks and media with 5-FU was replaced in the treatment flasks. The control MCF7 cells and cells treated with different concentrations of 5-FU (1µM and 50µM) for 48 hours were observed using a light microscope with a CCD camera.

Cell proliferation assays

The different inhibitory concentrations of 5-FU were then determined by generating dose-response curves after treating the cells with increasing concentration of 5-FU and analyzing the cell sensitivity using the Trypan blue dye exclusion and the MTT [3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide] dye reduction assays.

For the trypan blue dye exclusion assay, the cells were seeded at $2.5x10^4$ cells/cm^2 ($9.5x10^4$ cells/well) in flat-bottom 12-well plates in 1ml of complete media. After 24 hours, the media was replaced with fresh media containing different 5-FU concentrations (0-2000µM); a negative control (containing cells and only media but no drug) was also included. After 48 hours, the cells were detached by trypsinization, appropriately diluted with 0.4% trypan blue dye (Sigma-Aldrich, St. Louis, MO) and 10µl of this mixture was loaded on the counting chambers of the Hematocytometer. The viable cells were then visually counted using a microscope and the percentage of unstained treated cells compared to the unstained control cells was calculated. The cells were counted in three individual experiments in triplicates. The following formula was used to determine the number of viable cells/well:

Number of viable cells/ml = (Total number of viable cells counted/4) * Dilution factor * 10,000

The dose-response for 5-FU was confirmed using the MTT dye reduction assay. The technique originally developed by (Mosmann 1983) was followed. Briefly, cells were seeded at $1.5x10^4$ cells/cm^2 (4800 cells/well) in flat-bottom 96-well plates in 100µl of complete media without phenol red. After 24 hours, they were treated with various concentrations of 5-FU for 48 hours. Negative controls (containing cells and only media but no drug) and blank controls (containing only media and no cells) were also included. After 48 hours of drug treatment, 20µl of 5 mg/ml MTT (Sigma-Aldrich, St. Louis, MO) in Phosphate-Buffered Saline (PBS) (12mM) was added to each well and incubated for 4 h at 37˚C with 5% CO_2. The drug and MTT containing media was then removed and 100µl of dimethyl sulfoxide (DMSO) was added to each well to dissolve the formazan crystals. The absorbance of the plates was measured at 540nm using a microplate reader (Multiscan MCC/340, Fisher Scientific, Pittsburg, PA) and the % cell viability was calculated compared to the negative control. All experiments were performed five separate times in triplicates.

% Cell viability = [(OD of treated group – OD of blank) / (OD of control group – OD of blank)] * 100

OD = Optical density obtained using the Microplate reader.

Detection of Apoptosis and Necrosis

The induction of apoptosis and necrosis in MCF7 cells by 5-FU was determined morphologically using a Fluorescent microscope after labeling the cells with acridine orange and ethidium bromide dyes to detect nuclear changes characteristic of apoptosis in the cells as described by (Duke 1992). Briefly, cells were treated with 0.01µM, 0.3µM, 2µM and 50µM5-FU solutions for 48 hours, floating and attached cells were then collected by trypsinization, washed in PBS and resuspended in 25µl of complete media. 100µg each of acridine orange and ethidium bromide was dissolved in 1ml of PBS and 5µl of this dye mixture was mixed with 25µ

of the cell suspension. Ten µl of this mixture was loaded on a microscopic glass slide and examined under the 20X dry objective of the Fluorescent microscope using the epillumination and a filter combination suitable for observing fluorescein. A minimum of 300 cells were scored for each sample preparation and each concentration was treated in triplicates.

Acridine orange intercalates into the DNA staining it green, and binds to the RNA making it appear red. Ethidium bromide is taken up only by dead (nonviable) cells and stains them orange-red (Duke 1992). Cells were scored as live normal (bright green nuclei with intact structure), live apoptotic (bright green nuclei with condensed chromatin), dead apoptotic (red/orange nuclei with condensed chromatin) and dead necrotic (red/orange nuclei with intact structure).

Flow cytometric analysis of cell cycle progression

5-FU induced changes in the cell cycle of MCF7 cells were determined by using BD FACScan™ Flow Cytometer (Becton-Dickinson, Franklin Lakes, NJ). Briefly, $5x10^5$ cells were seeded in a $25cm^2$ cell culture flask ($2.0x10^4$ cells/cm^2) in 5ml of complete media. After 24 hours, the media was discarded and fresh media without serum (FBS) was added. Cells were synchronized by serum starvation for 5 days (120 hours), after which they were treated with 0.01µM, 2µM and 50µM 5-FU solutions in complete media (different ICs of 5-FU) or with fresh complete media (control) for 48 hours. After 48 hours, the cells were detached by trypsinization, centrifuged, washed with PBS and fixed by passing through a 23G needle into cold 70% ethanol, followed by incubating at -20˚C for 30 mins. The cells were then resuspended in 1 ml PBS containing 10µg/ml RNase (Sigma-Aldrich, St. Louis, MO) and 10µg/ml Propidium iodide (PI) (Sigma-Aldrich, St. Louis, MO) and incubated in dark for 30 mins at room temperature (RT), after which they were refrigerated until analyzed on a FACScan Flow cytometer. Ten thousand

events were captured and the data was analyzed using the CellQuest software. Each concentration was repeated five times.

Statistical Analysis

All data are expressed as mean ± S.E.M. (Standard error of the mean). Statistical significance of the differences between the control and treated groups was determined by one-way analysis of variance (ANOVA) using the PASW (Predictive Analytics SoftWare) Statistics 17.0 (SPSS Inc., Chicago, IL). Differences were considered significant if $P < 0.05$.

Results

MCF7 growth curves

The MCF7 breast cancer cell line is a moderately aggressive adherent cell line which grows in monolayer. The normal growth curve of the cells was determined over a time period of 5 days using the Trypan blue dye exclusion assay. The cells were counted every 24 hours and the cells/well was calculated. Figure 2.1 shows the summarized growth curve for four independent experiments. As shown in the figure, the MCF7 cells entered log growth phase ~ 24 hours after plating and continued to grow exponentially even after 5 days. Our data showed that the doubling time of the cells was approximately 31 hours. Our data concurs with the ATCC guidelines, which state that the doubling time for MCF7 cells is about 29 hours.

Growth inhibitory effect of 5-FU on MCF7 cells

The effect of 5-FU on the growth of MCF7 cells was determined using the MTT dye reduction assay. For this purpose, the cells were treated with different 5-FU concentrations, the

optical density was measured at times 0, 24 and 48 hours (Figure 2.2a), and the actual cells/well was calculated by comparing the optical density to a standard curve (Figure 2.2b). Figures 2.2a and c show the dose-response curves of MCF7 cells at 24 and 48 hours. These figures illustrates that cells treated with 5-FU exhibit a dose- and time-dependent reduction in their proliferation rate. A reduction in cell growth compared to control untreated cells was observed at 24 hours itself. However, a significant reduction in the cell number was observed following 48 hours of exposure to 5-FU ($p < 0.05$). Concentration as low as 0.01µM of 5-FU was shown to be sufficient to inhibit cell growth. Further, a higher inhibition of cell growth was observed with increasing 5-FU concentrations. The growth inhibitory rate of 50µM 5-FU amounted to around 50% inhibition compared to control cells at 48 hours. Thus, the data suggest an increase in drug effect with higher drug concentrations and longer exposure.

Changes in cell morphology following 5-FU treatment

The changes in morphology of the MCF7 cells after 5-FU exposure was analyzed using phase contrast microscopy. The cells were treated with 1µM and 50µM 5-FU for 48 hours and observed directly using a light microscope. Figure 2.3a shows the control untreated adherent MCF7 cells and Figure 2.3b shows the control cells that have been detached by trypsinization. The untreated MCF7 cells are circular when unattached, and variously shaped when attached to the culture plate. Figures 2.3c and 2.3d show MCF7 cells treated with 1µM and 50µM respectively. As shown in the figures (arrow marks), cells exposed to 5-FU exhibited an obvious change in their morphological characteristics. Cells were shrunken and rounded which increased in a dose-dependent manner. Further, it was observed that 5-FU treated MCF7 cells exhibited compromised anchorage to the culture plate, and increased amount of floating cells were observed with higher 5-FU concentrations.

Dose-response curve of MCF7 cells after 5-FU treatment

To further study the anti-proliferative effects of 5-FU on MCF7 cells, the cells were treated with different 5-FU concentrations over a broad range (0-2000µM) for 48 hours and the cell viability was measured using the Trypan blue dye exclusion and the MTT dye reduction assays. The trypan blue dye exclusion assay distinguishes between live, viable cells with intact cell membrane which exclude the trypan blue dye, and the non-viable cells which will take up the dye. Alternatively, the MTT dye reduction assay considers the ability of the viable cells to metabolically reduce the MTT dye to an insoluble colored compound, which can then be measured spectrophotometrically. The viable cell counts were calculated compared to the control untreated MCF7 cells as described under *Methods and Materials* and the data were plotted vs. log drug concentrations to give the dose-response curves from which the Inhibitory concentrations (ICs) values for 5-FU were determined. The IC_{50} was defined as the drug concentration required to reduce the cell number by 50% as compared to the control untreated cells; the other ICs for 5-FU were defined similarly.

Figure 2.4 shows the dose-response curve of 5-FU treatment in MCF7 cells. As shown in the figure, treatment of MCF7 cells with increasing concentrations of 5-FU for 48 hours gave an atypical dose-dependent decrease in cell number ($p < 0.001$). It was observed that with increasing 5-FU concentrations, the cell numbers decreased initially (0-0.5µM), followed by an increase (0.7µM) and then again a decrease in the cell numbers (1-2000µM). Concentrations as low as 0.005µM and 0.01µM 5-FU induced a significant reduction in MCF7 cell numbers, ~ 35% and 72% inhibition respectively compared to the control cells ($p < 0.001$). 0.01-0.5µM 5-FU caused a significant decrease in MCF7 cell number (~ 75% inhibition), but little difference was observed among these treatments, thus creating a plateau phase in the curve. However,

treatment with 0.7µM 5-FU showed a significant increase in the cell counts, with only 30% cells inhibited compared to the control MCF7 cells ($p < 0.001$). This rise in the cell numbers was followed again by a decline in a dose-dependent manner with increasing 5-FU concentrations. The MCF7 cells were almost completed inhibited at concentrations greater than 50µM 5-FU. As a result of the bimodal dose-response curve, two separate IC_{50}s of 5-FU were calculated, 0.007µM and 2.8µM respectively. The results suggest that 5-FU is potent to inhibit growth of MCF7 cells even at an extremely low concentration. Table 2.1 gives the different ICs of 5-FU in MCF7 cells after 48 hours of treatment.

In parallel with this assay, the effects of 5-FU on MCF7 cells were also examined by the MTT dye reduction assay. The results obtained with the MTT dye reduction assay were strongly correlated with those obtained by the Trypan blue dye reduction assay (n = 24, $r = 0.779$, $p < 0.001$). Figure 2.5 shows the dose-response curve of 5-FU in MCF7 cells at 48 hours by the MTT dye reduction assay. The MCF7 cells exhibit a similar growth inhibition pattern as observed by the Trypan blue dye exclusion assay. 0-0.5µM 5-FU causes a partial reduction in the optical density of the cells, with highest inhibition of 25% at 0.5µM. A similar increase in the optical density of the cells was observed at 0.7µM, followed by a decline in a dose-dependent manner. This demonstrates that 5-FU significantly interferes with the metabolic activity of the MCF7 cells. The data is consistent with that from the Trypan blue dye exclusion assay. However, even very high 5-FU concentrations failed to completely inhibit the cellular metabolism.

Induction of apoptosis by 5-FU treatment

To determine if 5-FU induced apoptosis in MCF7 cells, the cells were exposed to varying concentrations of 5-FU (0-50µM) and evaluated for morphological changes characteristic of

apoptosis by AO/EB staining for fluorescence microscopy. Acridine orange (AO) enters live cells and stains them green, while Ethidium bromide (EB), a DNA-binding fluorescent dye, is taken up by only non-viable, dead cells and stains its DNA red. As shown in Figure 2.6, treatment of MCF7 cells with 5-FU led to four morphologically distinct populations of cells on AO/EB stained samples: uniformly green cells with normal nuclei (Live normal cells, Figure 2.6a), Live apoptotic cells (Figure 2.6b), Dead apoptotic cells (Figure 2.6c) and Dead necrotic cells (Figure 2.6d), as described in the Materials and Methods section. Figure 2.7 gives the response of MCF7 cells to different 5-FU concentrations. Live, normal cells were observed mainly in control cells and their numbers decreased significantly following 5-FU treatment. Presence of live and dead apoptotic cells was evaluated to indicate induction of apoptosis in MCF7 cells. At 48 hours, 5-FU induced significant numbers of apoptotic cells in a concentration-dependent manner. Early apoptotic cells were observed at concentrations as low as 0.002µM, and reaching 82% at 50µM (Figure 2.8). Alternatively, dead necrotic cells were not shown to be significantly increased following 5-FU treatment. Thus, both light microscopy and AO/EB staining assay together suggest that 5-FU is able to induce apoptosis in MCF7 cells even at low concentrations.

Effect of 5-FU on cell cycle of MCF7 cells

To determine the effect of 5-FU on the cell cycle of MCF7 cells, flow cytometric analysis was performed. Synchronized MCF7 cells were incubated with or without increasing concentrations of 5-FU for after 48 hours, and relative DNA content was analyzed by propidium iodide staining followed by flow cytometry. As shown in figures 2.9 and 2.10, no significant differences in cell cycle distribution were found after exposure to 5-FU ($p = 0.166$; $p = 0.290$; $p = 0.160$ for G1, S and G2 phases respectively). The data showed that control untreated sample

had a higher percentage of cells in the G0/G1 phase of the cell cycle. After treatment with increasing concentrations of 5-FU, the cells were still present in the G0/G1 phase of the cell cycle. All results were virtually indistinguishable from the control untreated cells. Further, no significant apoptosis was detected even at 50µM 5-FU.

Discussion

The fluoropyrimidine 5-FU has been used in clinical practice for almost 50 yrs, and it has been shown that a good correlation exists between 5-FU plasma levels and the biological effects of 5-FU treatment. Presently, the standard approach for calculating the 5-FU drug dosage has been to use body surface area (mg/m^2), which results in considerable variability in 5-FU plasma concentrations. 5-FU yields a 20% response rate in patients with metastatic breast cancer when given as a single drug by bolus i.v. injection (Ansfield, Klotz et al. 1977). (Finch, Bending et al. 1979) reported that i.v. administration of a single dose of 5-FU (370 to 560 mg/m^2) gives a peak plasma concentration of 13 to 130 µg/ml. Another study by (Muller, Mader et al. 1997) have demonstrated that administration of a single i.v. dose of 600 mg/m^2 5-FU for 5 mins in breast cancer patients achieved a tumor concentration of 16.4 (± 6.7) µg/ml and plasma concentration of 27.3 (± 4.1) µg/ml. The plasma half-life is approximately 6 to 20 min and varies significantly among patients. Within 6 hours of administration, plasma concentrations fall below 0.13 µg/ml. Further, the major urinary metabolite, α–fluoro-ß-alanine (FBAL), has a plasma elimination half-life of approximately 33 hours (Huan, Pazdur et al. 1989; Porter, Chestnut et al. 1992). However, single-agent bolus 5-FU does not seem to have much of a role in the treatment of breast cancer

(Rubens 1991), and this is partly related to the low response rates, about 29% (Cameron, Gabra et al. 1994).

The short half life and S phase-specificity (Pinedo and Peters 1988) of 5-FU makes it pharmacokinetically suitable for administration as a continuous infusion which ensures prolonged cancer cell exposure and thereby enhances 5-FU cytotoxicity. Continuous infusion of 5-FU is very well tolerated, as demonstrated by various studies. It is possible to administer doses of 1-1.4 g/m^2/day up to 5 days; 500 mg/m^2/day up to 30 days (Lokich, Bothe et al. 1981; McDermott, van den Berg et al. 1982; Hansen, Quebbeman et al. 1987; Chang, Most et al. 1989; Hatfield AK 1989; Huan, Pazdur et al. 1989; Jabboury, Holmes et al. 1989; J Berlie 1990; Lokich, Ahlgren et al. 1991; Cameron, Gabra et al. 1994; Ng, Cameron et al. 1994); and 250 mg/m^2/day for up to 3 weeks (Regazzoni, Pesce et al. 1996). (Petit, Milano et al. 1988) have reported a circadian rhythm-varying pattern in the plasma concentrations of 5-FU following a five-day continuous venous infusion. Some authors have reported that plasma peak levels of up to 584 ng/ml 5-FU were achieved by the continuous infusion of 450-955 mg/m^2/day (Petit, Milano et al. 1988); while others have reported that maximum dose of only 300 mg/m^2/day can be administered for a prolonged period of time without any side effects (Lokich, Ahlgren et al. 1991), and this results in 5-FU plasma concentration of only 27 ng/ml (Harris, Song et al. 1990). Thus, a large variability have been shown to exist in the plasma levels of 5-FU following i.v. infusions. However, an optimal therapeutic window has been recommended by several pharmacokinetic clinical studies to obtain improved clinical outcomes and reduced toxicity. A consistent target range of AUC (area under the concentration range) of 20-25 mg.h/L or a plasma concentration of 450-550 µg/L have been established as most optimal despite different

administration modes (bolus/ infusion i.v. or oral) and time schedules (Gamelin, Delva et al. 2008).

In the present study, we confirmed the anti-tumor properties of 5-FU in vitro and have shown that 5-FU inhibits the growth of MCF7 cells in a dose- and time-dependent manner. Treatment with different concentrations of 5-FU for 48 hours was observed to significantly inhibit MCF7 cell proliferation. A wide 5-FU concentration range, ranging from 0-2000µM, with small interval groups was selected to determine the minimum effective and maximum effective concentrations of 5-FU exposure. A unique dose-response curve was obtained following 5-FU treatment. The MCF7 cells displayed a bimodal dose-response curve after treatment with 5-FU for 48 hours. A significant decline in the cell number was observed at very low doses of 5-FU, followed by an increase in the cell number and further subsequent decrease as very high 5-FU concentrations were used. Thus two separate dose-response curves were obtained between 0-0.5µM and 0.7-2000µM 5-FU. Doses as low as 0.005µM and 0.01µM were capable of inhibiting 35% and 72% of MCF7 cell numbers respectively. A plateau stage was then observed between 0.01 – 0.5µM 5-FU doses, indicating that these doses were equally capable of inhibiting MCF7 growth (~ 75% inhibition).

Two separate sets of ICs of 5-FU were calculated because of the bimodal response curve. The IC_{50}s of 5-FU in MCF7 cells following 48 hours of exposure were calculated to be approximately 0.007 and 2.8µM. Several studies have been performed to determine the inhibitory concentrations of 5-FU on the MCF7 cells. (Wang, Cassidy et al. 2004) reported an IC_{50} of 1.2µM following exposure of MCF7 cells to 5-FU for 48 hours. Other studies have reported IC_{50} values of 10µM (Hernandez-Vargas, Ballestar et al. 2006) and 23µM (Raymond, Buquet-Fagot et al. 1997) following treatment for 48 hours. Different 5-FU IC_{50} have been

reported after treatment of MCF7 cells with 5-FU for longer period of time: 0.7µM after 3 days treatment (Akbulut, Tang et al. 2004); 13µM after 4 days treatment (Miwa, Ura et al. 1998); 6.3µM after 5 days treatment (Etienne, Ilc et al. 2004); and 1.03µM after 7 days treatment (Patterson, Zhang et al. 1995). It is easy to understand that the IC_{50} is decreased as the time of treatment increased, however the variations in the IC_{50} values of 5-FU might be caused by cell culture maintenance conditions, cell passage number or treatment conditions.

A bimodal response has not been previously reported for 5-FU. The reason for this could be because the effects of 5-FU has not been studied over a very large concentration range, and over small concentration intervals. In this study, 23 different 5-FU concentrations were analyzed covering the range of 0-2000µM, which helped get a detailed dose-response curve for 48 hours time point. Gurevich (Gurevich 2001) discussed that low doses of certain biologically active substances sometimes function with a different mechanism of action as compared with the clinically relevant doses. A similar low-dose response curve was obtained between 0-0.5µM, suggesting that a low-dose treatment with 5-FU might function with a different mechanism compared to the clinical doses. A similar bimodal response has been reported for some other compounds. The alkaloid sanguinarine was shown to act via a bimodal cell death, apoptosis and oncosis, in human cervical cancer cells (Ding, Tang et al. 2002). 5-FU might act with a dual mechanism of action, which is dose-dependent; low doses elicit a different response than the higher doses. Further work needs to be performed in order to support this hypothesis.

The MTT dye reduction assay was also used to determine the effect of 5-FU on MCF7 cell viability and proliferation. It is a spectrophotometric assay which helps to distinguish between metabolically active cells from the non-active cells depending on their ability to metabolically convert MTT. Thus, although there is an obvious difference in the MCF7 growth

curves obtained by Trypan blue dye exclusion assay and MTT assay, the MTT assay mainly demonstrates the effect of 5-FU on cell metabolism. The curves obtained by both the methods show the same trend in the growth curve and a bimodal response for 5-FU. Further, the results obtained with the MTT dye reduction assay show a strong correlation with those obtained by the Trypan blue dye reduction assay ($n = 24$, $r = 0.779$, $p < 0.001$). The results obtained from the morphological examination and AO/EB assay further confirm with these results. Higher concentrations of 5-FU are able to induce morphological changes and damage the ability of the cells to adhere. The results from the AO/EB assay look at the different stages of cell death induced after 5-FU exposure. A significant increase in the number of both live and dead apoptotic cells was observed following treatment with higher 5-FU concentrations. The study together confirms the ability of 5-FU to inhibit proliferation and induce apoptosis in MCF7 breast cancer cells.

Flow cytometry was used to analyze the effect of 5-FU treatment on the cell cycle progression of MCF7 cells. The results obtained in this study suggested that 5-FU does not have an influence on cell cycle progression in MCF7 cells. Contrasting results have been previously reported by other studies. (Grem, Nguyen et al. 1999) demonstrated that 5-FU caused an accumulation of MCF7 cells in S-phase, along with induction of p53 and p21 proteins and DNA strand breaks. (Hernandez-Vargas, Ballestar et al. 2006) also reported a G1 phase block following 5-FU treatment. Similar effects of 5-FU have also been described in other cancer cell line (Tokunaga, Oda et al. 2000). However, no significant accumulation of cells in G1-S phase was observed after 5-FU treatment as compared to the control MCF7 cells. The reason for this inconsistency could be because of the technique used for cell synchronization prior to flow cytometric analysis. Cell synchronization by serum starvation results in accumulation of cells in

the G1 phase. As a result, the control cells also showed cells accumulated in the G1 phase, and no significant difference could be observed in 5-FU treated samples compared to the control. The data represented here thus cannot conclude anything about the effect of 5-FU on the cell cycle progression of MCF7 cells.

In summary, this study confirmed the anti-tumor activity of 5-FU in human breast cancer cell *in vitro*. Our results indicated that 5-FU significantly inhibited cell proliferation of MCF7 cells in a dose-dependent manner, and with a plausible dual mechanism of action. Further work needs to be carried out to show this. Induction of apoptosis was shown to be one of the major mechanisms of cytotoxicity, as significant apoptosis was seen even at low doses of 5-FU. However, any effects on the cell cycle progression were not shown by our results. These experimental findings provide evidence of the global effects of 5-FU on human breast cancer cells *in vitro* and warrant further evaluation to study the molecular basis of its cytotoxicity.

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Table 2.1: Different inhibitory concentrations (ICs) of 5-FU in MCF7 breast cancer cells

IC values at 48hours	5-FU Concentrations (µM)
LEC	0.005
IC_5	0.001
IC_{10}	0.002
IC_{25}	0.004
IC_{50}	0.007, 2.8
IC_{75}	0.15, 0.5, 6
IC_{90}	50

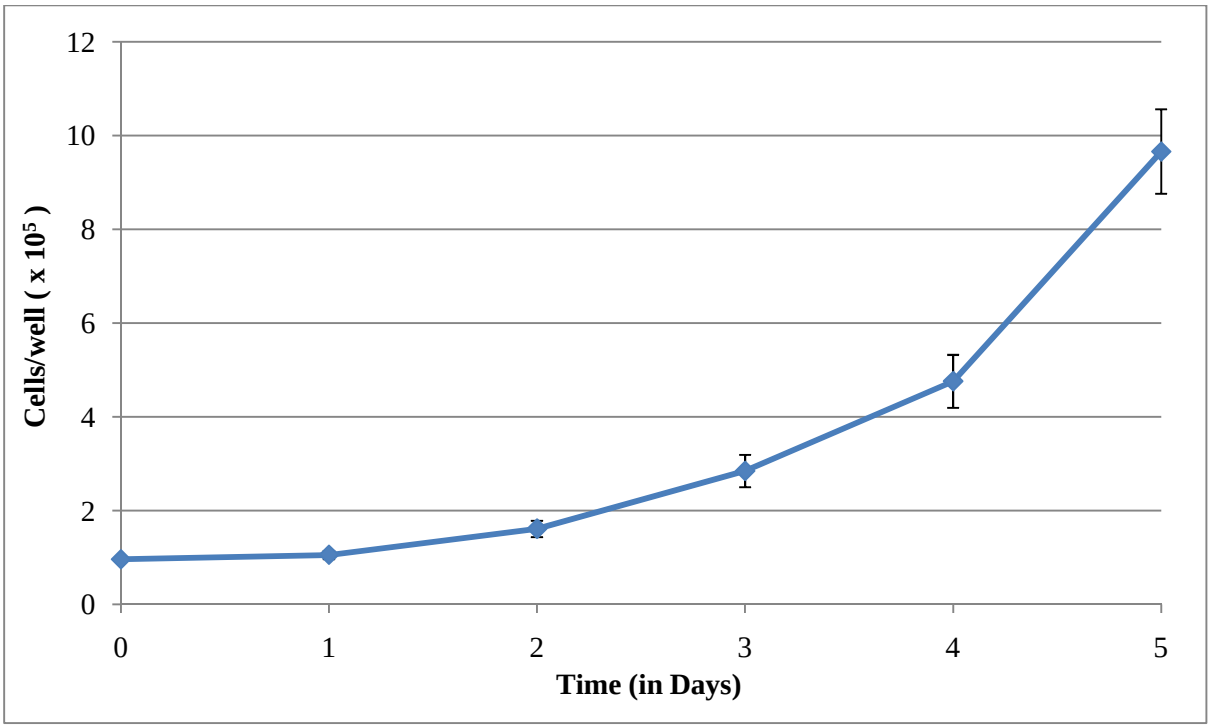
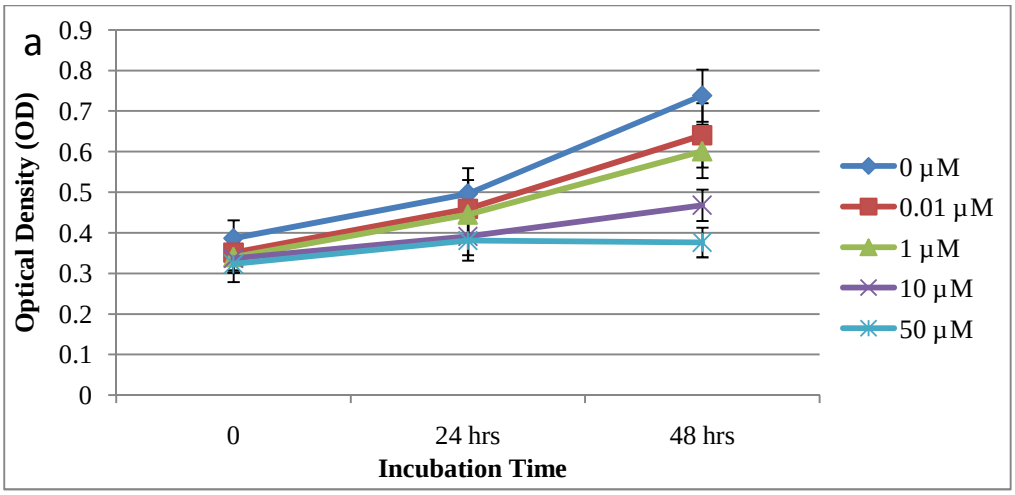


Figure 2.1: Growth curve of MCF7 cells. Cells were seeded in RPMI complete media, followed by harvesting them every 24 hours for 5 days and counted using a hematocytometer. The cells/well was calculated for each day and plotted against time to determine the growth curve of MCF7 cells. Results are mean ± SEM of four independent experiments.



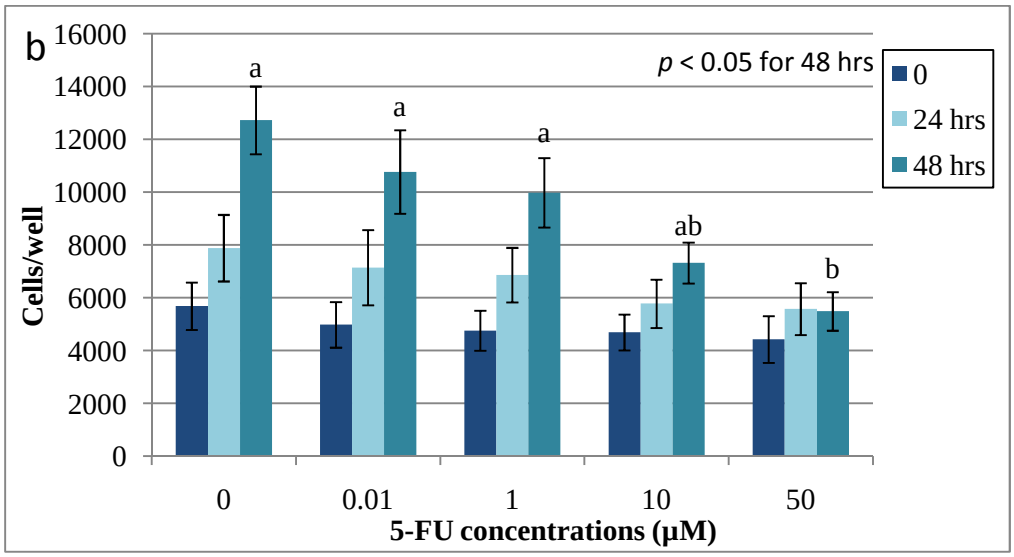


Figure 2.2 Effect of 5-FU on the growth and proliferation of MCF7 cells at 24 and 48 hours. a) OD vs. incubation time for different 5-FU concentration. The OD of each treatment was compared with the OD of control cells and plotted against time. The results are mean ± SEM for five different experiments. b) The cells/well vs. different 5-FU concentrations (µM). The cells/well for each treatment was determined and plotted against different 5-FU concentrations to investigate the effect of 5-FU on proliferation rate of MCF7 cells following 24 and 48 hours. Results are mean ± SEM for five separate trials. *p* values are 0.848, 0.589, 0.003 for 0, 24 and 48 hours of treatment with 5-FU respectively.

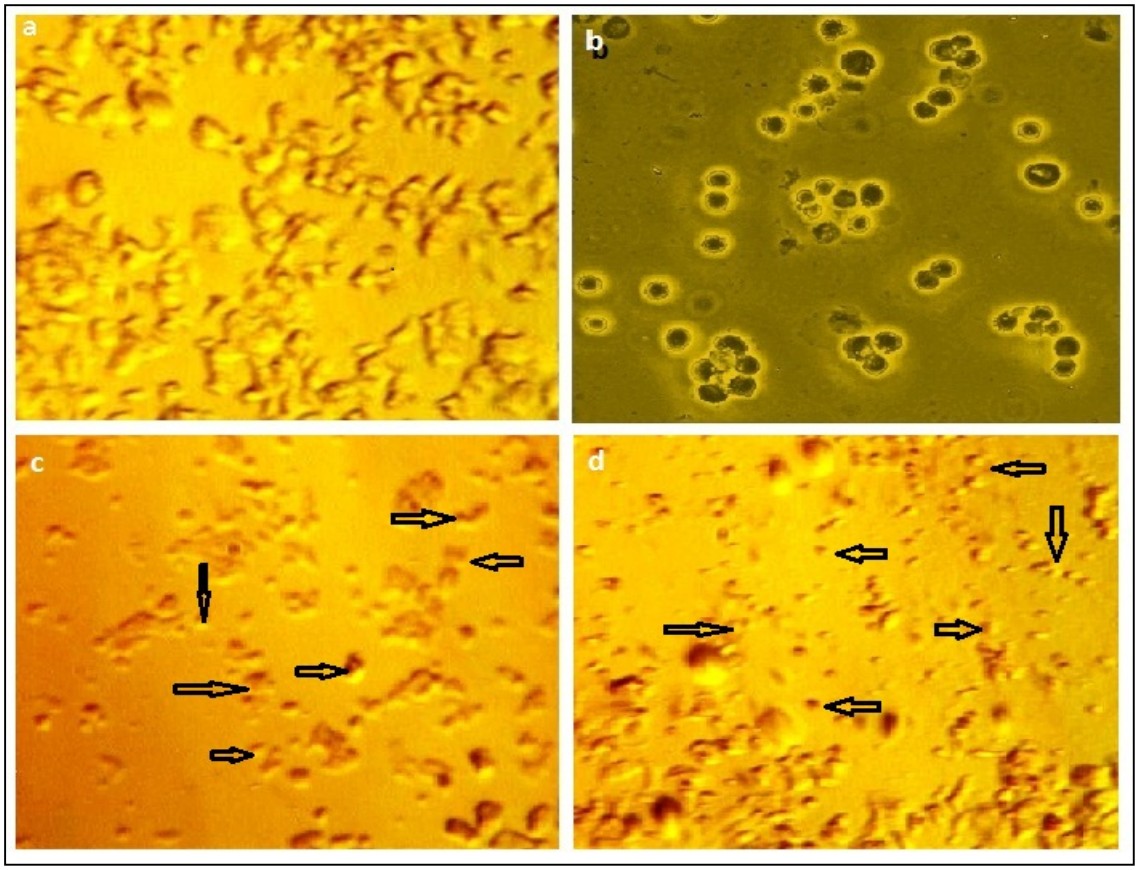


Figure 2.3: Morphological changes induced in MCF7 cells by 5-FU after 48 h treatment. Cells were seeded in a 5 cm^2 flask and treated with 5-FU (0, 1 and 50µM) for 48 hours. Cells were then observed using a light microscope and photographed after 48 hours. a) Control (untreated) MCF7 cells after 48 hours. Attached cells grow in monolayer and appear big and variously shaped. b) Control (untreated) MCF7 cells when unattached by trypsinization and observed using a glass slide. c) MCF7 cells treated with 1µM of 5-FU for 48 hours. The cells appeared shrunken and more were found detached following 5-FU treatment. Affected cells are marked with arrows. d) MCF7 cells treated with 50µM of 5-FU for 48 hours. The number of detached and shrunken increased, in a dose-dependent manner. Affected cells are marked with arrows.

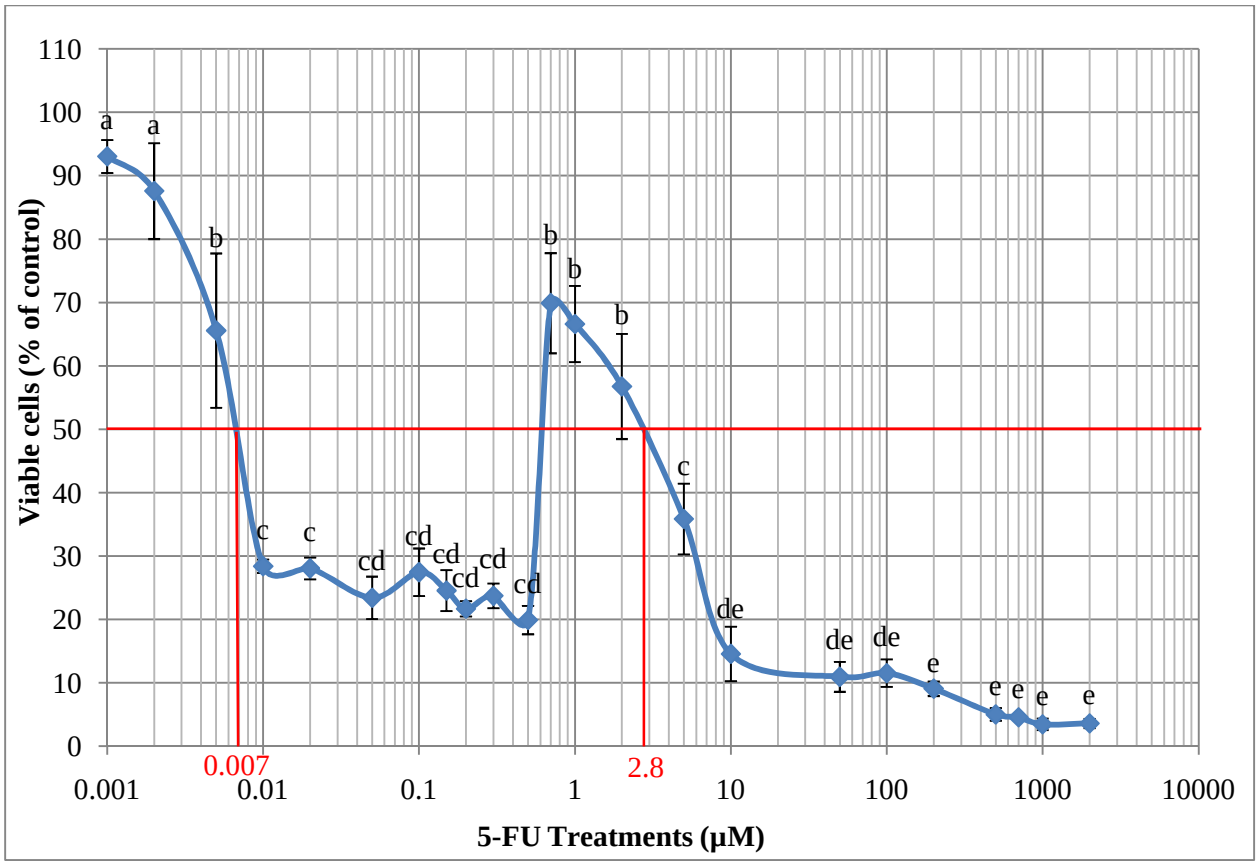


Figure 2.4: Dose-dependent effect of 5-FU on the growth of MCF7 breast cancer cells represented in a logarithmic graph. The cells were treated with different concentrations of 5-FU for 48 hours and IC values were calculated by using the Trypan blue dye exclusion assay. Percent inhibition was calculated compared to the control MCF7 cells. IC_{50} values have been shown in red in the graph. The results are mean ± SEM for triplicates in three different experiments ($p < 0.001$). Points with the same letter are not statistically different.

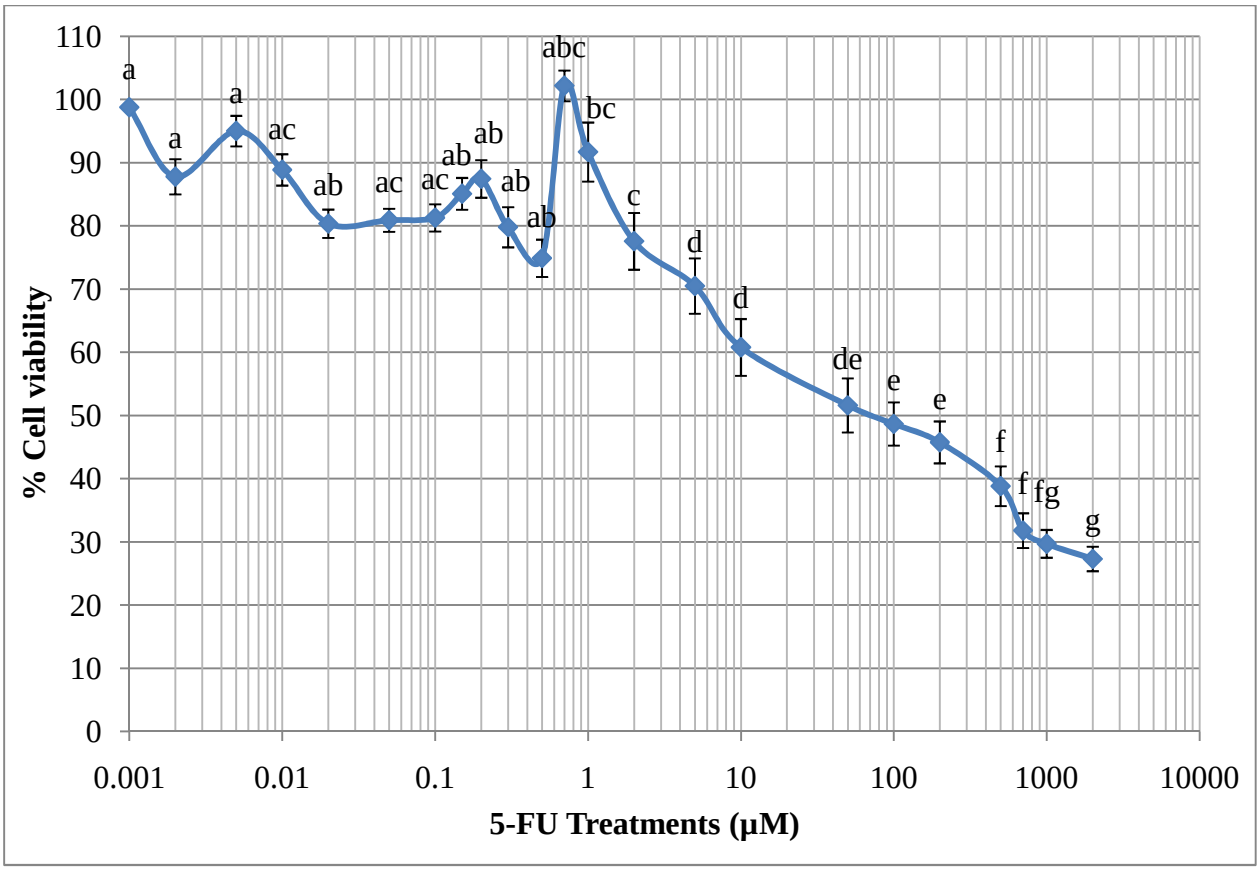


Figure 2.5: Dose-response curve for 5-FU in MCF7 breast cancer cells obtained from the MTT dye reduction assay and represented in a logarithmic graph. The cells were treated with different concentrations of 5-FU for 48 hours and the OD of each treated sample was compared to the control MCF7 cells to obtain the percent cell viability. The results are mean ± SEM for triplicates in five separate experiments ($p < 0.001$). Points with the same letter are not statistically different.

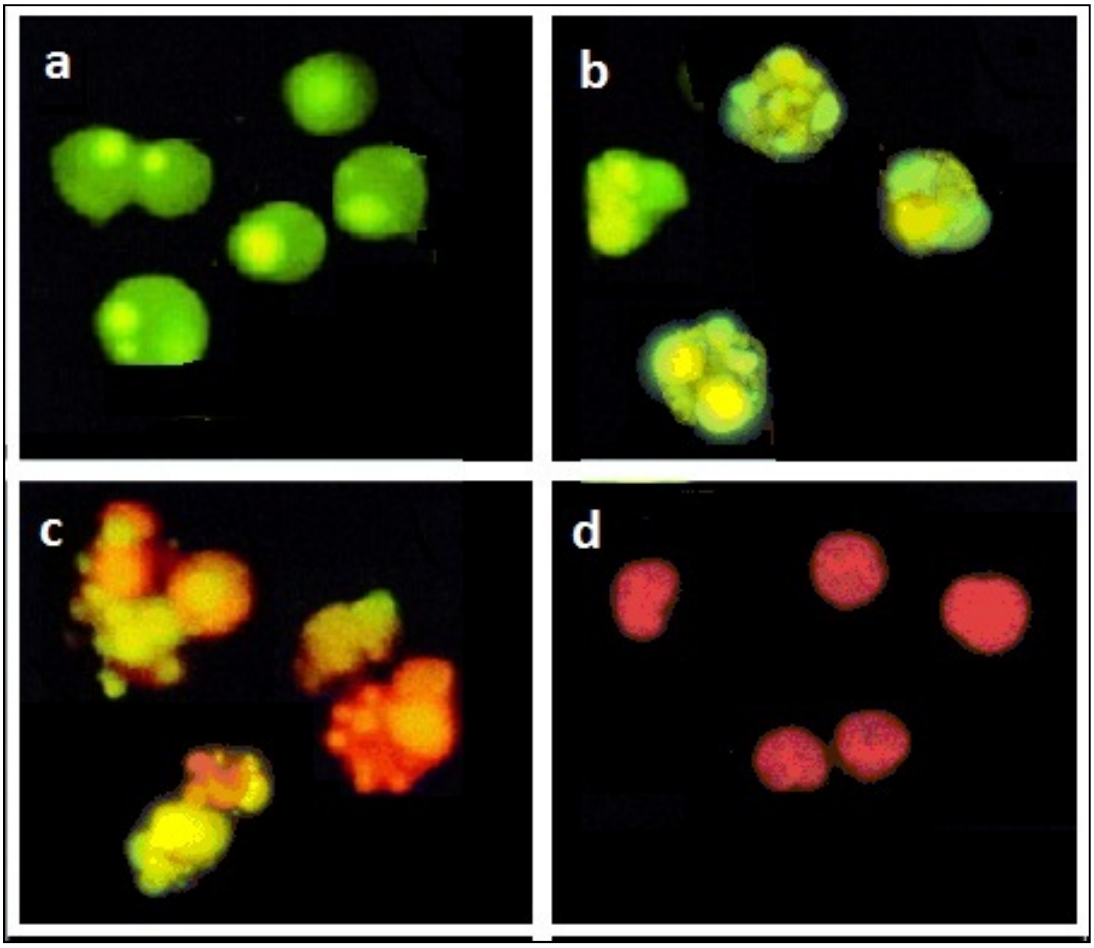


Figure 2.6: Morphological analysis of induction of apoptosis in MCF7 cells after treatment with 5-FU for 48 hours using the Acridine orange / Ethidium bromide (AO/EB) nuclei staining assay. a) Live normal cells - bright green with an intact nucleus; b) Live apoptotic cells - bright green nuclei with condensed chromatin; c) Dead apoptotic cells - red/orange nuclei with condensed chromatin; d) Dead normal cells - red/orange nuclei with intact structure.

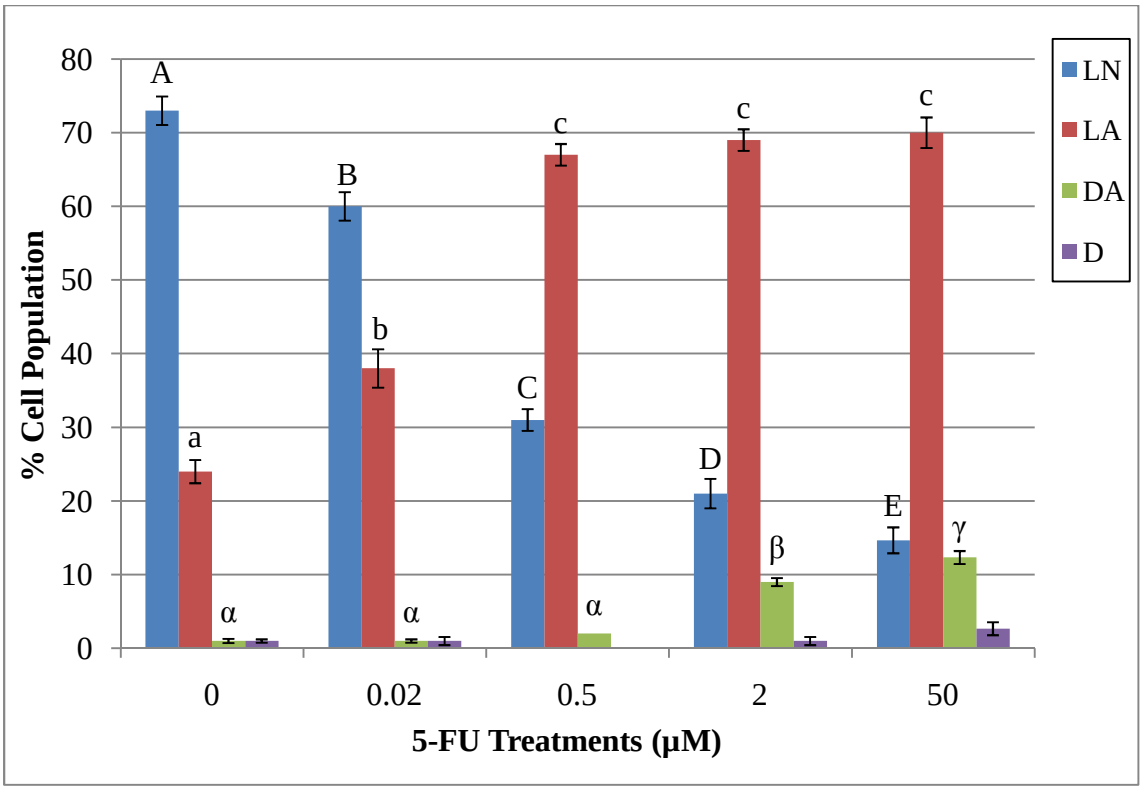


Figure 2.7: Acridine Orange/Ethidium Bromide Apoptosis analysis of MCF7 cells treated with 5-FU for 48 hours. LN= live normal, LA= live apoptotic, DA= dead apoptotic, DN = Dead necrotic. The results are represented as mean ± SEM. Statistically significant differences in 5-FU concentration are represented by single letters on the error bars ($p < 0.001$). Values that share a letter are not statistically significantly different.

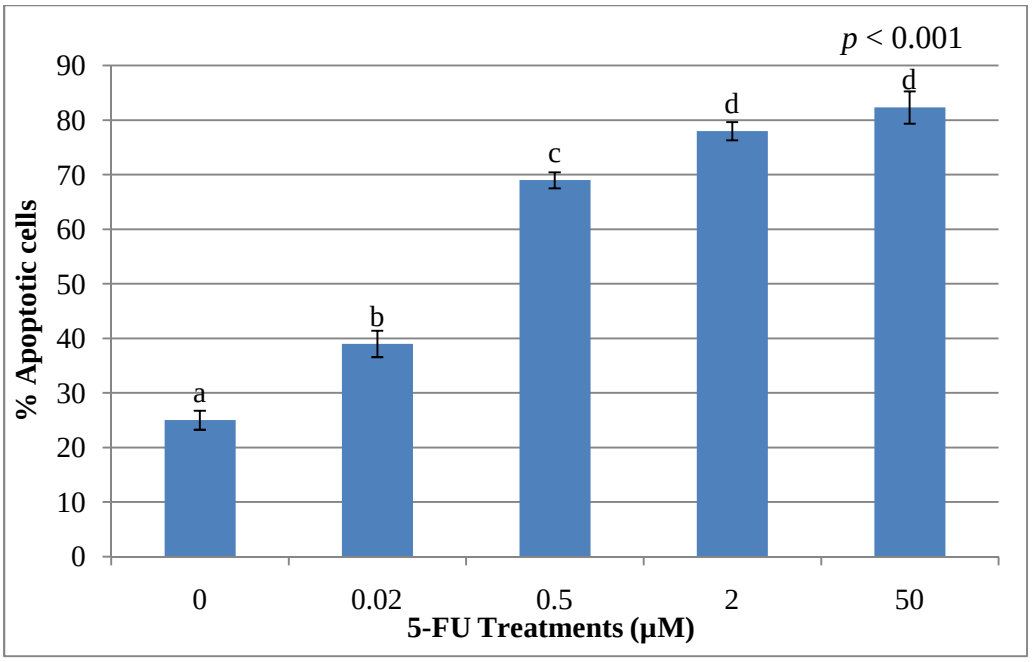


Figure 2.8: Total apoptosis induced following treatment of MCF7 cells with 5-FU for 48 hours. Both live apoptotic and dead apoptotic cells were included to estimate the total % apoptosis induced by 5-FU. The results are represented as mean ± SEM ($p < 0.001$). Values that share a letter are not statistically significantly different.

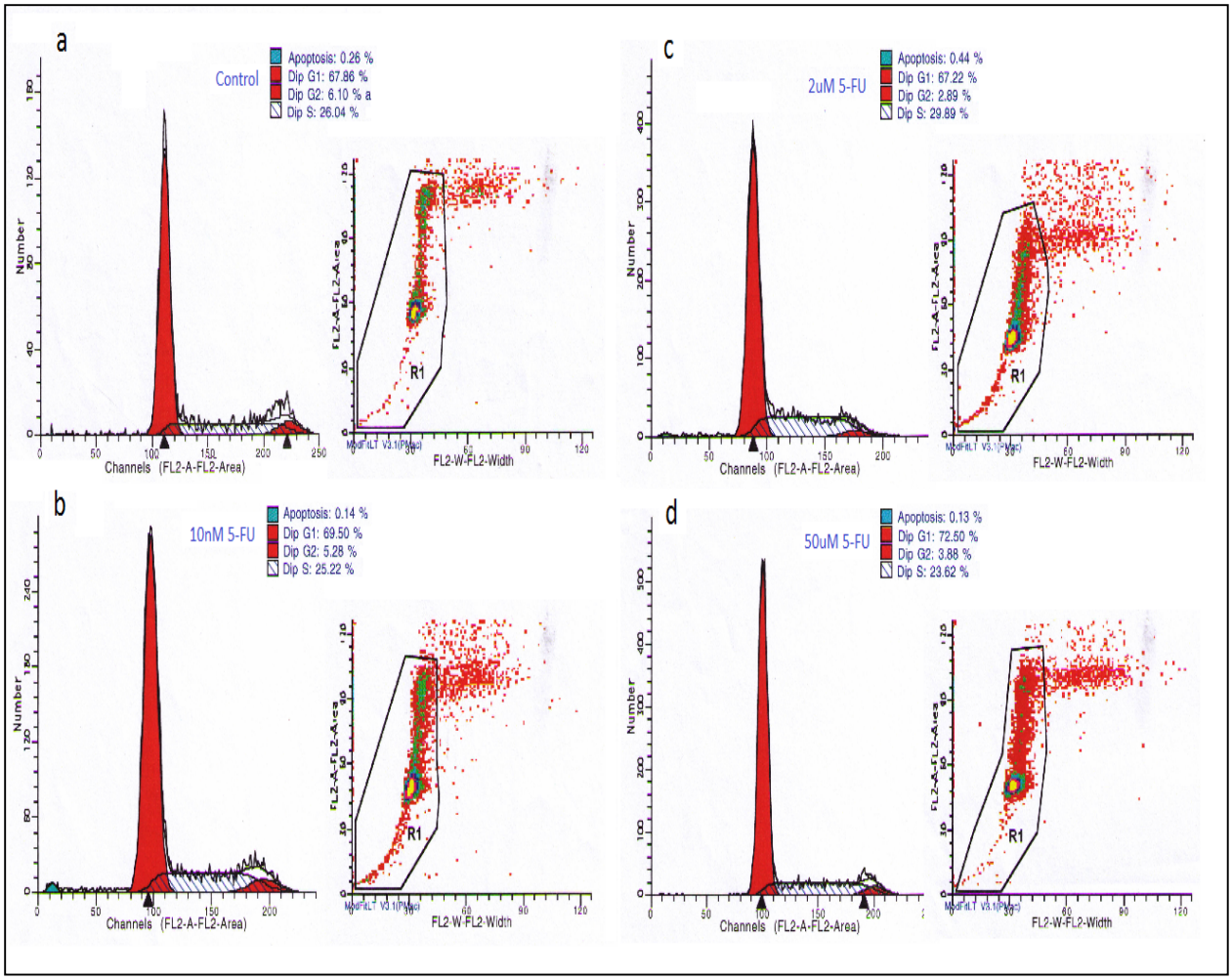


Figure 2.9: Flow cytometry analysis showing the DNA histogram and scatter plots for MCF7 cells following treatment with four different 5-FU concentrations for 48 hours: a) Control MCF7 cells; b) 10nM 5-FU; c) 2µM 5-FU; d) 50µM 5-FU

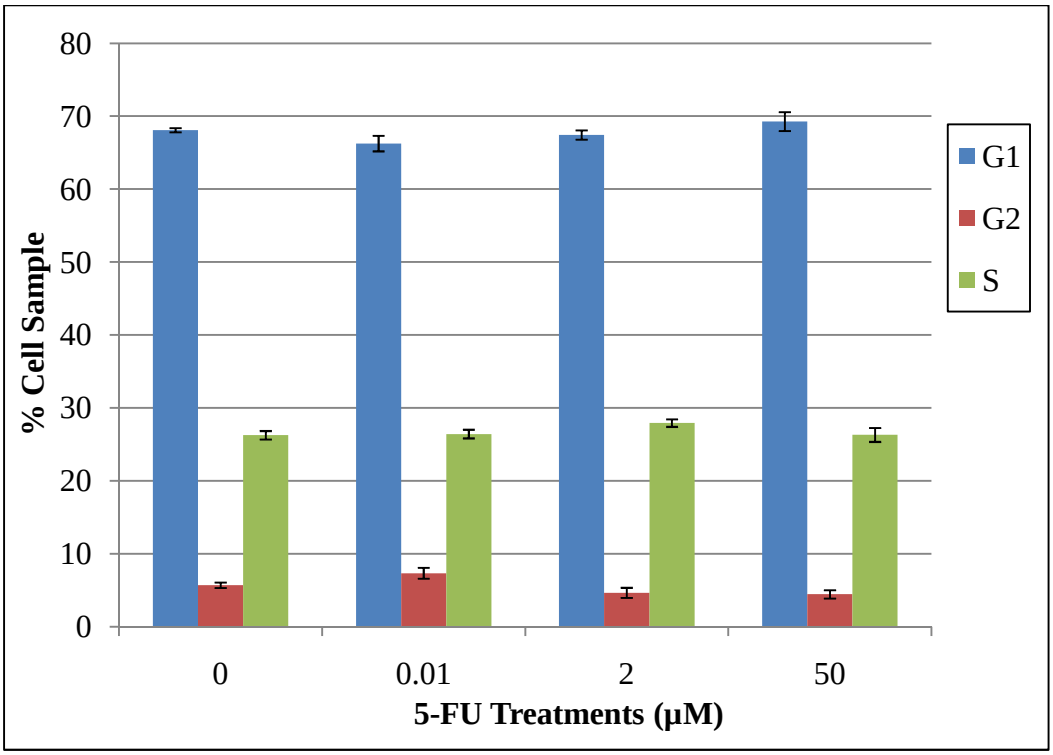


Figure 2.10: Cell cycle analysis of MCF7 cells treated with 5-FU for 48 hours. Cells were synchronized prior to 5-FU treatment using serum deprivation. Treated cells were then analyzed using the FACScan Flow cytometer. The results are represented as mean ± SEM of five individual experiments. (p = 0.166 for change in G1 phase; p = 0.290 for change in S phase; p = 0.160 for G2 phase respectively).

Chapter 3: Effects of 5-fluorouracil Drug Treatment on MicroRNA Expression Profile in MCF7 Breast Cancer Cells

Abstract

5-fluorouracil (5-FU) is a classic chemotherapeutic drug that has been widely used for breast cancer treatment. Although aberrant expression of protein-coding genes was observed after 5-FU treatment, the regulatory mechanism is unknown. In this study, by using a combined advanced microarray and quantitative real time PCR (qRT-PCR) technology, we found for the first time that 5-FU significantly alter the global expression profile of miRNAs, one class of recently identified small regulatory RNAs, in human breast cancer cells. After 48 hours of treatment with a low dosage (0.01μM), we observed that 42 miRNAs were differentially expressed in MCF7 cells. Of these, 23 miRNAs were up-regulated with up to a 4.59-fold change, while 19 were down-regulated with up to a 1.89-fold change. The most up-regulated miRNAs are miR-575 (4.59-fold change), miR-671-5p (3.25-fold change), miR-483-5p (3.07-fold change), miR-574-3p (2.52-fold change); the most down-regulated miRNAs are miR-365 (1.89-fold change) and miR-374b (1.62-fold change). A majority of miRNAs with differential expression are associated with cancer development, including breast cancer. Target prediction and GO analysis suggest that these differentially expressed miRNAs potentially targeted many oncogenes and tumor suppressor genes as well as protein-coding genes which are related to programmed cell death, activation of immune response and cellular catabolic process.

Key words: 5-fluorouracil, microRNA, human breast cancer, gene regulation, microarray, MCF7

Introduction

Chemotherapy with antineoplastic (or cytotoxic) drugs is the most extensively adopted practice for managing cancers. 5-fluorouracil (5-Fluoro-2,4(1H,3H)-pyrimidinedione or 5-FU) is one such chemotherapeutic drug which is widely used mainly for the treatment of breast (Fumoleau, Bonneterre et al. 2003), colorectal (Wils, O'Dwyer et al. 2001) and head and neck cancers (Posner, Colevas et al. 2000). 5-FU is a pyrimidine antagonist which is rapidly taken inside actively by dividing cancerous cells through facilitated transport similar to uracil (Wohlhueter, McIvor et al. 1980); in cancer cells, 5-FU is converted intracellularly into three cytotoxic metabolites: fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP) and fluorouridine triphosphate (FUTP). The cytotoxicity of 5-FU has been primarily attributed to the inhibition of enzyme thymidylate synthase (TS) activity by FdUMP, resulting in inhibition of dTMP de novo synthesis (Santi, McHenry et al. 1974; Sommer and Santi 1974) and subsequent imbalances in the deoxynucleotide pool, particularly dATP/dTTP ratio (Houghton, Tillman et al. 1995). This in turn inhibits DNA synthesis and repair, and thus causes DNA damage. In addition, 5-FU toxicity was also caused by misincorporation of FdUTP and FUTP into DNA (Aherne, Hardcastle et al. 1996) and RNA (Ghoshal and Jacob 1994) strands.

Despite the widespread clinical use of 5-FU for over 40 years, its molecular mechanism of cytotoxicity in cancer cells has only been recently understood. Recent evidence suggests that additional mechanisms could be involved in the cytotoxic activity of 5-FU, including gene regulation. 5-FU has been recently shown to modify the expression levels of protein-coding genes (Lowe, Bodis et al. 1994; Petak, Tillman et al. 2000; Hwang, Bunz et al. 2001; Longley, Harkin et al. 2003; Maxwell, Longley et al. 2003; Inokuchi, Uetake et al. 2004; Hernandez-

Vargas, Ballestar et al. 2006; Mauritz, van Groeningen et al. 2007; Rossi, Bonmassar et al. 2007), but the underlying regulatory mechanisms of these protein-coding genes are still unknown. The recently identified class of post-transcriptional gene regulators, microRNAs (miRNAs), may play an important role in 5-FU induced alteration of gene expression. miRNAs are a group of small (20-22 nt) endogenous non-protein-coding RNA molecules that negatively regulate gene expression (Ambros 2004). These miRNAs usually bind to the 3'-untranslated region (3'-UTR) of the target mRNAs for mRNA cleavage or translation inhibition (Ambros 2001; Zhang, Wang et al. 2007). It has been predicted that miRNAs may target more than 30% of protein coding genes (Lewis, Shih et al. 2003).

Extensive studies have indicated the significance of miRNAs in various biological processes, including developmental timing, organ development, stem cell maintenance and differentiation, disease, cell proliferation, apoptosis and response to different stresses (Ambros 2001; Alvarez-Garcia and Miska 2005; Hatfield, Shcherbata et al. 2005; Hwang and Mendell 2006; Zhang, Pan et al. 2006). miRNAs have also been reported to play a role in cancer initiation and progression (Calin and Croce 2006) and metastasis (Cheng, Byrom et al. 2005), with many miRNA genes being localized in cancer-associated genomic regions or in fragile sites (Calin, Sevignani et al. 2004). Aberrant miRNA expression levels have been reported in almost all human cancers (Iorio, Ferracin et al. 2005; Lu, Getz et al. 2005; Calin and Croce 2006; Blenkiron, Goldstein et al. 2007; Zhang, Pan et al. 2007). Many miRNAs targeting protein-coding genes are oncogenes and tumor suppressor genes, which are involved in tumorigenesis (Calin, Dumitru et al. 2002). Certain miRNAs have also displayed unique expression profiles in specific types of cancers (Lu, Getz et al. 2005), suggesting that miRNAs may be considered as a new biomarker for cancer diagnosis (Zhang and Farwell 2008). miRNA gene expression levels

are also aberrantly altered in breast cancer, miR-21, miR-10b are over-expressed (Ma, Teruya-Feldstein et al. 2007); whereas miR-125b and miR-145 are down-regulated (Iorio, Ferracin et al. 2005). Further, miRNAs also modulate breast cancer initiation, invasion and metastasis (Ma, Teruya-Feldstein et al. 2007; Huang, Gumireddy et al. 2008; Tavazoie, Alarcon et al. 2008). These studies highlight the importance of miRNAs as both stimulators and inhibitors in breast cancer.

Currently, a growing body of evidence has suggested the importance of miRNAs in modulating the chemosensitivity and chemoresistance of tumor cells (Meng, Henson et al. 2006; Blower, Verducci et al. 2007; Blower, Chung et al. 2008). It is reported that suppression of miR-21 sensitized MCF7 cells to anticancer drug topotecan (Si, Zhu et al. 2007). Similar studies have been reported for the drugs gemcitabine, doxorubicin and tamoxifen (Kovalchuk, Filkowski et al. 2008; Miller, Ghoshal et al. 2008; Zhao, Lin et al. 2008) illustrating the importance of miRNAs in drug sensitivity and resistance. Further, miRNAs such as miR-15b and miR-16 have been shown to modulate multidrug resistance by targeting the anti-apoptotic bcl2 gene (Xia, Zhang et al. 2008). These studies reveal the intrinsic role of miRNAs in managing the efficiency of chemotherapy in several human cancers.

In a recent study, 5-FU was reported to modify the expression of several miRNAs in colon cancer cells (Rossi, Bonmassar et al. 2007), indicating the potential role of 5-FU in altering miRNA expression. However, there is no report on the effect of 5-FU on miRNAs in human breast cancer. Considering the critical role of miRNAs in cancer and drug chemosensitivity, we hypothesized that the cytotoxicity of 5-FU in breast cancer may be partially elicited by regulation of miRNA expression levels. In this study, we have used miRNA microarray technology and

quantitative real time PCR (qRT-PCR) to investigate the effect of 5-FU exposure on the global expression profile of miRNAs in human breast cancer cell line MCF7.

Materials and Methods

Cell line and cell culture

All cell culture reagents were purchased from Invitrogen Inc (Carlsbad, CA). The human breast adenocarcinoma cell line MCF7 was obtained from the American Type Culture Collection (ATCC). They were cultured in RPMI 1640 media containing L-glutamine and 25 mM HEPES, and supplemented with 10% fetal bovine serum (FBS), 10 µg/ml gentamicin and 4 µg/ml insulin. The cells were maintained at 37˚C in a humidified incubator with 5% CO_2.

Drug treatment and cell viability assay

5-fluorouracil (5-FU) was purchased from Sigma-Aldrich (St. Louis, MO) and stored at 4˚C, away from light and moisture. For cell treatments, a 10 mM stock solution of 5-FU was prepared in RPMI complete media and stored at 4˚C for not more than two weeks. The different inhibitory concentrations of 5-FU were determined by generating dose-response curves after treating the cells with increasing concentrations of 5-FU and analyzing the cell sensitivity using trypan blue dye exclusion assays. Briefly, the cell suspension was appropriately diluted with 0.4% trypan blue dye (Sigma-Aldrich, St. Louis, MO) and a hematocytometer was used to estimate the percentage of unstained treated cells compared to the control cells. The IC_{10} (concentration of 5-FU to produce 10% cell inhibition) was determined and used for further analysis. All experiments were carried in triplicate.

RNA isolation

Cells were seeded at 50,000 cells/cm^2 (480,000 cells/well) in flat-bottom 6-well plates in 2 ml of complete media. After 24 h, media was replaced with fresh media (control group) or with 0.01µM 5-FU in complete media (treatment group) and cells were incubated for 48 hours, a traditional time points for most drug treatment. Total RNA was extracted from both groups with the mirVana™ miRNA Isolation Kit (Ambion, Austin, TX) according to manufacturer's instructions. Briefly, the cells were detached from the wells by trypsinization and washed in cold PBS (phosphate buffer saline – 1X, pH-7.4, without Calcium chloride and magnesium chloride). Cells were lysed by adding 500 µl of Lysis/Binding Solution, followed by vortexing. 50 µl of miRNA Homogenate Additive was then added, mixed by vortexing and cells were incubated on ice for 10 min. The RNA was extracted by adding 500 µl of Acid-Phenol: Chloroform, vortexing for 60 sec and centrifuging for 5 min at 10,000 X g to separate the aqueous and organic phases. The upper (aqueous) phase (400 µl) was transferred to another 2ml micro-centrifuge tube and 500 µl (1.25 volumes) of 100% ethanol at room temperature was added to precipitate the RNA. The total RNA was then filtered onto a filter cartridge by centrifugation, followed by multiple washings with Wash solutions 1 and 2/3. Finally, the total RNA was eluted with 50 µl of pre-heated (95˚C) nuclease-free water. All the steps were performed on ice. RNA quantity and quality was analyzed using NanoDrop ND1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE) and immediately stored at -80˚C. The experiment was performed a minimum of three times.

MicroRNA microarray expression analysis

The miRNA Microarray Expression Analysis was performed by LC Sciences (Houston, Texas). The assay was performed on approximately 5 µg of total RNA sample. The total RNA

was size fractionated using a YM-100 Microcon centrifugal filter (Millipore, Billerica, MA) and RNA sequences with <30 nt were isolated. These small RNA were then extended at 3'-end with a poly(A) tail using poly(A) polymerase, followed by ligation of an oligonucleotide tag to the poly(A) tail for later fluorescent staining. Two different tags (Cy3 and Cy5) were used for the two different RNA samples (control and 5-FU treated RNA samples). The two RNA samples were then hybridized overnight on a µParaflo™ microfluidic chip using a microcirculation pump (Atactic Technologies Inc., Houston, TX). Each microfluidic chip contained detection probes, positive control probes and negative control probes. The detection probes were made in situ by photogenerated reagent (PGR) chemistry. These probes consisted of chemically – modified nucleotide coding sequences complementary to the target miRNAs (all 871 human miRNAs listed in the Sanger's miRNA miRBase, Release 13.0 http://microrna.sanger.ac.uk/sequences/) and a spacer segment of polyethylene glycol to extend the coding sequence away from the substrate. A total of 50 positive and negative control probes were included to ensure uniformity of assay conditions and sample labeling. Chemical modifications of the probes were done to balance the melting temperatures of hybridization. RNA Hybridization was performed using 100µl of 6X SSPE buffer (0.9 M NaCl, 6 mM EDTA, 60 mM Na_2HPO_4 , pH 6.8) containing 25% formamide at 34 ˚C. After hybridization, control and 5-FU treated cells were dye-stained using tag – conjugating dyes Cy3 and Cy5 respectively. An Axon GenePix 4000B Microarray Scanner (Molecular Device, Union City, CA) was used to collect the fluorescent images, which were then digitized using the Array-Pro image Analysis software (Media Cybernetics, Bethesda, MD). Dye switching between control and treated RNA samples was performed in order to avoid dye bias. Each miRNA was analyzed four times and the controls were repeated four to sixteen times.

Statistical Analysis of miRNA microarray data

The statistical analysis of the microarray data was also performed at LC Sciences. The microarray data was analyzed by subtracting the background and then the signals were normalized using a locally – weighed regression (LOWESS) filter as reported by Bolstad et al., 2003. Detectable miRNAs were selected based on the following criteria: signal intensity higher than 3X (background standard deviation); spot CV < 0.5 (where CV = standard deviation/signal intensity); and signals from at least two out of the four replicates are above the detection level. To identify miRNAs whose expression differs between control and 5-FU treated MCF7 cells, statistic analysis was performed. The ratio of the two sets of detected signals (control and treated) was calculated and expressed in $\log_2$ scale (balanced) for each miRNA. The miRNAs were then sorted according to their differential ratios. The p-values of the t – test were also calculated. miRNAs with *p*-values < 0.01 and $\log_2$ ratio > 0.5 were considered to be significantly differentially expressed.

Quantitative Real Time PCR (qRT-PCR) of miRNA expression

The data obtained from miRNA microarray was confirmed by performing qRT-PCR on selected differentially expressed miRNAs. Total RNA was isolated using the mirVana™ miRNA Isolation Kit (Ambion, Austin, TX) as previously described. Detection and quantification of the miRNAs was performed using TaqMan® MiRNA Assays (Applied Biosystems, Foster City, CA). A single-stranded cDNA for a specific miRNA was generated by reverse transcription of 500 ng of total RNA using a miRNA-specific stem-looped RT primer and the Applied Biosystems TaqMan® miRNA Reverse Transcription Kit. A Reverse transcription reaction mixture contains 500ng of total RNAs, 1.5μL 10X TR Buffer, 1mM of each dNTPs, 0.188μL RNase Inhibitor, 3μL 5X Taqman® miRNA RT primer for a specific miRNA and 1μL

MultiScribe™ Reverse Transcriptase (50U/μL). An Eppendorf Mastercycler Personal PCR (Westbury, NY) was used to conduct the reverse transcription reaction at the following temperature conditions: 16°C for 30 min, 42°C for 30 min followed by 85°C for 5 min and finally held at 4°C.

Following reverse transcription reaction, quantitative RT-PCR was performed with Applied Biosystems 7300 Real-Time PCR system using the Taqman® MiRNA Assay kit. A total of 20μL qRT-PCR reaction mixture contains 3μL RT PCR product (diluted 1:7 times), 10μL Taqman® 2X Universal PCR Master Mix (No AmpErase® UNG), 2μL Taqman® MiRNA Assays 20X Taqman® Assay (qRT-PCR primers). Nuclease free water was used to adjust the final volume to 20μL. The reactions were incubated in a 96-well optical plate at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 60 sec. RNU 48 was used as an endogenous reference gene for normalizing the results. The relative abundance of each miRNA was calculated using the comparative cycle threshold ($2^{-\Delta\Delta Ct}$) method. The results are presented as fold change of each miRNA in 5-FU treated cells relative to the control MCF7 cells. Individual samples were assayed in triplicate with five independent biological replicates.

Target prediction and function analysis

The miRNAs which showed most dysregulation after 5-FU exposure were selected for target prediction. Two different computational programs, TargetScan (Lewis, Shih et al. 2003; Lewis, Burge et al. 2005); (Release 5.1, http://www.targetscan.org/) and MicroCosm Targets (Griffiths-Jones, Saini et al. 2008) (Version 5, http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/) were used to predict the targets. The reason for selecting two computational programs is that all current programs over-predict potential miRNA targets and two different computational programs would reduce the potential false positives and increase the

accuracy of prediction (Zhang and Pan 2009). During target prediction, we first generated two potential target lists using both computational programs. Second, the top 200 targets predicted by each program were compared and the common targets were selected. Third, top 50 targets of each list were compared to the entire list of the other program and the common targets were selected. Finally, the top 10 targets of each list that were not already included were added to the final list. The targets were sorted according to their total score obtained after adding up the individual scores from each program. The higher the total score a predicted gene has, more likely it is to be the actual target of the specific miRNA.

The list of potential gene targets for each selected miRNA was compiled as explained above and the genes were then classified according to their biological function determined using the Gene Ontology system (http://www.geneontology.org/). To determine the possible overlapping of biological functions among these miRNAs, significantly overrepresented GO terms among all predicted gene targets for each individual miRNA were searched using the GOstat software (http://gostat.wehi.edu.au/cgi-bin/goStat.pl) (Falcon and Gentleman 2007). The program determines all the annotated GO terms associated with the target genes, and then counts the number of appearances of each GO term for these genes; a Fischer's exact test is then performed to give the p-value for each GO term, representing the probability that the observed counts could have been due to chance. In addition, Pathway analysis of the target genes was performed using the DAVID Bioinformatics Resources 2008 (http://david.abcc.ncifcrf.gov/) (Dennis, Sherman et al. 2003; Huang da, Sherman et al. 2009). The program groups together related annotations (GO terms) for a similar set of genes, compares the GO processes if they might be related in a biological network and compiles a list of potential pathways for the effects of the target genes.

Results

miRNA expression profile of MCF7 breast cancer cells

The MCF7 breast cancer cells were analyzed for the expression of 871 human miRNAs listed in the Sanger's miRNA miRBase, Release 13.0. A unique miRNA expression profile was observed in the cells. Of the 871 human miRNAs, only 223 (25.6%) miRNAs were detected (Figure 3.2, Table 3.1). Of these, majority of the miRNAs were expressed at low signal intensities. 148 (66%) miRNAs showed a signal intensity of less than 500. However, 11 miRNAs were highly expressed in human MCF7 breast cancer cells with signal intensities greater than 10,000; these include miR-21, let-7f, let-7a, miR-1826, let-7d, miR-1979, miR-200c, let-7c, miR-191, miR-342-3p, and miR-26a. Among all 871 analyzed miRNAs, miR-21, let-7f and let-7a showed the highest expression levels with signal intensities of 57,600, 21,201 and 20,206, respectively. The variation in the expression levels of miRNAs indicates that the abundance of these miRNAs varies significantly in MCF7 cells. Some miRNAs are expressed in many copies, while others are expressed only in few copies, thus exhibiting a distinct miRNA expression signature pattern in MCF7 breast cancer cells.

Effect of 5-FU on human breast cancer cell MCF7

To determine the sensitivity of MCF7 cells to anticancer drug 5-FU, we performed cell viability assays and generated a dose-response curve. From the data obtained, the concentration of 0.01µM of 5-FU was selected for determining the effect of 5-FU on the global expression levels of miRNAs in MCF7 cells. Since genetic changes are more sensitive as compared to cellular changes, a low concentration of 5-FU was selected to avoid the possibility of observing

alterations in miRNA expression levels due to cellular changes such as apoptosis or necrosis, which are generally induced at high 5-FU doses.

Alteration of miRNA expression profiles in MCF7 cells after 5-FU treatment

5-FU treatment significantly altered the miRNA expression profile in human breast cancer cell line MCF7. Of the 871 human miRNAs analyzed, a total of 309 miRNAs were detected (either in control or treated cells). The control cells expressed 223 miRNAs, while the treated cells expressed 289 miRNAs. This result shows that 5-FU exposure resulted in the expression of higher number of miRNAs as compared to the control. Among these, 193 miRNAs were expressed in both the control and treated cells, while 20 miRNAs were detected only in control cells, and 96 miRNAs were detected only in the treated cells (Table 3.2). Of the total 309 miRNAs that were detected (either in control or treated cells), 55 were miRNA* sequences. miRNA* are usually degraded during miRNA biogenesis. The reason for detection of these miRNA* sequences after 5-FU exposure is unclear.

For most of the detected miRNAs, the expression levels were low, which is evident by their low signal intensities (less than 1000) during microarray analysis (Figures 3.3 and 3.4). Of the 223 miRNAs detected in control cells, 144 miRNAs emitted signals less than 1000, and only 19 miRNAs gave signal more than 10,000. On the other hand, of the 289 miRNAs detected in treated cells, 219 gave signals below 1000, while 19 gave signals higher than 10,000. The signal intensities of the 20 miRNAs expressed only in control cells and the 96 miRNAs expressed only in treated cells were not high enough to consider them as being differentially expressed between control and treated cells, and hence will not be considered for further analysis.

Along with the difference in number of detectable miRNAs, the expression levels of these miRNAs were also significantly altered after 5-FU exposure. Of the total 193 miRNAs that

were differentially expressed between the control and treatment groups, 115 were up-regulated, while 78 were down-regulated (Table 3.2). Statistical analysis of these differentially expressed miRNAs showed that 42 of these miRNAs (22%) were significantly dysregulated with $p < 0.01$ and with more than one fold change, while the remaining 151 miRNAs were not significantly different (Figure 3.4). Fold-change comparisons of the significantly altered miRNAs are shown in Figure 3.5. Among these 42 miRNAs, 23 (55%) miRNAs were up-regulated and 19 (45%) miRNAs were down-regulated (Table 3.4). miRNAs showing at least two-fold difference (i.e. $\log_2$-fold change ≥ 1) in their expression levels between control and treated groups were selected for further analysis using qRT-PCR technique. For the up-regulated miRNAs, only four showed a more than two-fold increase: miR-575 (4.6 fold), miR-671-5p (3.25 fold), miR-483-5p (3.00 fold) and miR-574-3p (2.5 fold). Since all the down-regulated miRNAs showed a less than two-fold decrease in their expression, we selected miRNAs with at least a 1.5 fold decrease in their expression (i.e. $\log_2$-fold change ≥ 0.56) for further analysis. These include following two miRNAs: miR-365 (1.9 fold) and miR-374b (1.6 fold).

Validation of miRNA expression levels by qRT-PCR

To validate the data obtained from miRNA microarray, qRT-PCR was performed on five differentially expressed miRNAs (two up-regulated: miR-575 and miR-574-3p; and three down-regulated: miR-374b, miR-15a, miR-27a) and the results from microarray and qRT-PCR were compared. As shown in Figure 3.6, miR-15a, miR-27a and miR-374b were down-regulated, while miR-575 and miR-574-3p were up-regulated according to the qRT-PCR data. Our qRT-PCR data is comparable with the microarray data and thus validated the results for these miRNAs obtained from miRNA microarray.

Prediction of target genes of differentially expressed miRNAs

In order to determine the probable biological function of the differentially expressed miRNAs, we predicted the potential miRNA targets of six most 5-FU-regulated miRNAs (miR-575, miR-671-5p, miR-483-5p, miR-574-3p, miR-365 and miR-374b) by using two different computational programs, TargetScan and miRBase Targets. After prediction by these programs as described, a total of 318 potential targets were identified for the five miRNAs with most differential expression after 5-FU treatment; about 50-60 protein-coding targets were predicted for each miRNA (Table 3.4). Generally speaking, 5-FU-up-regulated miRNAs targets many oncogenes, such as *HMGA2*, *KRAS* and *MYC* while 5-FU-down-regulated miRNAs targets many tumor suppressor genes. This suggests the role of 5-FU treatment in chemotherapy by targeting miRNA-mediated gene regulation.

The basic biological function of each gene was also classified using the Gene Ontology system. Since a single gene is associated with many GO terms, the overrepresented GO terms for each miRNA were identified by GOstat software. Table 3.5 gives a few representative biological processes associated with each miRNA as predicted by the GOstat software. The targets of these miRNAs were further used for pathway analysis by DAVID Bioinformatics Resources. The program provides potential pathways of function for the target genes of the 5-FU responsive miRNAs. Based on the target analysis, we found that several important biological processes, such as programmed cell death, activation of the immune response, neurotransmitter metabolic process and cellular catabolic process were included as putative biological functions of the predicted potential genes. This result suggests an important role of these miRNAs in human health and disease regulation. Further, a pathway analysis suggests an important regulatory role of these miRNAs in different biological processes (Tables 3.5 and 3.6).

Discussion

miRNAs are an important class of gene regulators which have the potential to function as a diagnostic and prognostic tool for a variety of human cancers (Blenkiron and Miska 2007; Tricoli and Jacobson 2007; Lowery, Miller et al. 2008; Zhang and Farwell 2008). They constitute a novel target system for cancer treatment as each miRNA has the ability to regulate the expression of several hundred target genes, including several important oncogenes or tumor suppressor genes. Extensive research is currently being focused on identifying differential expressed miRNAs that play primary roles in cancer development and therapy. Thus, a study of the possible effects of chemotherapeutic drug treatment on the expression profile of miRNAs is of prime importance for cancer therapy and resistance.

In this study, the effect of chemotherapeutic drug 5-FU on miRNA expression profile was investigated for the first time in human breast cancer cells. In this study, we found that 23 miRNAs were up-regulated while 19 were down-regulated. Among the differentially expressed miRNAs, several miRNAs were well characterized and have been previously implicated in different cancers (Table 3.7). Of particular interest are the miRNAs that showed maximum dysregulation in their expression levels. Of the 42 differentially expressed miRNAs after 5-FU treatment, 26 miRNAs (62%) have been previously confirmed to be linked with different human cancers, most of them being differentially expressed between tumors and normal cells. Identification of such a high proportion of cancer-related miRNAs may suggest that these miRNAs may be involved in anti-cancer treatment.

Many up-regulated miRNAs function as tumor suppressor genes by targeting oncogenes or genes involved in cell cycle and apoptosis. After 5-FU treatment, both miR-15 and miR-16

were overexpressed. Pervious study shows that miR15/16 induces apoptosis by targeting the bcl2 gene (Cimmino, Calin et al. 2005), and also cause cell cycle arrest by regulating multiple cell cycle genes (Xia, Qi et al. 2009). miR-15b and miR-16 also modulate multidrug resistance by targeting the bcl2 gene (Xia, Zhang et al. 2008). Further, miR-200c regulates the expression of ZEB1 and ZEB2, which play an important function in breast cancer progression (Gregory, Bert et al. 2008). miRNA-23a and -23b on the other hand modulate the expression of proto-oncogene c-Met (Salvi, Sabelli et al. 2009). Additionally, miRNA let-7a has been found to be a tumor suppressor gene by targeting the oncogenes HMGA2 (High mobility group AT-hook 2), KRAS and Myc (Johnson, Grosshans et al. 2005; Lee and Dutta 2007; Sampson, Rong et al. 2007).

In our study, 5-FU also reduced the expression of several important cancer-associated miRNAs, such as miR-21, miR-203, miR-24, miR-25 and miR-27a. All of these miRNAs target different tumor suppressor genes; for example, miR-21 targets PTEN (Phosphatase and tensin homolog) (Meng, Henson et al. 2007), TPM1 (Tropomyosin-1) (Zhu, Si et al. 2007) and PDCD4 (Programmed cell death protein 4) (Asangani, Rasheed et al. 2008) while as p16 and p57 have been found to be modulated by miRNAs miR -24 (Lal, Kim et al. 2008) and miR -25 (Kim, Yu et al. 2009) respectively. Additionally, miR-27a is reported to be an oncogene targeting specificity transcription protein in breast cancer cells (Mertens-Talcott, Chintharlapalli et al. 2007). The down-regulation of these miRNAs after 5-FU treatment aids the function of the tumor suppressor genes, and thus helps control cancer progression and metastasis. Further, several miRNAs are reported to be differentially expressed in breast cancer cells in comparison with normal breast cells. Our data identified 13 such miRNAs that are usually dysregulated in breast cancer. These include miR-16, let-7a, let-7d, let-7f and miR-200b, which are usually down-regulated; and miR-23a, miR-23b, miR-203, miR-21, miR-24, miR-27a, miR-30a and

miR-365, which are usually up-regulated. Most of these miRNAs, except miR-23a, miR-23b and miR-200b, have been shown to be inversely expressed in 5-FU treated cells compared to untreated breast cancer cells (Table 3.7).

Previous studies have reported p53 to be an important molecular effector in 5-FU cytotoxicity. p53-mutated cells have demonstrated higher resistance to 5-FU treatment suggesting its role in 5-FU sensitivity (Lowe, Bodis et al. 1994). Further, p53 has been suggested to induce apoptosis and cause Fas upregulation after 5-FU treatment (Petak, Tillman et al. 2000). Moreover, several p53 regulated genes have been shown to be modified by 5-FU. Thus, there is evidence for the importance of the p53 regulatory process in 5-FU toxicity, but the molecular mechanism is still unclear. In this study, we found that 5-FU induced differential expression of several miRNAs that putatively target several p53-regulated genes. These genes include *CASP8, SERPINB5, CASP9, CCND2, CCND3, CASP3, PTEN, CDK6, CCNE1* and *CCND1* (Table 3.7). This observation suggests that miRNAs might be involved in the p53-5-FU interaction. Further investigations of the regulatory mechanism of miRNAs on these p53 -regulated genes are required to better understand the 5-FU regulated p53 pathway.

The widespread deregulation of the miRNAome global expression profiles and the identification of several cancer-related deregulated miRNAs after low dose 5-FU treatment indicates that the cytotoxicity of 5-FU may be partially due to alteration in the miRNA expression levels. The observation that an antimetabolite drug is able to increase the expression of certain miRNAs is inconsistent with its DNA-synthesis-inhibiting mechanism. However, it has been previously suggested that incorporation of 5-FU into RNA is a major mechanism of cytotoxicity in MCF7 breast cancer cells (Kufe and Major 1981). Further, it is established that the 5-FU active metabolites, FdUTP and FUTP, get actively incorporated into growing DNA and

RNA molecules. Incorporation into RNA results in disruption of the further synthesis and processing of mRNAs, tRNAs and rRNAs (Randerath, Tseng et al. 1983; Kanamaru, Kakuta et al. 1986). Additionally, 5-FU has also been shown to be incorporated into the uridine-rich U2 snRNA at pseudouridylation sites, thus inhibiting post-transcriptional conversion of uridine to pseudouridine in these RNAs (Samuelsson 1991), and greatly affecting the splicing mechanism. Assimilating together this knowledge of 5-FU activity, it can be hypothesized that the drug might be incorporated into the miRNA gene transcript (as FdUTP) or produce fluorinated mature miRNA, thus causing misexpression of miRNAs and altering their primary function. However, further work is needed to test this hypothesis and to elucidate the exact molecular mechanism.

Determination of the effects of 5-FU on the expression profile of miRNAs in the human breast cancer cell can help to identify miRNAs that play a role in chemosensitivity and resistance of 5-FU in breast cancer cells in particular, and other chemotherapeutic drugs in general. This information might be helpful in improving the efficacy of 5-FU treatment, by reducing its side-effects or by decreasing the incidence of 5-FU resistant cancers. The data also could be extrapolated to other chemotherapeutic drugs which could target these specific miRNAs. The research could be extended to predict the protein targets of the miRNAs whose expression levels are mainly altered by 5-FU treatment. On a broad consideration, the data could also be used to determine the oncogenic miRNAs or Oncomirs associated with breast cancer after 5-FU treatment. Specific targeted deliveries, such as artificial miRNAs or siRNAs that target these miRNAs and alter their expression levels in tumor cells can be synthesized, thus down-regulating the expression of these oncomirs and prevent breast cancer initiation and metastasis. Examples of such delivery systems that are currently under development include antisense oligonucleotide

(ASOs), siRNAs, miRNA mimics and antagomirs. Thus, our data demonstrates the potential of miRNAs as novel targets for cancer therapy.

In summary, a low dosage of 5-FU induced the differential expression of a set of miRNAs, which in turn regulated the expression of oncogenes and tumor suppressor genes, potentially by multiple mechanisms. A proposed model showing the functional mechanism of action of 5-FU involving miRNA activity is shown in Figure 3.7. Although the mechanism of how 5-FU regulates miRNA expression is unclear, our results provide some interesting clues for chemotherapeutic drugs and their effects on miRNAs. Further investigation on the regulation mechanism of miRNAs on gene expression in breast cancer cells will provide new insights into cancer chemotherapy and designing new anti-cancer drugs.

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Table 3.1: miRNA expression profile in MCF7 breast cancer cells

Microarray signal intensity	Number	miRNAs
> 10,000	11	miR-21, let-7f, let-7a, miR-1826, let-7d, miR-1979, miR-200c, let-7c, miR-191, miR-342-3p, miR-26a
5,000-10,000	14	let-7e, miR-1977, miR-15b, miR-23a, miR-23b, miR-16, miR-1308, let-7b, miR-125a-5p, let-7i, miR-638, miR-1974, miR-25, miR-182
1,000-5,000	34	miR-26b, miR-200b, miR-92a, let-7g, miR-361-5p, miR-1975, miR-151-5p, miR-103, miR-203, miR-320a, miR-99b, miR-107, miR-24, miR-1978, miR-93, miR-320c, miR-1280, miR-454, miR-27a, miR-27b, miR-183, miR-106b, miR-320b, miR-7, miR-1915, miR-375, miR-425, miR-423-5p, miR-30b, miR-185, miR-320d, miR-98, miR-30d, miR-92b
< 1,000	164	miR-20a, miR-1275, miR-30c, miR-17, miR-151-3p, miR-128, miR-195, miR-197, miR-663, miR-15a, miR-106a, miR-720, miR-149*, miR-193a-5p, miR-374b, miR-125b, miR-365, miR-424, miR-130b, miR-1246, miR-342-5p, let-7d*, miR-1469, miR-181a, miR-877, miR-625, miR-30a, miR-421, miR-574-5p, miR-29a, miR-200a, miR-1180, miR-503, miR-34a, miR-324-5p, miR-192, miR-378, miR-484, miR-301a, miR-1268, miR-498, miR-489, miR-340, miR-652, miR-548m, miR-574-3p, miR-148a, miR-20b, miR-744, miR-222, miR-126, miR-28-5p, miR-99a, miR-141, miR-193b*, miR-132, miR-532-5p, miR-148b, miR-22, miR-345, miR-429, miR-625*, miR-29c, miR-140-3p, miR-1281, miR-18a, miR-486-5p, miR-505*, miR-149, miR-424*, miR-940, miR-194, miR-1307, miR-331-3p, miR-362-5p, miR-152, miR-629, miR-7-1*, miR-101, miR-125a-3p, miR-1277, miR-575, miR-16-2*, miR-27b*, miR-103-2*, miR-25*, miR-200b*, miR-671-5p, miR-548d-3p, miR-1259, miR-548h, miR-30b*, miR-23a*, miR-210, miR-181b, miR-605, miR-483-5p, miR-933, miR-455-3p, miR-30e, miR-765, miR-15b*, miR-302f, miR-938, miR-500*, miR-106b*, let-7b*, miR-1289, miR-1825, miR-425*, miR-30e*, miR-32*, miR-708, miR-138-1*, miR-30a*, miR-616*, miR-1973, miR-32, miR-19b, miR-760, miR-451, miR-296-5p, miR-501-5p, miR-191*, miR-183*, miR-550*, miR-361-3p, miR-193a-3p, miR-589*, miR-24-2*, miR-133a, miR-29c*, miR-1249, miR-208a, miR-30c-2*, miR-200a*, miR-330-3p, miR-30c-1*, miR-936, let-7f-1*, miR-30d*, miR-221, miR-374a, miR-31*, miR-31, miR-2052, miR-629*, miR-28-3p, miR-1247, miR-302e, miR-365*, miR-2054, miR-150*, miR-1913, miR-302d, miR-302c, miR-146b-5p, miR-105, miR-376a, miR-29b-2*, miR-302b, miR-513a-3p, miR-202, miR-1470

Table 3.2: Comparison of miRNA expression profiles between control and 5-FU treated groups

miRNA category	Number	miRNAs
Up-regulated miRNAs	115	miR-181b, miR-151-5p, miR-421, miR-7-1*, miR-720, miR-320b, let-7i, let-7b, miR-107, miR-484, miR-361-5p, miR-1975, miR-486-5p, miR-125b, miR-17, miR-425, miR-26a, let-7d, miR-106b, miR-1978, miR-148b, miR-1308, miR-744, miR-1977, miR-183, miR-1979, let-7f, miR-28-5p, miR-149, miR-148a, let-7a, miR-625, miR-130b, miR-342-3p, miR-532-5p, miR-15b, miR-28-3p, miR-126, miR-16, miR-1180, miR-99a, miR-708, miR-200c, miR-22, miR-23b, miR-192, miR-191, miR-365*, miR-342-5p, miR-141, miR-200b*, miR-18a, miR-222, miR-1275, let-7b*, miR-23a, miR-16-2*, miR-362-5p, miR-652, miR-503, miR-425*, miR-1307, miR-1826, miR-132, miR-625*, miR-30e, miR-125a-3p, miR-302f, miR-106b*, miR-498, miR-345, miR-1246, miR-505*, miR-489, miR-1259, miR-194, miR-629, miR-140-3p, miR-936, miR-19b, miR-25*, miR-1915, miR-1281, miR-548m, miR-1268, miR-1469, miR-638, miR-331-3p, miR-193a-3p, miR-940, miR-221, miR-24-2*, miR-574-5p, miR-550*, miR-500*, miR-27b*, miR-501-5p, miR-23a*, miR-424*, miR-210, miR-149*, miR-152, miR-663, miR-200a*, miR-183*, miR-1277, miR-330-3p, miR-629*, miR-455-3p, miR-548h, miR-574-3p, miR-1289, miR-671-5p, miR-483-5p, miR-575
Down-regulated miRNAs	78	miR-30a*, miR-374a, miR-1249, miR-365, miR-374b, miR-361-3p, miR-1913, miR-30b*, miR-589*, miR-1825, miR-150*, miR-146b-5p, miR-27a, miR-15a, miR-30a, miR-296-5p, miR-29b-2*, miR-877,miR-21, miR-197 , miR-26b, miR-765, miR-29c*, miR-15b*, miR-1247, miR-101, miR-98, miR-200b, let-7e, miR-7, miR-454, let-7g, miR-27b, miR-760, let-7d*, miR-203, miR-29c, miR-30b, miR-30c, miR-20b, miR-1280, miR-423-5p, miR-103-2*, let-7f-1*, miR-1974, miR-25, miR-193a-5p, miR-181a, miR-200a, miR-193b*, miR-24, miR-93, miR-424, miR-378, miR-340, miR-106a, miR-125a-5p, miR-99b, miR-301a, miR-29a, miR-92a, miR-92b, miR-195, miR-375, miR-182, let-7c, miR-30d, miR-320a, miR-128, miR-320d, miR-103, miR-20a, miR-185, miR-324-5p, miR-34a, miR-429, miR-320c, miR-151-3p
miRNAs	20	miR-105, miR-1470, miR-202, miR-2052, miR-2054, miR-208a,

expressed only in control group		miR-302b, miR-302c, miR-302d, miR-302e, miR-30c-1*, miR-30c-2*, miR-30d*, miR-30e*, miR-31, miR-31*, miR-32, miR-32*, miR-376a, miR-513a-3p
miRNAs expressed only in treatment group	96	hsa-let-7a-2*, hsa-let-7c*, hsa-let-7g*, hsa-miR-100, hsa-miR-1181,, hsa-miR-1200, hsa-miR-1207-5p, hsa-miR-1224-3p, hsa-miR-1224-5p, hsa-miR-1226, hsa-miR-1227, hsa-miR-1228, hsa-miR-1228*, hsa-miR-1229, hsa-miR-1231, hsa-miR-1233, hsa-miR-1234, hsa-miR-1237, hsa-miR-1238, hsa-miR-1260, hsa-miR-1267, hsa-miR-1271, hsa-miR-1285, hsa-miR-1287, hsa-miR-1292, hsa-miR-1293, hsa-miR-129-3p, hsa-miR-1300, hsa-miR-1301, hsa-miR-1303, hsa-miR-130a, hsa-miR-130b*, hsa-miR-133a, hsa-miR-138-2*, hsa-miR-17*, hsa-miR-184, hsa-miR-18b, hsa-miR-1908, hsa-miR-191*, hsa-miR-1910, hsa-miR-1911*, hsa-miR-1973, hsa-miR-2110, hsa-miR-212, hsa-miR-215, hsa-miR-23b*, hsa-miR-299-3p, hsa-miR-299-5p, hsa-miR-323-5p, hsa-miR-324-3p, hsa-miR-328, hsa-miR-329, hsa-miR-331-5p, hsa-miR-335, hsa-miR-339-3p, hsa-miR-339-5p, hsa-miR-33b*, hsa-miR-340*, hsa-miR-34a*, hsa-miR-34b*, hsa-miR-34c-5p, hsa-miR-362-3p, hsa-miR-371-5p, hsa-miR-378*, hsa-miR-380*, hsa-miR-409-3p, hsa-miR-448, hsa-miR-450a, hsa-miR-485-3p, hsa-miR-497, hsa-miR-500, hsa-miR-502-3p, hsa-miR-502-5p, hsa-miR-517b, hsa-miR-548f, hsa-miR-550, hsa-miR-553, hsa-miR-584, hsa-miR-605, hsa-miR-627, hsa-miR-628-3p, hsa-miR-654-5p, hsa-miR-658, hsa-miR-659, hsa-miR-660, hsa-miR-664*, hsa-miR-665, hsa-miR-671-3p, hsa-miR-766, hsa-miR-769-3p, hsa-miR-769-5p, hsa-miR-93*, hsa-miR-933, hsa-miR-935, hsa-miR-943, hsa-miR-96

Table 3.3: Significantly dysregulated miRNAs after 5-FU exposure

Probe_ID	Control Signal	Treatment Signal	Log2 (Treatment/ Control)	Fold Change	p-value
Up-regulated (n=23)					
hsa-miR-575	31.68	145.42	2.22	4.59	4.11E-03
hsa-miR-671-5p	42.18	136.96	1.70	3.25	3.60E-03
hsa-miR-483-5p	43.41	133.14	1.62	3.07	2.14E-03
hsa-miR-574-3p	190.02	479.10	1.40	2.52	3.94E-05
hsa-miR-149*	761.77	1,380.01	0.78	1.81	4.72E-05
hsa-miR-663	750.55	1,290.55	0.81	1.72	6.87E-07
hsa-miR-1268	167.69	280.34	0.76	1.67	4.95E-03
hsa-miR-1915	891.28	1,359.02	0.59	1.52	1.02E-04
hsa-miR-574-5p	351.25	532.08	0.61	1.51	4.99E-03
hsa-miR-638	5,497.66	8,256.06	0.56	1.50	5.55E-17
hsa-miR-1246	770.27	1,043.87	0.45	1.36	4.28E-03
hsa-miR-1826	22,510.52	27,941.79	0.33	1.24	2.07E-12
hsa-miR-23a	10,898.44	13,085.57	0.23	1.20	1.32E-05
hsa-miR-191	14,586.74	16,888.33	0.21	1.16	1.72E-05
hsa-let-7a	24,215.14	27,545.79	0.19	1.14	1.91E-07
hsa-miR-342-3p	15,599.63	17,665.54	0.18	1.13	1.01E-08
hsa-miR-23b	10,734.11	12,153.14	0.15	1.13	1.06E-03
hsa-miR-16	12,622.24	14,011.40	0.15	1.11	1.18E-04
hsa-miR-1979	12,024.04	13,317.24	0.15	1.11	1.48E-04
hsa-miR-15b	12,855.28	14,081.31	0.14	1.10	2.85E-03
hsa-miR-200c	16,146.22	17,485.41	0.16	1.08	6.66E-04
hsa-let-7d	19,259.32	20,608.62	0.10	1.07	2.37E-03
hsa-let-7f	24,515.34	25,713.03	0.07	1.05	9.87E-04
Down-regulated (n=19)					
hsa-miR-365	394.04	208.43	-0.83	-1.89	2.06E-03
hsa-miR-374b	1,116.20	688.88	-0.76	-1.62	1.21E-04
hsa-miR-30a	626.23	421.31	-0.57	-1.49	2.93E-04
hsa-miR-15a	1,433.70	1,018.10	-0.50	-1.41	1.06E-03
hsa-miR-197	1,231.67	906.19	-0.44	-1.36	1.46E-03
hsa-miR-27a	4,197.58	3,093.67	-0.44	-1.36	1.43E-08
hsa-miR-21	57,202.28	44,077.27	-0.38	-1.30	0.00E+00
hsa-miR-26b	8,444.64	6,784.69	-0.33	-1.24	3.51E-06
hsa-miR-203	5,749.60	4,644.42	-0.27	-1.24	1.78E-03

hsa-miR-1280	3,838.92	3,152.21	-0.21	-1.22	4.02E-04
hsa-miR-454	4,618.90	3,860.29	-0.25	-1.20	7.08E-04
hsa-miR-200b	8,059.98	6,745.94	-0.24	-1.19	7.45E-05
hsa-miR-7	3,475.24	2,930.62	-0.25	-1.19	2.78E-03
hsa-miR-27b	4,034.72	3,443.66	-0.21	-1.17	1.81E-03
hsa-let-7g	7,585.57	6,538.32	-0.23	-1.16	2.50E-04
hsa-let-7e	13,719.05	12,008.35	-0.25	-1.14	1.57E-04
hsa-miR-25	8,505.37	7,512.26	-0.18	-1.13	1.03E-03
hsa-miR-24	4,427.72	3,926.62	-0.17	-1.13	1.31E-03
hsa-miR-125a-5p	11,138.55	10,205.68	-0.13	-1.09	4.05E-03

Table 3.4: Predicted targets of the selected miRNAs

Gene name	Description	Gene name	Description
miR-575		**miR-671-5p**	
DENND1C	DENN/MADD domain containing 1C	CD79A	B-cell antigen receptor complex-associated protein alpha-chain precursor (Ig-alpha)
SLC7A7	solute carrier family 7 [Y+L amino acid transporter 1 (y(+)L-type amino acid transporter 1)]	CPNE2	Copine-2 (Copine II).
FBXO15	F-box only protein 15	ZNF668	Zinc finger protein 668
TMEM81	transmembrane protein 81 (TMEM81)	POLR3D	DNA-directed RNA polymerase III subunit D
NENF	Neudesin precursor (Neuron-derived neurotrophic factor).	FES	Proto-oncogene tyrosine-protein kinase Fes/Fps
CPNE2	Copine-2 (Copine II)	LGALS3BP	Galectin-3-binding protein precursor (Lectin galactoside-binding soluble 3-binding protein)
GPX3	Glutathione peroxidase 3 precursor	CHCHD2	Coiled-coil-helix-coiled-coil-helix domain-containing protein 2
MASP2	Mannan-binding lectin serine protease 2 precursor	MS4A8B	Membrane-spanning 4-domains subfamily A member 8B
FAM22G	Protein FAM22G precursor	CA7	Carbonic anhydrase 7
KIR2DL4	Killer cell immunoglobulin-like receptor 2DL4 precursor (MHC class I NK cell receptor KIR103AS)	VPS25	Vacuolar protein sorting-associated protein 25
HAGH	Hydroxyacylglutathione hydrolase	DGKZ	Diacylglycerol kinase zeta
DPH5	Probable diphthine synthase	PITX3	Pituitary homeobox 3 (Homeobox protein PITX3).
DDT	D-dopachrome decarboxylase	C1QA	Complement C1q subcomponent subunit A precursor.
VEGFB	Vascular endothelial growth factor B precursor	SIPA1L1	Signal-induced proliferation-associated 1-like protein 1
KIR2DL1	Killer cell immunoglobulin-like receptor 3DL2 precursor (MHC class I NK cell receptor)	HOOK2	Hook homolog 2
IARS2	Isoleucyl-tRNA synthetase, mitochondrial precursor	KRT9	Keratin, type I cytoskeletal 9
NEU4	Sialidase-4	HTR3E	5-hydroxytryptamine receptor 3 subunit E
IL3RA	Interleukin-3 receptor alpha chain precursor	HOXB1	Homeobox protein Hox-B1 (Hox-2I).
CDC45L	CDC45 cell division cycle 45-like (S. cerevisiae)	ACO2	Aconitate hydratase, mitochondrial precursor
DPP8	Dipeptidyl peptidase 8	CCDC114	coiled-coil domain containing 114
FMO1	flavin-containing monooxygenase 1	SLC46A3	solute carrier family 46, member 3
CSTA	Cystatin-A	ADCK2	Uncharacterized aarF domain-containing

			protein kinase 2
HSPBAP1	HSPB1-associated protein 1 (27 KdA heat shock protein-associated protein 1)	C14orf124	UPF0105 protein C14orf124.
RDH12	Retinol dehydrogenase	SPTBN2	Spectrin beta chain, brain 2 (Spectrin, non-erythroid beta chain 2)
KIR3DL1	Killer cell immunoglobulin-like receptor 3DL1 precursor (MHC class I NK cell receptor)	HYAL1	Hyaluronidase-1 precursor
RAB25	Ras-related protein Rab-25	C2orf16	Uncharacterized protein C2orf16
ACTL6A	Actin-like protein 6A	PCBP4	Poly (rC)-binding protein 4 (Alpha-CP4).
TPT1	Translationally-controlled tumor protein (TCTP)	DVL3	Segment polarity protein disheveled homolog DVL-3 (Dishevelled-3) (DSH homolog 3).
PMM1	Phosphomannomutase 1	SIRT3	NAD-dependent deacetylase sirtuin-3, mitochondrial precursor
MRPL12	39S ribosomal protein L12, mitochondrial precursor	KCNA6	Potassium voltage-gated channel subfamily A member 6
SPAG11A	sperm associated antigen 11B isoform H precursor	USP36	Ubiquitin carboxyl-terminal hydrolase 36
FOXRED1	FAD-dependent oxidoreductase domain-containing protein 1	SSBP1	Single-stranded DNA-binding protein, mitochondrial precursor
ALS2CR4	Amyotrophic lateral sclerosis 2 chromosomal region candidate gene 4 protein	C6orf129	OTTHUMP00000016329 (Chromosome 6 open reading frame 129).
TFPI	Tissue factor pathway inhibitor precursor	C10orf25	Uncharacterized protein C10orf25 precursor.
BID	BH3-interacting domain death agonist	BCAP31	B-cell receptor-associated protein 31 (BCR-associated protein Bap31)
CCDC74A	Coiled-coil domain-containing protein 74A	SPTB	Spectrin beta chain, erythrocyte (Beta-I spectrin).
WDR57	WD repeat protein 57 (Prp8-binding protein)	RAB32	Ras-related protein Rab-32.
GALR1	Galanin receptor type 1	TRIM47	Tripartite motif-containing protein 47
RB1CC1	RB1-inducible coiled-coil protein 1	CRTC3	transducer of regulated CREB protein 3 isoform a
ST8SIA1	ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 1	MYOM3	myomesin family, member 3
RAB6IP1	RAB6 interacting protein 1	ST8SIA5	Alpha-2,8-sialyltransferase 8E
EPB41L5	erythrocyte membrane protein band 4.1 like 5	SYPL2	Synaptophysin-like protein 2.
UBAP2	ubiquitin associated protein 2	ACBD6	Acyl-CoA-binding domain-containing protein 6.
PCDH19	protocadherin 19	SATB2	DNA-binding protein SATB2 (Special AT-rich sequence-binding protein 2).
TRAF3IP1	TNF receptor-associated factor 3	SLC30A6	solute carrier family 30 (zinc transporter),

	interacting protein 1
ZNF142	zinc finger protein 142
POLR3B	polymerase (RNA) III (DNA directed) polypeptide B
STIL	SCL/TAL1 interrupting locus
GCLC	GRIP and coiled-coil domain containing 2
GCM2	glial cells missing homolog 2 (Drosophila)
IGSF6	immunoglobulin superfamily, member 6
P2RY14	purinergic receptor P2Y, G-protein coupled, 14
NCAPH	Condensin complex subunit 2 (Non-SMC condensin I complex subunit H)
ST7L	suppression of tumorigenicity 7-like isoform 3
ZYG11B	zyg-11 homolog B (C. elegans)
BLID	BH3-like motif containing, cell death inducer
FLJ40296	FLJ40296 protein
MICA	MHC class I polypeptide-related sequence A
C20orf57	Dual specificity protein phosphatase 15

miR-483-5p	
ACBD6	Acyl-CoA-binding domain-containing protein 6.
RUSC1	RUN and SH3 domain-containing protein 1
BACE2	Beta-secretase 2 precursor
TACC3	Transforming acidic coiled-coil-containing protein 3 (ERIC-1).
ACO2	Aconitate hydratase, mitochondrial precursor
SLC7A3	Cationic amino acid transporter 3

	member 6
AAK1	AP2-associated protein kinase 1 (Adaptor-associated kinase 1).
CFL2	Cofilin-2 (Cofilin, muscle isoform).
TGOLN2	Trans-Golgi network integral membrane protein 2 precursor
LIN9	Lin-9 homolog (huLin-9) (hLin-9) (Beta-subunit associated regulator of apoptosis)
BCR	Breakpoint cluster region protein
SH3TC2	SH3 domain and tetratricopeptide repeats-containing protein 2.
ANKRD33	Ankyrin repeat domain-containing protein 33.
PIK4CB	Phosphatidylinositol 4-kinase beta
NP_001017927.1	hypothetical protein LOC447937
MAT1A	S-adenosylmethionine synthetase isoform type-1

miR-574-3p	
C9orf19	chromosome 9 open reading frame 19
COL7A1	collagen, type VII, alpha 1 (epidermolysis bullosa, dystrophic, dominant and recessive)
LAT	linker for activation of T cells
CSE1L	CSE1 chromosome segregation 1-like (yeast)
CBX8	chromobox homolog 8 (Pc class homolog, Drosophila)
MAPK11	mitogen-activated protein kinase 11
MADD	MAP-kinase activating death domain
TRPC4AP	transient receptor potential cation channel, subfamily C, member 4 associated protein
ADAMTS17	ADAM metallopeptidase with thrombospondin type 1 motif, 17
DEAF1	deformed epidermal autoregulatory factor 1 (Drosophila)
EXOC3	exocyst complex component 3

	(CAT-3) (Solute carrier family 7 member 3)		
CBS	Cystathionine beta-synthase	LRRC23	Leucine-rich repeat-containing protein 23
IL1R1	Interleukin-1 receptor type I precursor	GABBR1	gamma-aminobutyric acid (GABA) B receptor, 1
CYP2F1	Cytochrome P450 2F1	MAGEA6	melanoma antigen family A, 6
LAIR2	Leukocyte-associated immunoglobulin-like receptor 2 precursor (LAIR-2) (CD306 antigen).	MAGEA3	melanoma antigen family A, 3
CCDC9	Coiled-coil domain-containing protein 9.	CSDC2	cold shock domain containing C2, RNA binding
PHOX2A	Paired mesoderm homeobox protein 2A (Paired-like homeobox 2A)	MUM1	melanoma associated antigen (mutated) 1
MEA1	Male-enhanced antigen 1 (MEA-1).	IL6	interleukin 6 (interferon, beta 2)
PARL	Presenilins-associated rhomboid-like protein, mitochondrial precursor	ELL2	elongation factor, RNA polymerase II, 2
RSU1	Ras suppressor protein 1 (Rsu-1) (RSP-1).	NKG7	natural killer cell group 7 sequence
CUTA	Protein CutA precursor (Brain acetylcholinesterase putative membrane anchor)	CLRN3	clarin 3
MLLT1	Protein ENL (YEATS domain-containing protein 1).	MMP3	matrix metallopeptidase 3 (stromelysin 1, progelatinase)
C19orf52	Uncharacterized protein C19orf52	SOHLH1	spermatogenesis and oogenesis specific basic helix-loop-helix 1
KLHDC3	Kelch domain-containing protein 3 (Protein Peas) (Testis intracellular mediator protein).	ZDHHC18	zinc finger, DHHC-type containing 18
PIP5K1A	Phosphatidylinositol-4-phosphate 5-kinase type-1 alpha	CUL2	cullin 2
NXF1	Nuclear RNA export factor 1 (Tip-associating protein)	SLC2A11	solute carrier family 2 (facilitated glucose transporter), member 11
KIFC3	Kinesin-like protein KIFC3.	TPRX1	tetra-peptide repeat homeobox 1
ACAD9	Acyl-CoA dehydrogenase family member 9, mitochondrial precursor	C15orf27	chromosome 15 open reading frame 27
CRTAP	Cartilage-associated protein precursor.	SLC41A3	solute carrier family 41, member 3
MYOM2	Myomesin-2 (M-protein)	EPHB3	EPH receptor B3
IQSEC2	IQ motif and Sec7 domain-containing protein 2.	DAK	dihydroxyacetone kinase 2 homolog (S. cerevisiae)
SHROOM 2	Apical-like protein (Protein APXL).	TMEM49	transmembrane protein 49
TMEM143	transmembrane protein 143 (TMEM143), mRNA	MIZF	MBD2-interacting zinc finger
TNN	Tenascin-N precursor (TN-N).	SSX3	synovial sarcoma, X breakpoint 3
ACHE	Acetylcholinesterase precursor	STRN3	striatin, calmodulin binding protein 3

PDGFD	Platelet-derived growth factor D precursor
UGT3A1	UDP glycosyltransferase 3 family, polypeptide A1
ICAM4	Intercellular adhesion molecule 4 precursor.
ADRBK1	Beta-adrenergic receptor kinase 1
MAPK3	Mitogen-activated protein kinase 3
ALCAM	CD166 antigen precursor (Activated leukocyte-cell adhesion molecule) (ALCAM).
GPT2	Alanine aminotransferase 2
PLA2G5	Calcium-dependent phospholipase A2 precursor
IQCE	IQ domain-containing protein E.
WDR92	WD repeat domain 92
CLCN3	Chloride channel protein 3 (ClC-3).
SPAG11B	Sperm-associated antigen 11 precursor (EP2 protein) (Sperm antigen HE2).
SPAG11A	sperm associated antigen 11B isoform H precursor
AMICA1	Junctional adhesion molecule-like precursor
miR-365	
COL7A1	Collagen alpha-1(VII) chain precursor
BEST3	bestrophin 3
TBCEL	Tubulin-specific chaperone cofactor E-like protein (EL)
PSMD8	26S proteasome non-ATPase regulatory subunit 8
SETX	senataxin
PTCRA	pre T-cell antigen receptor alpha
ITGAD	Integrin alpha-D precursor (Leukointegrin alpha D) (ADB2) (CD11d antigen)
CPT2	carnitine palmitoyltransferase II
MUS81	Crossover junction endonuclease
NAP1L4	nucleosome assembly protein 1-like 4
DKK3	dickkopf homolog 3 (Xenopus laevis)
SSX1	synovial sarcoma, X breakpoint 1
PMP22	peripheral myelin protein 22
SSX4B	synovial sarcoma, X breakpoint 4B
SSX4	synovial sarcoma, X breakpoint 4
SSX6	synovial sarcoma, X breakpoint 6
ZSWIM5	zinc finger, SWIM-type containing 5
FBXO34	F-box protein 34
MTM1	myotubularin 1
CCDC39	coiled-coil domain containing 39
TMCC1	transmembrane and coiled-coil domain family 1
TMPRSS11D	transmembrane protease, serine 11D
CLTC	clathrin, heavy chain (Hc)
DCP1A	DCP1 decapping enzyme homolog A (S. cerevisiae)
B4GALT5	UDP-Gal:betaGlcNAc beta 1,4-galactosyltransferase, polypeptide 5
EP300	E1A binding protein p300
TCF25	Transcription factor 25 (Nuclear localized protein 1)
Q5JVI1_HUMAN	Novel protein (Fragment). [Source:Uniprot/SPTREMBL;Acc:Q5JVI1]
AIFM1	Apoptosis-inducing factor 1, mitochondrial precursor
ESR2	Estrogen receptor beta (ER-beta)
NP_945352.1	RIKEN cDNA 2310002J15 gene
PLEKHG3	pleckstrin homology domain containing, family G, member 3
SLC22A7	solute carrier family 22 member 7 isoform a
XR_01778	annexin A2 pseudogene 2 (ANXA2P2),

	MUS81
UBAC2	UBA domain containing 2
MGA	MAX gene associated
NUDT1	7,8-dihydro-8-oxoguanine triphosphatase
KIAA0391	Uncharacterized protein KIAA0391
DYNLL1	Dynein light chain 1, cytoplasmic
HAPLN2	Hyaluronan and proteoglycan link protein 2 precursor
FCRL1	Fc receptor-like 1
STYXL1	Serine/threonine/tyrosine-interacting-like protein 1
TMEM106C	transmembrane protein 106C
PRPF40A	PRP40 pre-mRNA processing factor 40 homolog A (S. cerevisiae)
CYP2C9	cytochrome P450, family 2, subfamily C, polypeptide 9
KCNQ1	potassium voltage-gated channel, KQT-like subfamily, member 1
NLRP1	NLR family, pyrin domain containing 1
RAPGEF4	Rap guanine nucleotide exchange factor (GEF) 4
RHEBL1	Ras homolog enriched in brain like 1
CYP24A1	cytochrome P450, family 24, subfamily A, polypeptide 1
USP22	ubiquitin specific peptidase 22
DLAT	dihydrolipoamide S-acetyltransferase
ADD3	adducin 3 (gamma)
CCDC55	coiled-coil domain containing 55
MAK	male germ cell-associated kinase
IHPK2	inositol hexaphosphate kinase 2
USP48	ubiquitin specific peptidase 48
ZNF680	zinc finger protein 680
NUFIP2	nuclear fragile X mental retardation protein interacting protein 2
C14orf142	chromosome 14 open reading frame 142
DCP2	DCP2 decapping enzyme homolog
4.1	misc RNA
miR-374b	
MYC	Myc proto-oncogene protein (c-Myc) (Transcription factor p64)
GNL3	Guanine nucleotide-binding protein-like 3 (Nucleolar GTP-binding protein 3)
NETO1	neuropilin (NRP) and tolloid (TLL)-like 1
ACTR10	Actin-related protein 10 (hARP11)
SMPDL3A	Acid sphingomyelinase-like phosphodiesterase 3a precursor
PSMC3IP	TBP-1 interacting protein isoform 1
MEP1B	Meprin A subunit beta precursor
TIGD4	Tigger transposable element-derived protein 4
GRAMD1A	GRAM domain containing 1A
CCL18	Small inducible cytokine A18 precursor (CCL18)
CCL11	chemokine (C-C motif) ligand 11
COPB2	Coatomer subunit beta
NOG	Noggin precursor
C11orf71	Uncharacterized protein C11orf71
NSUN3	NOL1/NOP2/Sun domain family, member 3
TRMT11	tRNA guanosine-2'-O-methyltransferase TRM11 homolog
BPNT1	3'(2'), 5'-bisphosphate nucleotidase 1
ANKRD32	Ankyrin repeat domain-containing protein 32
PCNX	pecanex homolog (Drosophila)
SFRS15	Splicing factor, arginine/serine-rich 15 (CTD-binding SR-like protein RA4)
PAXIP1	PAX interacting protein 1
OCIAD1	OCIA domain containing 1 isoform 1
ADD3	adducin 3 (gamma)
NCK1	NCK adaptor protein 1

	(S. cerevisiae)		
USP33	ubiquitin specific peptidase 33	DBR1	debranching enzyme homolog 1 (S. cerevisiae)
NSL1	NSL1, MIND kinetochore complex component, homolog (S. cerevisiae)	STK38L	serine/threonine kinase 38 like
LUC7L2	LUC7-like 2 (S. cerevisiae)	RANBP6	RAN binding protein 6
TRAM1	translocation associated membrane protein 1	DPY19L4	dpy-19-like 4 (C. elegans)
TMOD3	tropomodulin 3 (ubiquitous)	7A5	putative binding protein 7a5
MTMR2	myotubularin related protein 2	ACVR2B	activin A receptor, type IIB
TBK1	TANK-binding kinase 1	KIAA0999	KIAA0999 protein
RBM11	RNA binding motif protein 11	VGLL3	vestigial like 3 (Drosophila)
ACVR1	activin A receptor, type I	SYT14L	synaptotagmin XIV-like
LPAR5	lysophosphatidic acid receptor 5	AHI1	Abelson helper integration site 1
SGTB	small glutamine-rich tetratricopeptide repeat (TPR)-containing, beta	DCDC2	doublecortin domain containing 2
MGC13057	hypothetical protein MGC13057	TMPRSS11B	transmembrane protease, serine 11B
PRUNE2	prune homolog 2 (Drosophila)	DNAJC12	DnaJ homolog subfamily C member 12 (J domain-containing protein 1)
NFIB	nuclear factor I	SLC25A5	ADP/ATP translocase 2 (Adenine nucleotide translocator 2)
MYCBP	c-myc binding protein	VPS36	Vacuolar protein sorting-associated protein 36
UBOX5	U-box domain containing 5	SLC26A5	Prestin (Solute carrier family 26 member 5)
NP_057218.1	plasma glutamate carboxypeptidase	Q7Z2R7_HUMAN	MSTP131
THBS3	Thrombospondin-3 precursor	ATF2	Cyclic AMP-dependent transcription factor ATF-2
XR_015893.1	similar to zinc finger protein 611 (LOC731301), mRNA	CYorf15A	Putative testis protein CYorf15A
NP_001006948.1	RIKEN cDNA E030041M21 gene	C4orf20	Ufm1-specific protease 2

Table 3.5: Analysis of biological processes of the predicted miRNAs targets by GOstat

miRNAs	GO process	Target genes	Count	Total	*p*-value
miR-575	GO:0065008: Regulation of biological quality	*vegfb rdh12 rb1cc1 tpt1 actl6a gclc tfpi gcm2*	8	953	0.0281
	GO:0048518: Positive regulation of biological process	*nenf masp2 vegfb bid rb1cc1 gclc mrpl12*	7	1062	0.056
	GO:0012501: Programmed cell death	*bid rb1cc1 tpt1 gclc blid gcm2*	6	862	0.056
	GO:0009308: Amine metabolic process	*ddt iars2 rb1cc1 gclc slc7a7*	5	636	0.056
miR-671-5p	GO:0051129: Negative regulation of cellular component organization and biogenesis	*sptbn2 sptb*	2	52	0.0611
	GO:0002253 Activation of immune response	*c1qa cd79a*	2	71	0.079
miR-483-5p	GO:0042133: Neurotransmitter metabolic process	*ache phox2a*	2	32	0.0296
	GO:0009306: Protein secretion	*bace2 ache*	2	57	0.0345
miR-574-3p	GO:0050789: Regulation of biological process	*lat deaf1 csdc2 il6 cul2 tcf25 plekhg3 ell2 ssx4 ep300 cbx8 tprx1 aifm1 esr2 ssx1 dkk3 ssx3 mizf madd ssx6 pmp22 gabbr1 sohlh1*	23	6731	0.0185
	GO:0010468: Regulation of gene expression	*deaf1 csdc2 ssx1 il6 mizf ssx3 ssx6 tcf25 ell2 ssx4 ep300 tprx1 cbx8 esr2 sohlh1*	15	3833	0.0211
	GO:0048468: Cell development	*deaf1 madd il6 cul2 ephb3 ep300 aifm1 cse1l*	8	1242	0.0306
	GO:0048856: Anatomical structure development	*deaf1 col7a1 il6 dkk3 ephb3 mtm1 tcf25 ep300 pmp22 esr2*	10	2005	0.045
	GO:0012501: Programmed cell death	*ep300 madd il6 cul2 aifm1 cse1l*	6	862	0.0541
miR-365	GO:0043687: Post-translational protein modification	*acvr1 usp48 tbcel usp33 ubox5 mtmr2 mak rapgef4 styxl1 tbk1 usp22*	11	2815	0.0678

	GO:0044248: Cellular catabolic process	*dlat dcp2 usp33 usp48 usp22*	5	809	0.0912
	GO:0043067: Regulation of programmed cell death	*acvr1 ihpk2 nlrp1 prune2*	4	576	0.0912
	GO:0006512: Ubiquitin cycle	*usp33 ubox5 usp48 usp22*	4	549	0.0912
	GO:0048522: Positive regulation of cellular process	*acvr1 ihpk2 nlrp1 tbk1 prune2*	5	954	0.0951
miR-374b	GO:0000287: Magnesium ion binding	*acvr2b stk38l kiaa0999 bpnt1*	4	447	0.101
	GO:0006968: Cellular defense response	*dcdc2 ccl11*	2	72	0.101
	GO:0042379: Chemokine receptor binding	*ccl18 ccl11*	2	77	0.101

Table 3.6: Pathway analysis of the selected miRNAs using DAVID Bioinformatics software

Category	Term	Count	%	PValue	Genes
miR-575					
KEGG_ PATHWAY	hsa04650:Natural killer cell mediated cytotoxicity	5	8.47%	2.77E-03	KIR2DL4, MICA, KIR2DL1, KIR3DL1, BID
KEGG_ PATHWAY	hsa04612:Antigen processing and presentation	3	5.08%	5.42E-02	KIR2DL4, KIR2DL1, KIR3DL1
miR-483-5p					
KEGG_ PATHWAY	hsa04510:Focal adhesion	3	6.67%	1.23E-01	PDGFD, TNN, MAPK3
miR-574-3p					
KEGG_ PATHWAY	hsa05040:Huntington's disease	2	3.77%	6.68E-02	EP300, CLTC
KEGG_ PATHWAY	hsa05211:Renal cell carcinoma	2	3.77%	1.48E-01	CUL2, EP300
miR-374b					
KEGG_ PATHWAY	hsa04350:TGF-beta signaling pathway	3	6.98%	1.47E-02	ACVR2B, NOG, MYC
KEGG_ PATHWAY	hsa04060:Cytokine-cytokine receptor interaction	3	6.98%	9.98E-02	ACVR2B, CCL11, CCL18

Table 3.7: Significantly dysregulated miRNAs and the associated human cancers

miRNAs (up / down regulation)	Associated cancers		Chromosome Location	Validated Gene targets
	Down regulation	Up regulation		
miR-125a-5p↓	Lung cancer (Wang, Mao et al. 2009)		19q13.41	
miR-15a↓	Chronic Lymphocytic Leukemia (CLL) (Calin, Liu et al. 2004; Calin, Pekarsky et al. 2007; Calin, Cimmino et al. 2008) Pituitary adenoma (Bottoni, Piccin et al. 2005) HCC (Budhu, Jia et al. 2008) Prostate cancer (Bonci, Coppola et al. 2008)	Kidney cancer (Chow, Youssef et al. 2009)	13q14.2	BCL2 (Cimmino, Calin et al. 2005; Calin, Pekarsky et al. 2007), CDC25A (Lee, Masyuk et al. 2008), CCND1(Bandi, Zbinden et al. 2009), CCND2(Bandi, Zbinden et al. 2009), CCNE1(Bandi, Zbinden et al. 2009), WNT3A(Bonci, Coppola et al. 2008), RARS(Calin, Dumitru et al. 2002; Bottoni, Piccin et al. 2005)
miR-15b↑	CLL (Calin, Liu et al. 2004; Calin, Pekarsky et al. 2007) Gastric cancer (Xia, Zhang et al. 2008)	Colorectal cancer (Volinia, Calin et al. 2006)	3q25.33	BCL2 (Xia, Zhang et al. 2008), CCNE1 (Xia, Qi et al. 2009), eIF-4A (Xi, Shalgi et al. 2006)
miR-16↑	Breast cancer (Iorio, Ferracin et al. 2005) CLL (Calin, Pekarsky et al. 2007) Gastric cancer (Xia, Zhang et al. 2008) Pituitary adenoma (Bottoni, Piccin et al. 2005) Prostate cancer (Porkka, Pfeiffer et al. 2007; Schaefer, Jung et al. 2009)	Lung cancer (Yanaihara, Caplen et al. 2006)	*miR-16-1:* 13q14.2; *miR-16-2:* 3q25.33	BCL2 (Cimmino, Calin et al. 2005; Xia, Zhang et al. 2008), Caprin-1 (Kaddar, Rouault et al. 2009), CGI-38 (Kiriakidou, Nelson et al. 2004), CCND1 (Liu, Fu et al. 2008; Bandi, Zbinden et al. 2009), CCND3 (Liu, Fu et al. 2008), CCNE1 (Liu, Fu et al. 2008), CDK6 (Liu, Fu et al. 2008),

				HMGA1 (Kaddar, Rouault et al. 2009), WNT3A(Bonci, Coppola et al. 2008), RARS(Calin, Dumitru et al. 2002)
miR-191↑	Kidney cancer (Chow, Youssef et al. 2009)	Colorectal cancer (Volinia, Calin et al. 2006; Xi, Formentini et al. 2006; Xi, Shalgi et al. 2006) Gastric cancer (Volinia, Calin et al. 2006) Lung cancer (Volinia, Calin et al. 2006; Yanaihara, Caplen et al. 2006) Pancreatic cancer (Volinia, Calin et al. 2006) Pituitary adenoma (Bottoni, Piccin et al. 2005) Prostate cancer (Volinia, Calin et al. 2006; Prueitt, Yi et al. 2008) Glioblastoma (Ciafre, Galardi et al. 2005)	3p21.31	
miR-197↓	Glioblastoma (Ciafre, Galardi et al. 2005) OSCC (Kozaki, Imoto et al. 2008) Uterine leiomyeomas (Wang, Zhang et al. 2007)	Lung cancer (Yanaihara, Caplen et al. 2006) Pituitary adenoma (Bottoni, Piccin et al. 2005; Bottoni, Zatelli et al. 2007)	1p13.3	FUS1 (Du, Schageman et al. 2009), ACVR1(Weber, Teresi et al. 2006), TSPAN3(Weber, Teresi et al. 2006)
miR-200b↓	Breast cancer (Gregory, Bert et al. 2008) CLL (Calin, Liu et al. 2004) HCC (Murakami, Yasuda et al. 2006) Kidney cancer (Chow, Youssef et al. 2009) OSCC (Kozaki, Imoto et al. 2008)	Colorectal cancer (Bandres, Cubedo et al. 2006) Ovarian cancer (Iorio, Visone et al. 2007) Cholangiocarcinoma (Meng, Henson et al. 2006)	1p36.33	SIP1 (ZEB2) (Korpal, Lee et al. 2008), ZEB1(Korpal, Lee et al. 2008), ZFHX1B (Christoffersen, Silahtaroglu et al. 2007), PDGFD (Kong, Li et al. 2009), PTPN12 (Meng,

				Henson et al. 2006)
miR-200c↑	Breast cancer (Hurteau, Carlson et al. 2007; Korpal, Lee et al. 2008) Lung cancer (Hurteau, Carlson et al. 2007) OSCC (Kozaki, Imoto et al. 2008)	Ovarian cancer (Iorio, Visone et al. 2007)	12p13.31	SIP1 (ZEB2) (Korpal, Lee et al. 2008), ZEB1 (Korpal, Lee et al. 2008), TUBB3 (Cochrane, Spoelstra et al. 2009)
miR-203↓	Acute Lymphoblastic Leukemia (Bueno, Perez de Castro et al. 2008) Chronic Myeloid Leukemia (Bueno, Perez de Castro et al. 2008) Esophageal cancer (Feber, Xi et al. 2008) OSCC (Kozaki, Imoto et al. 2008) uterine leiomyoma (Wang, Zhang et al. 2007)	Bladder cancer (Gottardo, Liu et al. 2007) Breast cancer (Iorio, Ferracin et al. 2005) Colorectal cancer (Schetter, Leung et al. 2008) Lung cancer (Yanaihara, Caplen et al. 2006) Pancreatic cancer (Greither, Grochola et al. 2009)	14q32.33	ABL1 (Bueno, Perez de Castro et al. 2008), SOCS3 (Sonkoly, Wei et al. 2007), p63 (TP63) (Yi, Poy et al. 2008)
miR-21↓		Breast cancer (Iorio, Ferracin et al. 2005; Yan, Huang et al. 2008) Cervical cancer (Lui, Pourmand et al. 2007) Cholangiocarcinoma (Meng, Henson et al. 2006) CLL (Marton, Garcia et al. 2008) Colorectal cancer (Volinia, Calin et al. 2006) Diffuse large B cell lymphoma (DLBCL)(Lawrie, Gal et al. 2008) Esophageal cancer (Feber, Xi et al. 2008) Glioblastoma (Chan, Krichevsky et al. 2005; Ciafre, Galardi et al.	17q23.2	BTG2 (Liu, Wu et al. 2009), MARCKS (Li, Li et al. 2009), SERPINB5 (Zhu, Wu et al. 2008), PDCD4 (Asangani, Rasheed et al. 2008; Chen, Liu et al. 2008; Yao, Xu et al. 2009), PTEN (Meng, Henson et al. 2007), TPM1 (Zhu, Si et al. 2007; Zhu, Wu et al. 2008), HNRPK (Papagiannakopoulos, Shapiro et al. 2008), TAp63 (Papagiannakopoulos, Shapiro et al. 2008), LRRFIP1 (Li, Li et al.

		2005; Chen, Liu et al. 2008) Head & Neck cancer (Tran, McLean et al. 2007; Chang, Jiang et al. 2008) HCC (Meng, Henson et al. 2007) Kidney cancer (Chow, Youssef et al. 2009) Lung cancer (Yanaihara, Caplen et al. 2006) OSCC (Kozaki, Imoto et al. 2008) Ovarian cancer (Iorio, Visone et al. 2007) Pancreatic cancer (Bloomston, Frankel et al. 2007) Prostate cancer (Volinia, Calin et al. 2006; Prueitt, Yi et al. 2008) Stomach cancer (Volinia, Calin et al. 2006) Uterine leiomyomas (Wang, Zhang et al. 2007)		2009) JAG1 (Hashimi, Fulcher et al. 2009), WNT1 (Hashimi, Fulcher et al. 2009)
miR-23a↑	CLL (Calin, Liu et al. 2004) Prostate cancer (Volinia, Calin et al. 2006; Porkka, Pfeiffer et al. 2007) OSCC (Kozaki, Imoto et al. 2008)	Bladder cancer (Gottardo, Liu et al. 2007) Breast cancer (Iorio, Ferracin et al. 2005) Glioblastoma (Ciafre, Galardi et al. 2005) Head & Neck cancer (Tran, McLean et al. 2007) Pancreatic cancer (Bloomston, Frankel et al. 2007) Stomach cancer (Volinia, Calin et al. 2006)	19p13.12	RING1 (Lin, Murtaza et al. 2009), CXCL12 (Lewis, Shih et al. 2003), FLJ13158 (Kiriakidou, Nelson et al. 2004)
miR-23b↑	Prostate cancer (Porkka, Pfeiffer et al. 2007) Renal cancer (O'Rourke, Swanson et al. 2006)	Bladder cancer (Gottardo, Liu et al. 2007) Breast cancer (Iorio, Ferracin et al. 2005) CLL (Calin, Ferracin et al. 2005)	9q22.32	uPA (Salvi, Sabelli et al. 2009), c-met/MET (Salvi, Sabelli et al. 2009), MOR1 (Wu, Law et al. 2008)

	OSCC (Kozaki, Imoto et al. 2008)	Cervical cancer (Lui, Pourmand et al. 2007) Colon cancer (Volinia, Calin et al. 2006) Glioblastoma (Ciafre, Galardi et al. 2005) Head & Neck cancer (Tran, McLean et al. 2007) Pancreatic cancer (Volinia, Calin et al. 2006; Bloomston, Frankel et al. 2007) Stomach cancer (Volinia, Calin et al. 2006) Uterine leiomyomas (Wang, Zhang et al. 2007)		
miR-24↓		Breast cancer (Iorio, Ferracin et al. 2005)	*miR-42-1:* 9q22.32; *miR-24-2:* 19p13.13	DHFR (Mishra, Humeniuk et al. 2007), E2F2, MYC, AURKB, CCNA2, CDC2, CDK4 (Lal, Navarro et al. 2009), p16 (Lal, Kim et al. 2008)
miR-25↓	CLL (Calin, Liu et al. 2004)	Pancreatic cancer (Volinia, Calin et al. 2006) Prostate cancer (Volinia, Calin et al. 2006) Stomach cancer (Volinia, Calin et al. 2006) Glioblastoma (Ciafre, Galardi et al. 2005) HCC (Li, Tan et al. 2009)	7q22.1	p57 (Kim, Yu et al. 2009), BIM (Li, Tan et al. 2009)
miR-26b↓	CLL (Calin, Pekarsky et al. 2007) OSCC (Kozaki, Imoto et al. 2008) Prostate cancer (Porkka, Pfeiffer et al. 2007)	Bladder cancer (Gottardo, Liu et al. 2007) Pituitary adenoma (Bottoni, Zatelli et al. 2007)	2q35	
miR-27a↓	OSCC (Kozaki, Imoto et al. 2008)	Breast cancer (Mertens-Talcott, Chintharlapalli et al. 2007; Guttilla and White 2009)	19p13.12	Myt1 (Mertens-Talcott, Chintharlapalli et al. 2007), MDR1(Zhu, Wu et al.

		Kidney cancer (Chow, Youssef et al. 2009) Gastric cancer (Liu, Tang et al. 2009) Prostate cancer (Porkka, Pfeiffer et al. 2007) Uterine leiomyomas (Wang, Zhang et al. 2007)		2008) , FOXO1 (Guttilla and White 2009), PHB (prohibitin) (Schaar, Medina et al. 2009), ZBTB10 (Mertens-Talcott, Chintharlapalli et al. 2007)
miR-27b↓	Lung cancer (Yanaihara, Caplen et al. 2006) OSCC (Kozaki, Imoto et al. 2008) Prostate cancer (Porkka, Pfeiffer et al. 2007; Prueitt, Yi et al. 2008)		9q22.32	CYP1B1 (Tsuchiya, Nakajima et al. 2006)
miR-30a↓	HCC (Budhu, Jia et al. 2008)	Uterine leiomyomas (Wang, Zhang et al. 2007)	6q13	
miR-342-3p↑		Kidney cancer (Chow, Youssef et al. 2009)	14q32.2	
miR-365↓		Breast cancer (Yan, Huang et al. 2008)	*miR-365-1:* 16p13.12; *miR-365-2:* 17q11.2	
miR-7↓	Glioblastoma (Kefas, Godlewski et al. 2008)	Bladder cancer (Veerla, Lindgren et al. 2009) Lung cancer (Crawford, Batte et al. 2009)	miR-7-1: 9q21.32; miR-7-2: 15q26.1; miR-7-3: 19p13.3	PAK1 (Reddy, Ohshiro et al. 2008), EGFR (Kefas, Godlewski et al. 2008), SNCA (Junn, Lee et al. 2009)
let-7a↑	Breast cancer (Iorio, Ferracin et al. 2005) CLL (Marton, Garcia et al. 2008) Lung cancer (Takamizawa, Konishi et al. 2004) Prostate cancer (Porkka, Pfeiffer et al. 2007) Burkitt's Lymphoma (Sampson, Rong et al. 2007)	Cholangiocarcinoma (Meng, Henson et al. 2007)	*let-7a-1:* 9q22.32; *let-7a-2:* 11q24.1; *let-7a-3:* 22q13.31	MYC (Sampson, Rong et al. 2007), caspase 3 (CASP3) (Tsang and Kwok 2008), Integrinβ3 (Muller and Bosserhoff 2008), NIRF (UHRF2), NF2 (He, Duan et al. 2009), HMGA2 (Motoyama, Inoue et al. 2008), PRDM1 (Blimp-1) (Nie, Gomez et al. 2008)

let-7d↑	Breast cancer (Iorio, Ferracin et al. 2005) Head & Neck Squamous Cell Carcinoma (Childs, Fazzari et al. 2009) OSCC (Kozaki, Imoto et al. 2008)	Prostate cancer (Porkka, Pfeiffer et al. 2007)	9q22.32	
Let-7e↓	OSCC (Kozaki, Imoto et al. 2008) Ovarian cancer (Dahiya, Sherman-Baust et al. 2008) Pituitary adenoma (Bottoni, Zatelli et al. 2007)	Acute myeloid leukemia (Dixon-McIver, East et al. 2008) DLBCL (Roehle, Hoefig et al. 2008)	19q13.33	SMC1L1 (SMC1A) (Kiriakidou, Nelson et al. 2004)
let-7f↑	Breast cancer (Yan, Huang et al. 2008) Ovarian cancer (Dahiya, Sherman-Baust et al. 2008) Prostate cancer (Porkka, Pfeiffer et al. 2007)		*let-7f-1:* 9q22.32; *let-7f-2:* Xp11.22	
let-7g↓	HCC (Budhu, Jia et al. 2008) OSCC (Kozaki, Imoto et al. 2008) Prostate cancer (Porkka, Pfeiffer et al. 2007)	Colon cancer (Nakajima, Hayashi et al. 2006) Lung cancer	9q22.32	HMGA2 (Kumar, Erkeland et al. 2008)

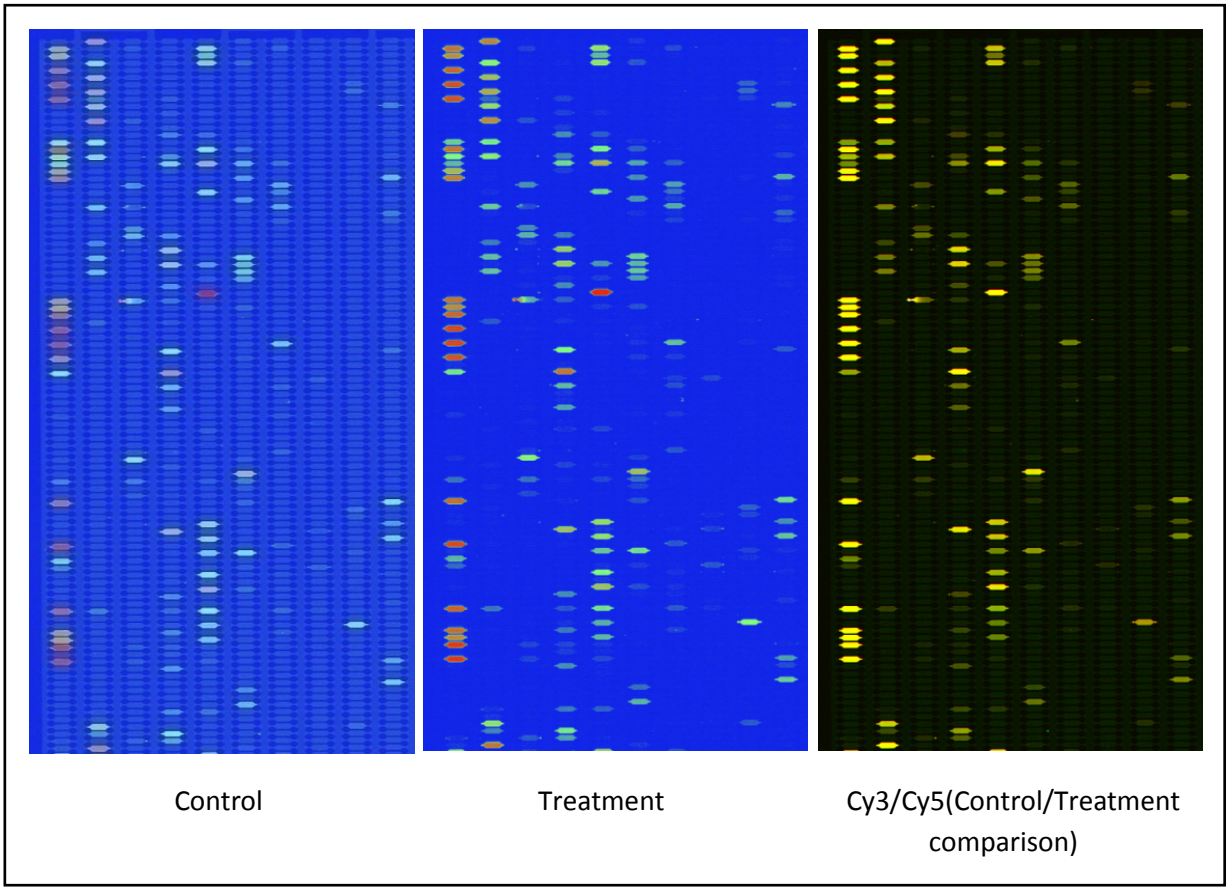

Figure 3.1: Representative heat map diagram of miRNA expression levels in control and 5-FU treated MCF7 cells. The image was displayed in pseudo colors to expand visual dynamic range, and the signal intensity increases from blue to green, to yellow, and to red.

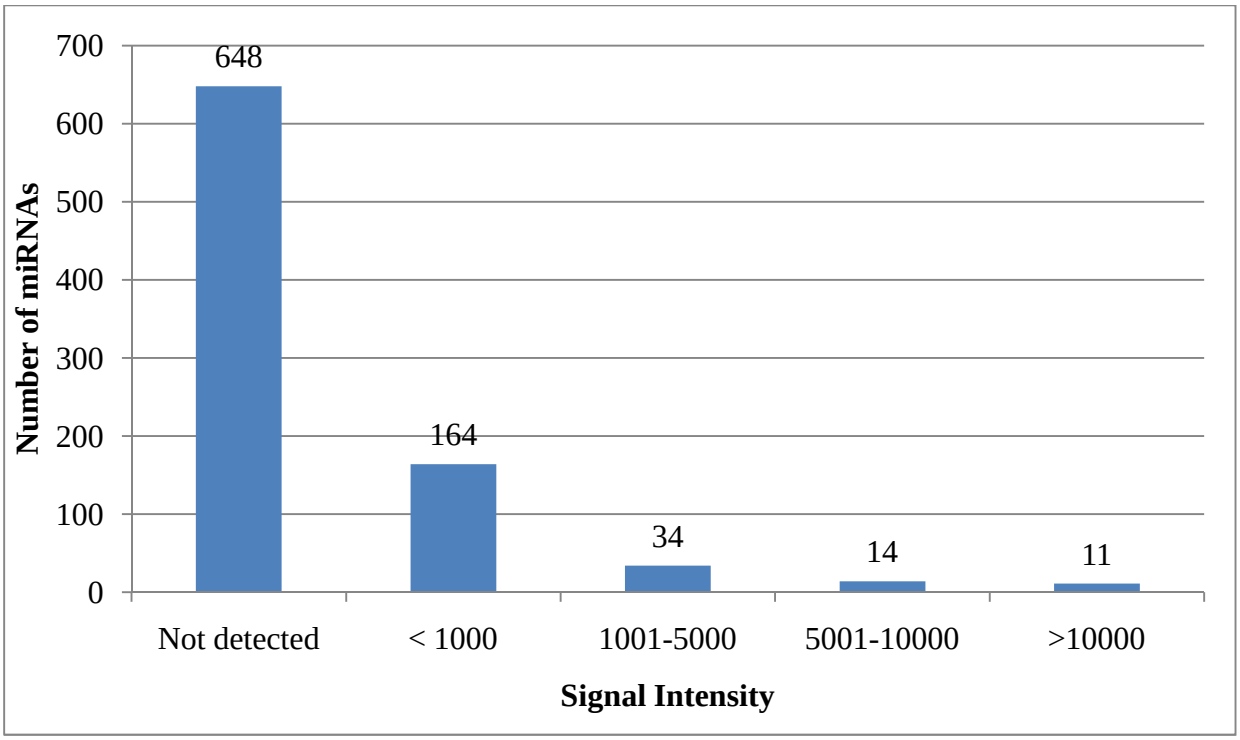

Figure 3.2: miRNA expression profile in human MCF7 breast cancer cell line.

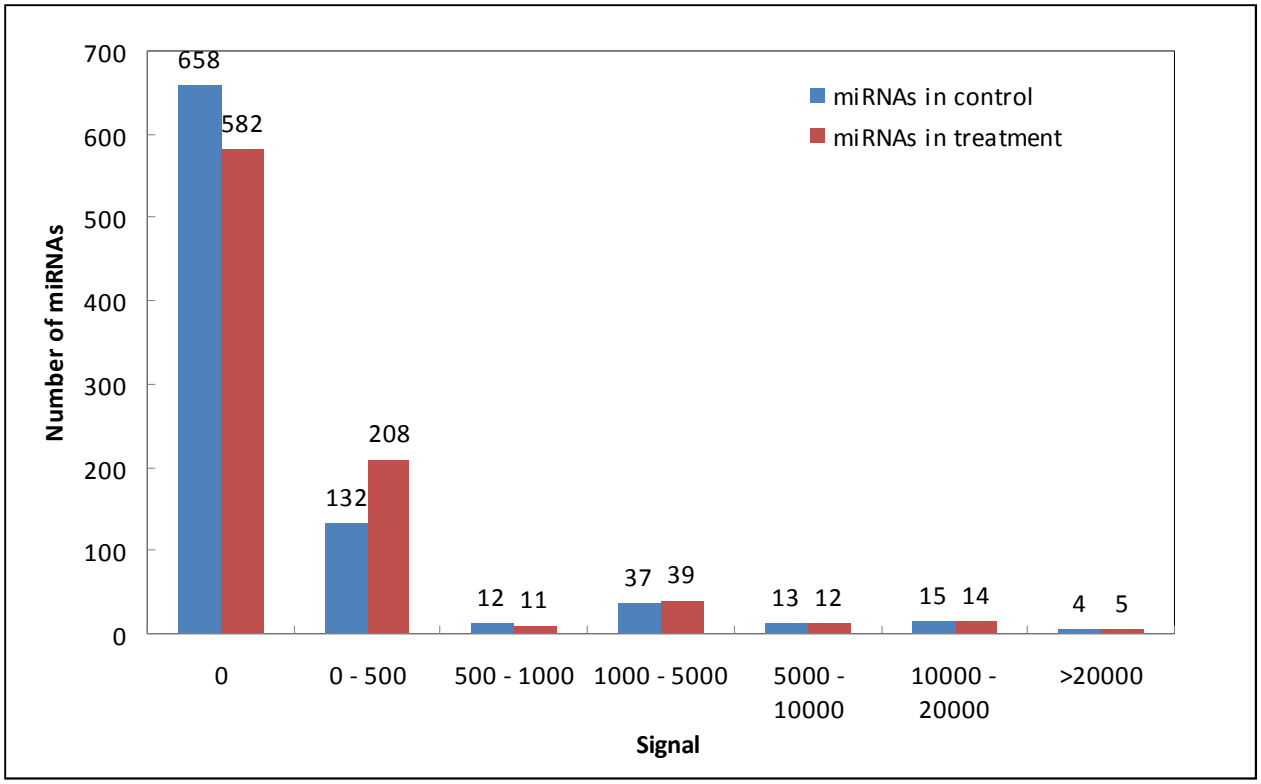

Figure 3.3: Signal distribution of all analyzed miRNAs in MCF7 cells by miRNA microarray assay.

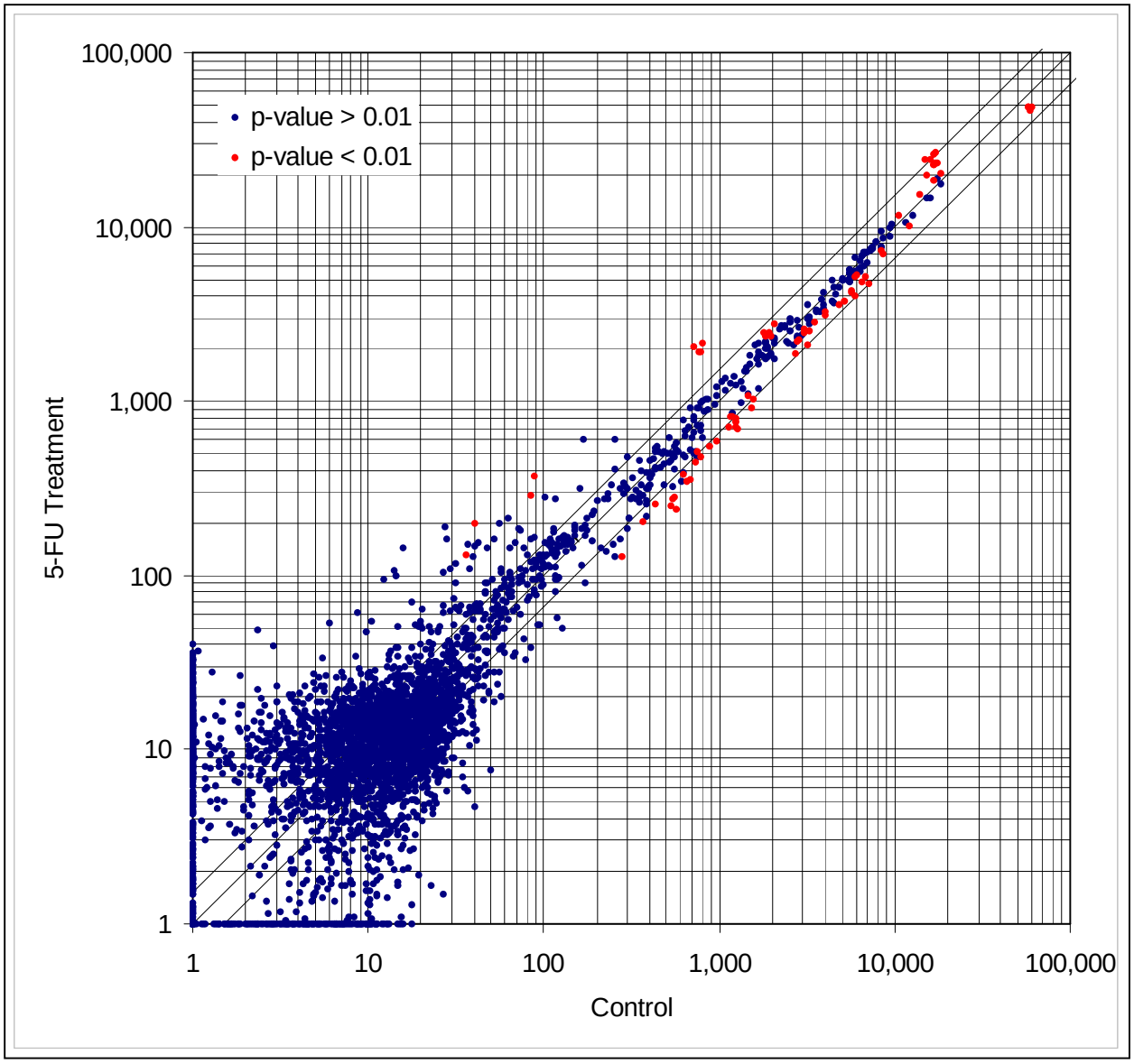

Figure 3.4: Comparison of signal distribution between control and 5-FU-treatment human breast cancer cell MCF7.

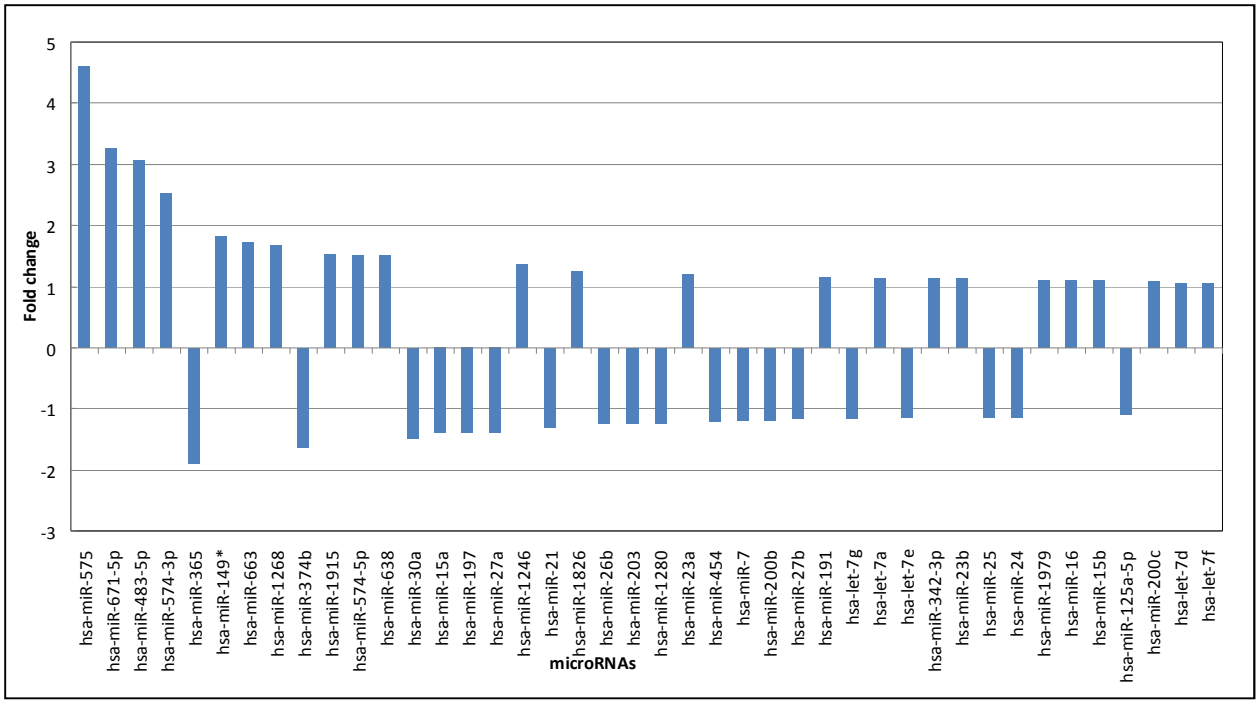

Figure 3.5: Fold change in the expression of significantly altered miRNA after 5-FU exposure

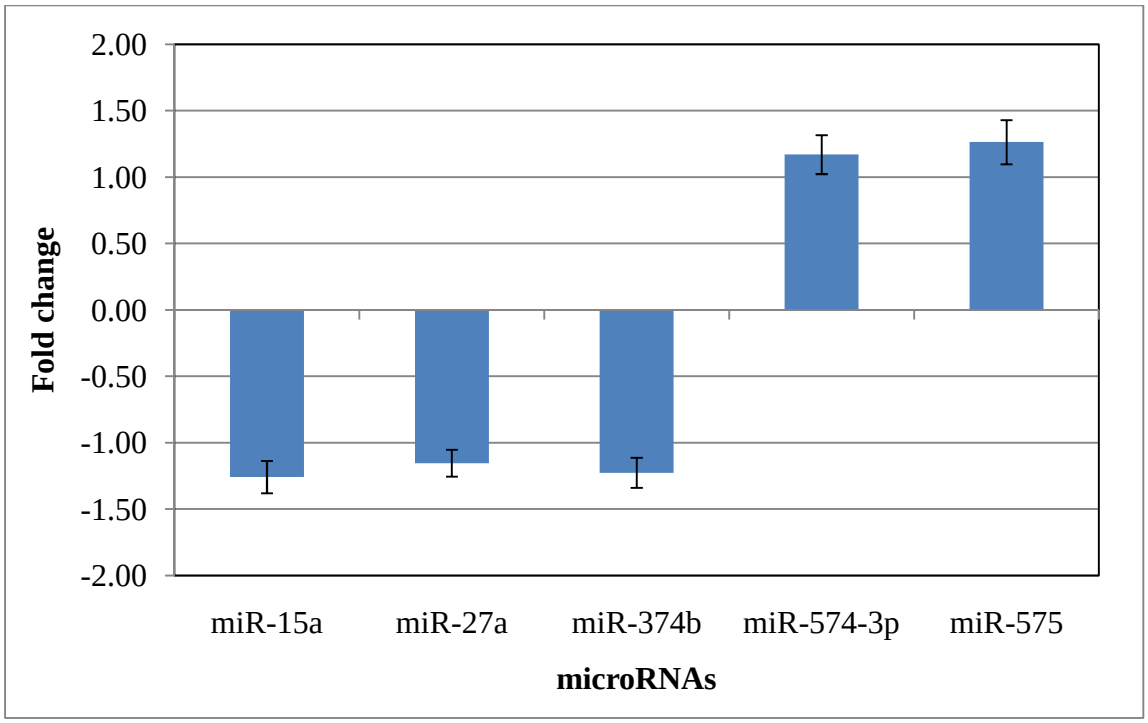

Figure 3.6: Confirmatory studies of selected miRNAs by TaqMan Real-Time PCR. The results are represented as mean ± SEM of triplicate samples.

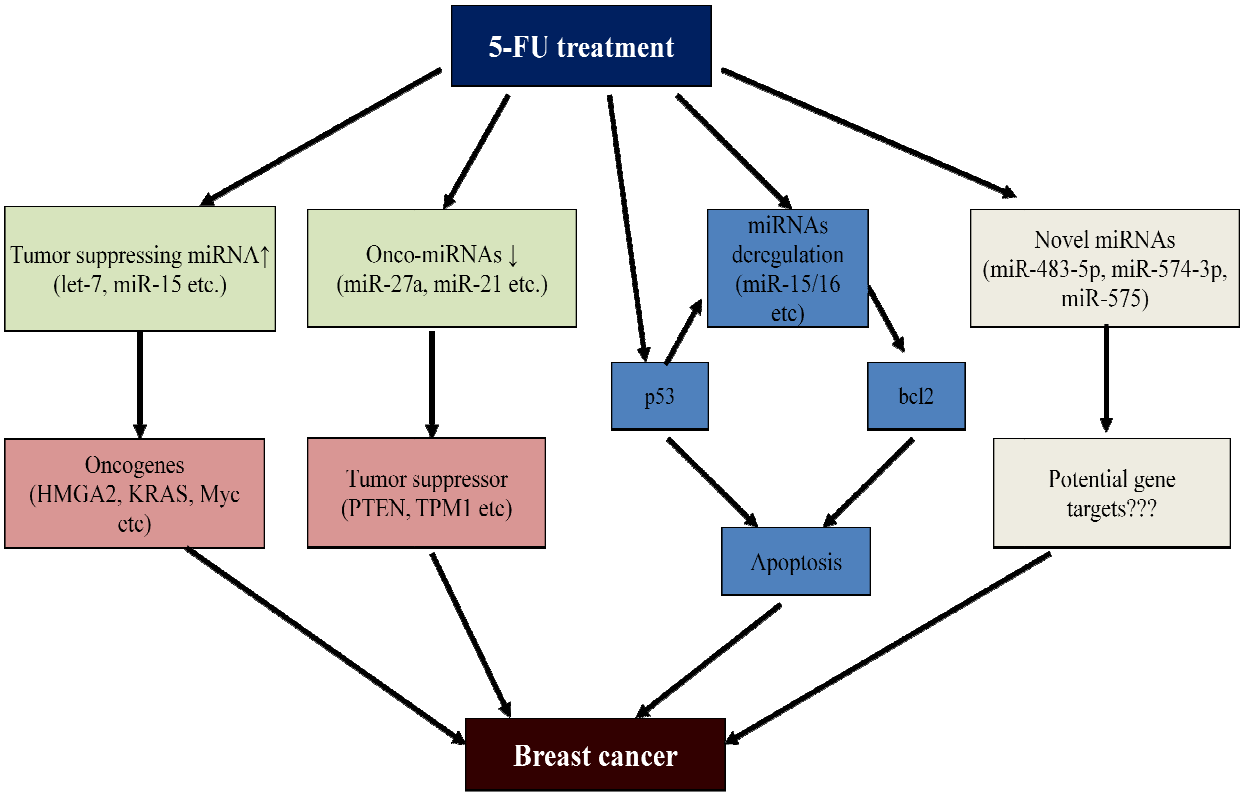

Figure 3.7: A proposed model showing the functional mechanism of 5-FU-mediated miRNAs in breast cancer treatment.

Chapter 4: Effect of 5-fluorouracil on the Expression of Selected MicroRNAs in a Dose- and Time- Dependent Manner

Abstract

MicroRNAs (miRNAs) are a newly identified class of gene regulators which have been shown to play an important role in human cancers. The ability of 5-fluororacil to alter the expression levels of miRNAs in MCF7 cells was previously observed. In this study, we further investigate the dose- and time-dependent modification in miRNA expression levels after 5-FU treatment. Eleven miRNAs previously identified in the microarray to be differentially expressed after treatment were selected to analyze their responsiveness to eight different 5-FU dosages of 0.001, 0.005, 0.01, 0.1, 0.7, 1, 5 and 10µM. The selected miRNAs included miR-365, miR-374b, miR-483-5p, miR-574-3p and miR-575, which were shown to be highly dysregulated in the microarray analysis. Additionally, let-7g, miR-10b, miR-15a, miR-16, miR-21, miR-27a were investigated based on previous reports of their connection to breast cancer. Of these, miR-10b, miR-21, miR-365 and miR-483-5p were found to be significantly regulated in a beneficial way for cancer treatment. Time-response data was also generated for miR-10b, miR-21, miR-483-5p, miR-574-3p and miR-575 following 12, 24, 36, 48, 60 and 72 hours of treatment with 0.1 (low), 0.7 (moderate) and 10 (high) µM 5-FU concentrations. At 0.7µM for short treatment duration, expression levels of miR-10b, miR-21, miR-574-3p and miR-575 were significantly altered; while treatment with high 5-FU dose for shorter time points showed a significant down-regulation of miR-21and miR-574-3p. The data suggests that miRNA expression in MCF7 cells is sensitive to 5-FU therapy at low doses and shorter treatment durations. The down-regulation of an important oncomir, miR-21; and alteration in the expression of three new miRNAs with no

previous breast cancer association, miR-483-5p, miR-574-3p and miR-575 indicates that miRNA might play an important role in 5-FU therapy.

Keywords: 5-fluorouracil, microRNA, qRT-PCR, breast cancer, MCF7

Introduction

Breast cancer is one of the leading causes of deaths worldwide, claiming more than 40,000 deaths in US in the year 2009 (Jemal, Siegel et al. 2009), and is expected to account for 27% of all new cancer cases among women for 2009 (Jemal, Siegel et al. 2009). The breast cancer cases are principally graded based on the cellular histology and the expression pattern of specific proteins such as estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2/neu). The expression profiles of these proteins play an important role in differentiating between benign and malignant breast tumors. About 65% of breast cancer cases are ER+ (Teixeira, Reed et al. 1995), which is important in predicting treatment options and final prognosis. The absence of estrogen receptor in primary breast tumors is associated with early recurrence and poor prognosis (Knight, Livingston et al. 1977; Hahnel, Woodings et al. 1979). Approximately 15-20 % of breast cancers have an amplification of the *HER2/neu* gene or over-expression of its protein product (Slamon, Clark et al. 1987; Slamon, Godolphin et al. 1989); which, in turn, is associated with increased disease reoccurrence and poor prognosis (Slamon, Clark et al. 1987). Similarly, several other oncogenes and tumor suppressor genes have been identified which show a differential expression in breast tumors compared to normal breast tissues (Chapter I, Table 1.1).

Several treatment options are available for breast cancer, mainly surgery, radiation therapy and chemotherapy. A combination of these approaches is usually adopted to achieve maximum therapeutic efficiency. However, the prevalent side effects due to chemotherapeutic drugs and the associated low quality of life after chemotherapy have urged an extensive need for more targeted and effective treatment options. Several drugs targeting the oncogenes and tumor suppressor genes have been designed. Trastuzumab (Herceptin®), a humanized monoclonal

antibody against HER-2/neu, has been shown to benefit patients with HER-2/neu-positive metastatic breast cancer (Slamon, Leyland-Jones et al. 2001; Vogel, Cobleigh et al. 2002; Baselga, Carbonell et al. 2005; Marty, Cognetti et al. 2005). Hormonal therapy with tamoxifen, a selective estrogen receptor modulator, showed a better prognosis in advanced metastatic breast cancer patients (Mouridsen, Palshof et al. 1978; Legha, Buzdar et al. 1979; Frasor, Weaver et al. 2009). Recently, genetic screening tools, such as the Onco*type* DX™, have also been used as diagnostic tools to predict recurrence prognosis and therapeutic response in breast cancer cases (Cronin, Sangli et al. 2007). Further research to identify novel intracellular targets and to design tumor specific drugs is needed to improve the effectiveness of cancer therapy.

MicroRNAs (miRNAs) are small endogenous non-coding single-stranded RNA molecules which act as post-transcriptional gene regulators and cause a decrease in gene expression (Bartel 2004). As a new level of gene regulation mechanism, several diverse functions of miRNAs have been identified, including cellular differentiation, proliferation and apoptosis (Cheng, Byrom et al. 2005; Croce and Calin 2005). Thus, deregulation of miRNAs could lead to a variety of disorders, including cancers. For instance, miRNAs have been reported to play a role in cancer initiation, progression (Calin and Croce 2006) and metastasis (Cheng, Byrom et al. 2005), with about 50% of miRNA genes being localized in cancer-associated genomic regions or in fragile sites (Calin, Sevignani et al. 2004). Aberrant miRNA expression levels have been reported in almost all human cancers (Iorio, Ferracin et al. 2005; Lu, Getz et al. 2005; Calin and Croce 2006; Blenkiron, Goldstein et al. 2007; Zhang, Pan et al. 2007). Certain miRNAs have also displayed unique expression profiles in specific types of cancers (Lu, Getz et al. 2005), making them important biomarkers for classifying these cancers. Many miRNAs target protein-coding genes which act as oncogenes or tumor suppressor genes, and are involved in

tumorigenesis (Zhang and Farwell 2008). For example, miR-21 targets important tumor suppressor genes such as PTEN (Meng, Henson et al. 2007) and TPM1 (Zhu, Si et al. 2007; Zhu, Wu et al. 2008); while miR-15a and miR-16 have been shown to regulate BCL2 (Cimmino, Calin et al. 2005; Calin, Pekarsky et al. 2007). These studies highlight the importance of miRNAs as both stimulators and inhibitors in breast cancer.

5-fluorouracil (5-FU), an antimetabolite, is a clinically useful chemotherapeutic agent approved for treatment of breast (Fumoleau, Bonneterre et al. 2003), colorectal (Wils, O'Dwyer et al. 2001) and head and neck cancers (Posner, Colevas et al. 2000). It is a pyrimidine antagonist which inhibits the activity of enzyme thymidylate synthase (TS), resulting in arrest of the *de novo* DNA synthesis pathway (Santi, McHenry et al. 1974; Sommer and Santi 1974). In addition, 5-FU toxicity is also caused by misincorporation of FdUTP and FUTP into DNA (Mitrovski, Pressacco et al. 1994; Aherne, Hardcastle et al. 1996) and RNA (Randerath, Tseng et al. 1983; Kanamaru, Kakuta et al. 1986; Santi and Hardy 1987; Doong and Dolnick 1988; Samuelsson 1991; Patton 1993; Ghoshal and Jacob 1994) strands respectively. The advancement in genetic analysis techniques have helped discover the ability of several chemotherapeutic drugs to alter gene expression profile in different tumors. For instance, p53 and FAS have been shown to be a potential target of 5-FU (Hernandez-Vargas, Ballestar et al. 2006) (Tillman, Petak et al. 1999). Similar studies have also been performed on other antineoplastic drugs (Kudoh, Ramanna et al. 2000; Daoud, Munson et al. 2003), suggesting a connection between disease prognosis, efficiency and resistance to chemotherapy and genetic expression profiles (Krajewski, Blomqvist et al. 1995; Thottassery, Zambetti et al. 1997). Currently, a growing body of evidence has suggested the importance of miRNAs in modulating the chemosensitivity and chemoresistance of tumor cells (Meng, Henson et al. 2006; Blower, Verducci et al. 2007; Blower, Chung et al.

2008). Si (Si, Zhu et al. 2007) reported that suppression of miR-21 sensitized MCF7 cells to anticancer drug topotecan. Similar studies have been reported for the drugs gemcitabine, doxorubicin and tamoxifen (Kovalchuk, Filkowski et al. 2008; Miller, Ghoshal et al. 2008; Zhao, Lin et al. 2008) illustrating the importance of miRNAs in drug sensitivity and resistance. Thus, it could be suggested that a distinct association is prevalent between cytotoxicity of chemotherapy drugs, their alteration of genetic profiles and the gene regulatory role of miRNAs.

In our previous study, we have demonstrated that miRNA expression profile in MCF7 breast cancer cells is sensitive to 5-FU treatment and a distinct set of miRNAs with differential expression after 5-FU treatment was identified using microRNA microarray and qRT-PCR (Chapter III). Further study was required to obtain a detailed understanding of the miRNA regulatory effects of 5-FU and to exploit its potential therapeutic implications. However, there has been no organized study examining the relevance of dosage and treatment duration of a chemotherapy drug on the expression profile of miRNAs. We have therefore investigated the miRNA expression profiles of MCF7 breast cancer cells in response to various 5-FU concentrations for different lengths of treatment using qRT-PCR analysis. We report a characteristic miRNA expression profile in response to 5-FU treatment, wherein miRNAs in 5-FU treated cells show a different profile at all 5-FU variables compared to the control MCF7 samples. Most importantly, miRNA regulation was shown to be highly sensitive to 5-FU treatment at low doses and shorter duration of exposure. This could be an important clinical consideration that is relevant in improving 5-FU therapy, and suggests a potential role of miRNA specific therapies in cancer treatment.

Materials and Methods

Cell line and Cell culture

All cell culture reagents were purchased from Invitrogen, Inc, Carlsbad, CA. The human breast adenocarcinoma cell line MCF7 (HTB-22™) was obtained from the American Type Culture Collection (ATCC, Rockville, MD). They were cultured in RPMI 1640 media containing L-Glutamine and 25 mM HEPES, and supplemented with 10% Fetal Bovine Serum (FBS), 10 µg/ml gentamicin and 4 µg/ml insulin and sterilized using a 0.22µM polyethersulfone filter (Corning Inc., Corning, NY). The cells were maintained at 37˚C in a humidified incubator with 5% CO_2. The media was replaced every 48 hours, and the cells were passaged once a week by trypsinization using 0.05% trypsin/0.02% EDTA (Sigma, St. Louis, MO).

5-fluorouracil drug treatment

5-fluorouracil (5-FU) was purchased from Sigma-Aldrich (St. Louis, MO) and stored at 4˚C, away from light and moisture. For cell treatments, a 10 mM stock solution of 5-FU was prepared in RPMI complete media and stored at 4˚C for not more than two weeks. The stock was filtered through a 0.22µM polyethersulfone filter prior to further dilution. Further dilutions were made from the stock solution as required.

Dose- and time-dependent miRNA response analysis

The dose-dependence of miRNA expression profile in MCF7 breast cancer cells was analyzed at eight different 5-FU concentrations. For this purpose, cells were seeded at 50,000 cells/cm^2 (480,000 cells/well) in flat-bottom 6-well plates in 2ml of RPMI 1640 complete media. After 24 hours, media was replaced with fresh media (control group) or with 5-FU in complete

media (treatment group) and cells were incubated for another 48 hours. The treatments included 0.001, 0.005, 0.01, 0.1, 0.7, 1, 5 and 10µM 5-FU. After 48 hours of incubation, the cells were detached by trypsinization and stored in RNAlater-ICE at -20º C till RNA extraction was performed. Each treatment was performed minimum in triplicates.

For time-dependent miRNA expression response, MCF7 cells were treated with 0.1, 0.7 and 10µM 5-FU and analyzed at six different time points: 12, 24, 36, 48, 60 and 72 hours. Briefly, cells were seeded at 50,000 cells/cm^2 (480,000 cells/well) in flat-bottom 6-well plates in 2ml of RPMI 1640 complete media. After 24 hours, media was replaced with fresh media (control group) or with 5-FU in complete media (treatment group) and cells were incubated. Cells were subsequently trypsinized after 12, 24, 36, 48, 60 and 72 hours respectively, and stored in RNAlater-ICE at -20º C till further analysis. Each treatment was repeated three times at each time point.

RNA isolation

Total RNA was extracted from both groups with the *mir*Vana™ miRNA Isolation Kit (Ambion, Austin, TX) according to manufacturer's instructions. Briefly, the cells were pelleted and RNAlater-ICE was removed by centrifugation. Cells were lysed by adding 500 µl of Lysis/Binding Solution, followed by vortexing. miRNA Homogenate Additive (50 µl) was then added, mixed by vortexing and cells were incubated on ice for 10 min. The RNA was extracted by adding 500 µl of Acid-Phenol: Chloroform, vortexing for 60 sec and centrifuging for 5 min at 10,000 X g to separate the aqueous and organic phases. The upper (aqueous) phase was transferred to another 2ml micro-centrifuge tube and its volume was noted. Room temperature 100% ethanol was added at 1.25 times the volume to precipitate the RNA. The total RNA was then filtered onto a filter cartridge by centrifugation, followed by multiple washings. The filter

cartridge was first washed with 700µl Wash solution 1, centrifuged briefly and the flow through was discarded. The washing was then repeated twice with 500µl of Wash Solution 2/3. Finally, the total RNA was eluted with 100 µl of pre-heated (95º C) nuclease-free water. All the steps were performed on ice. RNA quantity and quality was analyzed using NanoDrop ND1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE) and immediately stored at -80˚C until further analysis.

Quantitative Real Time PCR (qRT-PCR) of miRNA expression

Eleven different miRNAs were analyzed for changes in their expression level following 5-FU treatment. Primers for hsa-let-7g, hsa-miR-10b, hsa-miR-15a, hsa-miR-16, hsa-miR-21, hsa-miR-27a, hsa-miR-365, hsa-miR-374b, hsa-miR-483-5p, hsa-miR-574-3p, hsa-miR-575, and reference gene RNU48 were purchased from Applied Biosystems (Foster City, CA). The miRNAs were selected based on the microarray analysis (Chapter 3) and the importance of miRNAs in breast cancers. miR-365, miR-374b, miR-483-5p, miR-574-3p, miR-575 were shown to be differentially expressed with high fold change in the microRNA microarray data, while let-7g, miR-10b, miR-15a, miR-16, miR-21, miR-27a have been reported to be associated with breast cancer. A single-stranded cDNA for a specific miRNA was generated by reverse transcription of at least 500 ng of total RNA using a miRNA-specific stem-looped RT primer and the Applied Biosystems TaqMan® microRNA Reverse Transcription Kit. A Reverse transcription reaction mixture contains total RNAs, 1.5µL 10X TR Buffer, 1mM of each dNTPs, 0.188µL RNase Inhibitor, 3µL 5X Taqman® microRNA RT primer for a specific miRNA and 1µL MultiScribe™ Reverse Transcriptase (50U/µL). An Eppendorf Mastercycler Personal PCR (Westbury, NY) was used to conduct the reverse transcription reaction at the following

temperature conditions: 16°C for 30 min, 42°C for 30 min followed by 85°C for 5 min and finally held at 4°C.

Following reverse transcription reaction, quantitative RT-PCR was performed with Applied Biosystems 7300 Real-Time PCR system using the Taqman® MicroRNA Assay kit. The qRT-PCR reaction mixture contains 3μL RT PCR product (diluted 1:7 times), 10μL Taqman® 2X Universal PCR Master Mix (No AmpErase® UNG), 2μL Taqman® MicroRNA Assays 20X Taqman® Assay (qRT-PCR primers). The final volume was adjusted to 20μL with nuclease free water. The reactions were incubated in a 96-well optical plate at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 60 sec. RNU 48 was used as an endogenous reference gene for normalizing the results. Each sample was analyzed in duplicate (technique replicates). The relative abundance of each miRNA was calculated using the comparative cycle threshold ($2^{-\Delta\Delta Ct}$) method. The results are presented as fold change of each miRNA in 5-FU treated cells relative to the control MCF7 cells.

Statistical Analysis

All data are expressed as mean ± S.E.M. (Standard error of the mean). Statistical significance of the differences between the control and treated groups was determined by one-way analysis of variance (ANOVA) using the PASW (Predictive Analytics SoftWare) Statistics 17.0 (SPSS Inc., Chicago, IL). A two-way ANOVA analysis was also performed for the time-response study using the PASW (Predictive Analytics SoftWare) Statistics 17.0 (SPSS Inc., Chicago, IL) to determine if there was any significant interaction between the concentration of treatment and the duration of treatment on the expression level of miRNAs in MCF7 breast cancer cells. Differences were considered significant if $p < 0.05$.

Results

miRNA dose-response to 5-FU treatment

Eleven miRNAs (let-7g, miR-10b, miR-15a, miR-16, miR-21, miR-27a, miR-365, miR-374b, miR-483-5p, miR-574-3p and miR-575) were analyzed to determine the dose-dependency of their expression profiles at eight different 5-FU treatments (0.001, 0.005, 0.01, 0.1, 0.7, 1, 5 and 10µM) (Figures 4.1-4.11). Cells were treated with different 5-FU concentrations for 48 hours, followed by RNA extraction, reverse transcription and qRT-PCR to determine the relative miRNA expression levels. Of the eleven miRNAs analyzed, three miRNAs (miR-10b, miR-21 and miR-365) showed statistically significant alteration in their expression profile following treatment with different 5-FU concentrations. miR-10b showed up-regulation at 0.005 (40.4-fold), while no major alteration was observed at other 5-FU concentrations. Conversely, miR-21 showed a significant decrease in its expression levels in a dose-dependent manner ($p = 0.026$), with maximum down-regulation at 0.1µM (4.9-fold). This down-regulation decreased when treated with concentrations higher that 0.1µM 5-FU. miR-365 also showed a down-regulation compared to the control after 5-FU treatment, the highest down-regulation being at 0.005µM (4.4-fold, $p = 0.03$).

miR-15a, miR-16, miR-483-5p and miR-575 were all up-regulated compared to the control following 5-FU treatment. miR-15a and miR-16 show a very similar bimodal trend in their expression profiles, with the fold change showing a peak at two separate concentrations. miR-483-5p shows a remarkable up-regulation between 0.001-0.7µM, with the highest increase at 0.1µM 5-FU (56.3-fold). miR-575 also shows an up-regulation between 0.001-1µM (up to 17.7-fold). Let-7g, miR-27a, miR-374b and miR-574-3p showed a decrease in their expression

levels compared to control after treatment with 5-FU. let-7g and miR-27a both showed the highest down-regulation at 0.1µM (2-fold and 1.54-fold respectively). However, a slight up-regulation was seen at 0.005 and 5µM for let-7g, and at 0.005, 0.7 and 10µM for miR-27a. miR-374b, on the other hand, shows a dose-dependent increase in its down-regulation, with the maximum down-regulation between 0.01-0.7µM. The expression level however became similar to the normal control samples as the 5-FU concentration increased. miR-574-3p showed the highest decrease in its expression level at 0.005µM (1.9-fold) and at 10µM (2.7-fold) respectively. This study revealed that miRNA expression levels in MCF7 breast cancer cells were more sensitive to lower 5-FU concentrations, and these doses were sufficient to elicit an alteration in their expression levels.

miRNA time-response to 5-FU treatment

Based on the results obtained from the dose-dependent miRNA expression study, five miRNAs with high fold change (miR-10b, miR-21, miR-483-5p, miR-574-3p and miR-575) were selected to analyze any time-dependent changes in their expression. MCF7 cells were treated with 0.1, 0.7 and 10µM 5-FU for 12, 24, 36, 48, 60 and 72 hours, and analyzed for the expression levels of the selected miRNAs (Figure 4.12-4.26).

At 0.1µM 5-FU treatment, the selected miRNAs did not show a significant change in their expression at different time points. The expression levels after treatment were almost similar to the control samples. Further, it was observed that treatment for more than 48 hours was required to elicit a small change in the expression at 0.1µM 5-FU. On the other hand, treatment with 0.7µM 5-FU showed an atypical behavior compared to the other two treatments. Of the five miRNAs analyzed, four (miR-10b, miR-21, miR-574-3p and miR-575) showed a significant

differential expression at 0.7µM. For miR-10b, the expression level was up-regulated after treatment up to 48 hours; however, the expression was down-regulated if the treatment was continued for 72 hours. Similarly, miR-21 was up-regulated after 12 and 24 hours of treatment, but showed a down-regulation when treated for 36 and 48 hours. Conversely, the expression levels returned to normal control levels if the treatment was continued for 72 hours. Further, miR-483-5p showed the highest up-regulation after treatment for 12 hours, which reduced with longer duration of exposure. A decrease in the expression was observed after 36 hours of treatment; however, this change in expression never significantly varied from the control. A similar trend was also seen for miR-574-3p and miR-575. The miRNAs were significantly up-regulated after 12 and 24 hours of treatment, while the expression levels returned to normal if treated for longer duration of time.

At the 10µM treatment level, miR-10b showed an increase in expression at 24 and 48 hours of treatment, while the expression decreased after 60 and 72 hours of treatment, as was seen with the 0.1 and 0.7µM treatment. This suggests that expression of miR-10b is expressed differentially at different time points and is thus sensitive to duration of exposure to 5-FU. Similarly, miR-21 showed the highest down-regulation (7.1 fold) after 36 hours of treatment, while the expression decreased to control levels with increasing length of treatment. miR-483-5p, on the other hand, showed an up-regulation after 24 hours of treatment (2.4-fold), but the expression decreased significantly to 1.9-fold after 36 hours of treatment. These changes, however, were not significantly different from the control samples. Conversely, miR-574-3p showed a significant differential expression after treatment with 5-FU for different time durations ($p < 0.001$). The highest down-regulation was observed after 36 hours of treatment (4.4-fold), which reduced to 2-fold down-regulation after increasing length of treatment. Finally,

miR-575showed an up-regulation after treatment with 12 and 24 hours of treatment (2.3- and 4.2-fold respectively), which was decreased to a down-regulation of 2.5-fold if the treatment was continued for 72 hours.

The time-response data suggests that the expression of miRNAs in MCF7 breast cancer cells was more sensitive to shorter duration of treatment with 5-FU. The miRNAs showed a significant change in their expression levels when treated with 5-FU for shorter time length ($p < 0.01$ and $p < 0.05$ for 12 and 24 hours of treatment, respectively) as compared to increased time durations. A two-way ANOVA analysis to investigate the effect of various 5-FU concentrations and duration of treatment with these concentrations revealed that expression of each miRNA analyzed was significantly affected by both 5-FU dosage and treatment durations ($p < 0.05$ for each miRNA). This confirms that miRNA expression in MCF7 cells is responsive to 5-FU treatment. Further, a significant interaction between treatment dose and duration was also observed for miR-21, miR-574-3p and miR-575. This shows that alteration in the expression levels of different miRNAs may play an important role in the therapeutic response to 5-FU.

Discussion

miRNAs constitute a novel target system for cancer treatment as each miRNA has the ability to regulate the expression of several hundred target genes, including several important oncogenes or tumor suppressor genes, and have the potential to function as a diagnostic and prognostic tool for a variety of human cancers (Blenkiron and Miska 2007; Tricoli and Jacobson 2007; Lowery, Miller et al. 2008; Zhang and Farwell 2008). Extensive research is currently being focused on identifying differential expressed miRNAs that play primary roles in cancer

development and therapy. Thus, a study of the possible effects of chemotherapeutic drug treatment on the expression profile of miRNAs is of prime importance for cancer therapy and resistance. 5-FU is an important chemotherapeutic drug used for the treatment of breast cancer. Unfortunately, 5-FU is rarely used as a monotherapy now-a-days, and is mainly used in combination with other drug regimens. It has been shown to be a potent anti-proliferative agent capable of inducing apoptosis in MCF7 breast cancer cells (Chapter II). Further analysis to determine the role of miRNAs in the cytotoxicity of 5-FU in MCF7 cells showed that 5-FU is able to significantly dysregulate the expression levels of several miRNAs even at a low dose (Chapter III). In this study, we observed that higher and clinically relevant 5-FU doses also have a significant effect on the miRNA expression profiles in MCF7 breast cancer cells, but expression is more sensitive to lower treatment doses. The expression levels were also shown to be regulated by the time of exposure to 5-FU. The data presented here gives a detailed analysis of the expression profiles of miRNAs in response to 5-FU.

Eleven miRNAs were selected to study their expression profiles in response to different 5-FU concentrations. These include let-7g, miR-10b, miR-15a, miR-16, miR-21, miR-27a, miR-365, miR-374b, miR-483-5p, miR-574-3p and miR-575, which were treated with 0.001, 0.005, 0.01, 0.1, 0.7, 1, 5 and 10µM 5-FU. Of these, miR-10b, miR-15a, miR-16, miR-483-5p and miR-575 were consistently up-regulated; while miR-21, miR-365 and miR-374b were consistently down-regulated at all 5-FU treatments. These results further confirm the microRNA microarray data obtained previously (Chapter III). Conversely, let-7g, miR-27a and miR-574-3p were differentially expressed at various 5-FU concentrations. This suggests that different miRNAs respond to 5-FU treatment in different ways. Recently, miR-10b was identified to be highly expressed in metastatic breast cancer cells and positively regulate cell migration, invasion and

metastasis (Ma, Teruya-Feldstein et al. 2007). However, MCF7 cells, which have little if any metastatic ability, were not shown to have a high expression of this miRNA (Ma, Teruya-Feldstein et al. 2007). Up-regulation of miR-10b after 5-FU treatment was inconsistent with 5-FU cytotoxicity and could play an important role in the resistance of certain metastatic breast cancers to 5-FU treatment. Further time-based study was performed to understand the role of miR-10b in response of 5-FU. On the contrary, miR-21, miR-27a and miR-365 have been shown to be up-regulated in breast cancer cells (Iorio, Ferracin et al. 2005; Mertens-Talcott, Chintharlapalli et al. 2007; Yan, Huang et al. 2008; Guttilla and White 2009). Down-regulation of these miRNAs after 5-FU treatment further confirms the active function of miRNAs in 5-FU induced cytotoxicity. Further, miR-16 is highly down-regulated in breast cancer (Iorio, Ferracin et al. 2005), but was up-regulated following 5-FU treatment. Finally, let-7g, miR-15a, miR-374b, miR-483-5p, miR-574-3p and miR-575 have not been previously implicated in breast cancers, but show differential expression with 5-FU treatment, suggesting their potential role in 5-FU activity. Thus, based on their fold change levels or their novelty in breast cancers, we selected five miRNAs (miR-10b, miR-21, miR-483-5p, miR-574-3p and miR-575) for further time-dependent study.

An important miRNA that was chosen for dose- and time-dependent study is miR-10b. It was not classified to be differentially expressed between control and 5-FU treated samples after microRNA microarray analysis due to the low signal intensity; however, a significant variation in its expression profile was observed after 5-FU treatment. miR-10b has been previously implicated in several human cancers (Table 4.1), including breast cancer (Iorio, Ferracin et al. 2005; Ma, Teruya-Feldstein et al. 2007). Previous literature has also displayed a positive correlation between the expression of miR-10b and metastatic or invasive behavior of primary

breast carcinomas (Ma, Teruya-Feldstein et al. 2007), thus classifying it as an oncogenic miRNA or Oncomir. This has been partially correlated to the regulation of miR-10b by transcription factor Twist (Ma, Teruya-Feldstein et al. 2007), which is a master regulator of morphogenesis and plays an essential role in tumor metastasis (Yang, Mani et al. 2004). Several gene targets have also been identified for miR-10b. These targets include HOXD10 (Homeobox D10) (Ma, Teruya-Feldstein et al. 2007), which has been found to be progressively lost in breast tumors with increased malignancy (Carrio, Arderiu et al. 2005; Makiyama, Hamada et al. 2005) and regulates several genes that promote invasion, migration and tumor promotion, including uPAR and RhoC (Myers, Charboneau et al. 2002); and KLF4 (Krüppel-like factor 4) (Tian, Luo et al.), a zinc finger protein which is important in cell cycle regulation, differentiation, and in response to DNA damage (Shields, Christy et al. 1996; Zhang, Geiman et al. 2000).

miR-10b showed a highly differential expression following 5-FU treatment. A significant up-regulation was observed compared to the control after 5-FU treatment at different concentrations ($p < 0.01$) (Figure 4.2). The expression level showed a significant increase at 0.005µM (40-fold) compared to the control. Other treatments did not show considerable increase compared to the control. Up-regulation of an oncogenic miRNA after treatment with very low doses of 5-FU indicates that 5-FU could aggravate the disease state. Since miR-10b plays an important role in migration and invasion of breast cancer cells, this up-regulation could also suggest a role of the miRNA in acquired 5-FU resistance observed in certain metastatic breast cancer cases. Further, the return of the expression levels to normal at higher 5-FU concentrations implies that treatment with higher doses of 5-FU would be more beneficial for the therapeutic effect of 5-FU. A time-dependent analysis was carried out to further understand the expression of miR-10b in response to 5-FU.

A time-based response analysis was performed every 12 hours for 72 hours at concentrations 0.1, 0.7 and 10μM 5-FU to obtain further insights into the expression profile of miR-10b (Figures 4.12-4.14). At each 5-FU concentration, shorter duration of treatment (< 48 hours) showed a significant difference in the expression levels compared to the expression changes after longer treatments for 60 and 72 hours. This indicates that treatment with 5-FU for longer duration would lead to beneficial miRNA regulation, while miR-10b does not seem to play an important role in 5-FU cytotoxicity if treated for shorter durations. A two-way ANOVA analysis showed that there was a significant main effect of the concentration of the treatment [F (3, 56) = 3.192, $p < 0.05$] as well as the duration of treatment [F (6, 56) = 4.760, $p < 0.01$] on the expression level of miR-10b in MCF cells. Conversely, no significant interaction effect of the concentration and duration of treatment [F (18, 56) = 1.531, $p = 0.114$] was observed. Thus, treatment with high 5-FU doses for a higher time span (such as a continuous i.v. infusion for 3 or 4 days) would lead to a beneficial expression of miR-10b, while treatment for shorter length of time (a single dose i.v. infusion) could invoke potential problems.

miR-21 is another important miRNA whose expression levels are highly dysregulated after 5-FU treatment. Extensive study has been carried out on miR-21 and it stands out as a miRNA most often found over-expressed in solid tumors (Volinia, Calin et al. 2006), and also in most of the human cancers (Table 4.1). Furthermore, miR-21 have also been reported to play an imported role in cancer-related processes such as cell proliferation, migration, apoptosis and tumor growth in breast, gastric and hepatocellular cancers (Meng, Henson et al. 2007; Si, Zhu et al. 2007; Zhang, Li et al. 2008). In breast cancer, miR-21 is found to be highly up-regulated in tumors as compared to the normal breast tissues (Iorio, Ferracin et al. 2005). It has been directly implicated in promoting tumor growth (Si, Zhu et al. 2007) and is also associated with advanced

clinical stage lymph node metastasis and poor patient prognosis (Yan, Huang et al. 2008). It has been found to target several important genes (Table 4.1). For importance are the tumor suppressor genes PDCD4 (Programmed Cell Death 4) (Asangani, Rasheed et al. 2008; Chen, Liu et al. 2008; Yao, Xu et al. 2009); PTEN (phosphatase and tensin homolog) (Meng, Henson et al. 2007); and TPM1 (Tropomyocin 1) (Zhu, Si et al. 2007; Zhu, Wu et al. 2008). Based on its expression in cancers and its gene target, miR-21 can thus be classified as an oncogenic miRNA or oncomir. Down-regulation of an oncomir by 5-FU treatment suggests an important role of miRNA in 5-FU activity.

From our dose-dependent results, miR-21 was shown to be significantly down-regulated compared to the control after treatment with all the 5-FU concentrations ($p < 0.05$) (Figure 4.5). The highest down-regulation was observed between 0.01-1µM 5-FU, which continued even at higher concentrations. Further, the bimodal dose-response curve of the MCF7 cells in response to 5-FU (Chapter II) can also be partially explained by the miRNA expression response to different 5-FU doses. The initial 5-FU dose-dependent reduction in the MCF7 cell numbers between 0.001-0.5µM (Chapter II, Figure 2.4) can be correlated with the dose-dependent decrease in the expression level of miR-21 at the same concentration range, with the highest down-regulation observed at 0.01µM. This decrease in the fold change gradually reduced in a dose-dependent manner after treatment with higher concentrations of 5-FU, which gave a second dose-response curve in Figure 2.4. Thus, miR-21 was shown to be sensitive to different 5-FU doses and might play a role in the bimodality of 5-FU. Further, the effective concentration range for the regulation of miR-21 expression in response to 5-FU was shown to be 0.01-1µM, which is much lower than the doses required to elicit 5-FU cytotoxicity in cancerous tissues. This

shows that miRNA expression displays greater sensitivity to 5-FU treatment and this could be used to reduce 5-FU dosage and thus eliminate the side effects associated with it.

In order to further study the effect of 5-FU on miR-21, a detailed time-based response was performed at 0.1, 0.7 and 10µM 5-FU. The cells were treated with these 5-FU concentrations and the expression profile of miR-21 was analyzed every 12 hours for 3 days (Figures 4.15-4.17). Of these treatments, 0.7 and 10µM showed a significant alteration in the expression fold change as the time of exposure to 5-FU increased. Treatment with 0.1 and 10µM displayed a similar trend in the expression profile of miR-21. A decrease in the expression level compared to the control was observed till 48 hours of treatment, followed by an up-regulation if the treatment was continued till 72 hours. A significant difference in the expression between shorter and longer treatment durations was again observed. This shows that treatment with 5-FU concentration for shorter duration of time was enough to elicit a beneficial miR-21 regulation. Further, it also shows that a lower concentration of 5-FU (0.1µM) was capable of inducing a similar response as 10µM 5-FU. However, a different time-based response was observed following treatment of MCF7 cells with 0.7µM 5-FU. A significant up-regulation was observed at 12 and 24 hours of treatment, followed by a significant down-regulation between 36-48 hours, and finally the expression levels became similar to control if treatment was continued further till 72 hours. This distinct miRNA expression profile at 0.7µM provides additional explanation to the atypical increase in cell numbers of MCF7 cells observed during growth analysis at the same concentration. The two-way ANOVA analysis demonstrated that there was a significant main effect of the concentration of the treatment [F (3, 56) = 4.727, $p < 0.01$] as well as the duration of treatment [F (6, 56) = 4.454, $p < 0.01$] on the expression level of miR-21. A significant interaction effect of the concentration and duration of treatment was also observed on the

expression level of miR-21 [F (18, 56) = 4.759, $p < 0.01$]. Thus, both concentration and duration of treatment with 5-FU were shown to play an important role in the regulation of miR-21 expression levels in the MCF7 breast cancer cells.

Another miRNA whose expression levels were shown to be notably altered following 5-FU treatment is miR-483-5p. Recently, it has been identified to play an important role in differentiating between adrenocortical adenomas and adrenocortical carcinomas and also as a predictor of poor prognosis in adrenocortical cancers (Soon, Tacon et al. 2009). However, no definite role of miR483-5p has been recognized in breast cancers. Also, no potential targets of it have been identified yet. Thus, miR-483-5p has been identified for the first time in MCF7 breast cancer cells and alteration of its expression levels in response to 5-FU may indicate an important role for it in 5-FU efficacy.

miR-483-5p was previously identified as one of the most up-regulated miRNAs in the miRNA microarray analysis of MCF7 cells treated with 0.01µM 5-FU for 48 hours (Chapter III). Those results are further supported by the dose-dependent study. It continues to be the most up-regulated miRNA after treatment with various 5-FU concentrations (Figure 4.9). The highest up-regulations were observed at 0.01 (56.4-fold) and 0.001 (33.8-fold)µM respectively. The up-regulation became less substantial as the concentration increased, indicating that miR-483-5p expression in MCF7 cells was more sensitive to lower doses of 5-FU. The time-based study also showed similar results (Figures 4.18-4.20). 5-FU treatment for 12 hours was enough to elicit an up-regulation in its expression, suggesting that treatment for shorter time length was important for sensitivity to 5-FU. The expression levels were statistically significantly different after longer treatments compared to shorter treatments for each 5-FU concentration. On the contrary, a two-way ANOVA analysis showed that there was a significant main effect of the concentration of the

treatment [F (3, 56) = 5.401, $p < 0.01$] as well as the duration of treatment [F (6, 56) = 2.609, $p < 0.05$] on the expression level of miR-483-5p in MCF cells. No significant interaction effect of the concentration and duration of treatment [F (18, 56) = 1.188, $p = 0.302$] was observed.

miR-574-3p was identified as one of the most up-regulated miRNAs in the microRNA microarray analysis previously performed (Chapter III). It has been shown to play an important role in liver development (Tzur, Israel et al. 2009), non-alcohol fatty liver disease (Estep, Armistead et al.), and myocardial infarction (Bostjancic, Zidar et al. 2009). Recently, it has also been identified to show a stage specific differentially expression in B cell lymphomas (Malumbres, Sarosiek et al. 2009). However, no direct gene targets have been identified to explain the role of miR-574-3p in cancer development or expression. Modification in its expression levels after 5-FU treatment may serve to better understand the function of miR-574-3p in breast cancer in specific and human cancers in general.

Dose-response analysis revealed that miR-574-3p is differentially expressed through different 5-FU concentrations (Figure 4.10). However, the expression levels were not found to be significantly different compared to control level by One-way ANOVA. An independent samples t-test showed a significant down-regulation in the expression levels at 0.005 and 10µM 5-FU concentrations. Time-response analysis showed a significant effect of duration of treatment at 0.7µM and 10µM 5-FU (Figures 4.21-4.23). Higher down-regulation was observed with increased duration of treatment, indicating that longer exposure times were needed to induce an expression change in miR-574-3p after 5-FU treatment. The highest down-regulation was observed after 36 hours of treatment with both, 0.7µM (1.9-fold) and 10µM (4.4-fold) 5-FU respectively. However, a significant increase in the expression was observed after treatment with 0.7µM 5-FU for 12 and 24 hours. The two-way ANOVA analysis demonstrated that there was a

significant main effect of the concentration of the treatment [F (3, 56) = 15.555, $p < 0.001$] as well as the duration of treatment [F (6, 56) = 7.067, $p < 0.001$] on the expression level of miR-574-3p. A significant interaction effect of the concentration and duration of treatment was also observed on the expression level of miR-574-3p [F (18, 56) = 5.134, $p < 0.001$].

miR-575 also showed a 5-FU responsive increase in its expression level. It is a novel miRNA which has not been previously linked to human breast cancer. It has, however, been shown to be down-regulated in gastric cancer cell line (Hong-chun Luo 2009) and meningiomas (Saydam, Shen et al. 2009). It is also differentially induced by 4-hydroxynonenal (Pizzimenti, Ferracin et al. 2009), and in lupus nephritis (Dai, Sui et al. 2009) and myocardial infarction (Bostjancic, Zidar et al. 2009). In our previous study, miR-575 was shown to be highly up-regulated in the microRNA microarray of MCF7 cells after treatment with 0.01µM 5-FU. The dose-response data agree with the microarray data as miR-575 was consistently up-regulated at all 5-FU treatments (Figure 4.11). The maximum up-regulation was seen at 0.1µM (17.8-fold) and 0.7µM (17.6-fold) of 5-FU. The expression levels were however not found to be significantly different than normal control MCF7 cells. The time-response data also confirm with the dose-response data, as miR-575 was significantly up-regulated at 0.7µM and 10µM of 5-FU (Figures 4.24-4.26). However, expression was found to be more sensitive to short duration of treatment and the changes in the expression reduced if the treatment was continued for 72 hours. This is similar to expression profile of other miRNAs analyzed, suggesting that these miRNAs could help in improving 5-FU therapy in breast cancer patients. The two-way ANOVA analysis revealed a significant main effect of the concentration of the treatment [F (3, 56) = 6.279, $p < 0.01$], the duration of treatment [F (6, 56) = 5.720, $p < 0.001$] as well as the interaction effect among the two variables [F (18, 56) = 2.383, $p < 0.01$] on the expression level of miR-574-3p.

In our previous study, 5-FU was shown to have an atypical inhibitory on the proliferation of MCF7 breast cancer cells (Chapter II, Figures 2.4 and 2.5). An abnormal increase in cell number was observed at 0.7µM 5-FU treatment. We had hypothesized that 5-FU acts with a bimodal mechanism, wherein the lower 5-FU concentrations (0.001-0.1µM) act with a different method as compared to the higher doses (0.7-2000µM). This hypothesis was partially supported by the dose- and time-dependent data obtained in this study. Most of the miRNAs analyzed in the dose-response study showed a distinct expression profile at lower 5-FU doses than higher doses, for example, let-7g, miR-15a, miR-16 and miR-21. This could signify that a different inhibitory mechanism is adopted by 5-FU. The time-response results of some miRNAs further supported this observation. The time-based expression profile at low 5-FU dose (0.1µM) showed a different pattern as compared to the other two concentrations (0.7µM and 10µM), for example, miR-21, miR-574-3p and miR-575. Therefore, future studies will have to be performed to evidently explain the anomalous behavior of 5-FU in MCF7 breast cancer cells.

The results presented here provide a detailed analysis of the miRNA expression profile in MCF7 breast cancer cells in response to 5-FU treatment. Several important miRNAs which have been previously linked with different human cancers were identified to play an important role in response to 5-FU treatment, such as miR-10b and miR-21. More importantly, each of these miRNAs was shown to be beneficially regulated after 5-FU treatment as compared to control breast cancer cells. This suggests an important therapeutic potential of these miRNAs in breast cancer treatment. In addition, novel miRNAs were identified for the first time to play a role in breast cancer and breast cancer chemotherapy. These include miR-483-5p, miR-574-3p and miR-575. Further studies to determine the potential gene targets of these miRNAs are required to clearly understand their role in breast cancer chemotherapy. The dose- and time-dependent

response of miRNA expression illustrated that the expression profile changes were highly sensitive to 5-FU treatment, and that treatment with lower 5-FU doses for shorter durations was enough to obtain a beneficial miRNA response. This observation could be very helpful in improving 5-FU efficacy. The 5-FU drug regimen, which is rarely used as a monotherapy now-a-days due to serious side effects and increased incidence of resistance, could be monitored and used at lower concentrations for shorter time spans if targeted delivery could be achieved. This would ensure a significant miRNA expression change and a subsequent change in the target genes, while eliminating the side-effects associated with high 5-FU doses. Thus our data further substantiates the importance of miRNA in chemotherapy and the potential application of miRNAs as novel targets for cancer therapy.

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Table 4.1: Cancer association and verified gene targets of miRNAs selected for dose- and time-dependent study

miRNAs (up / down regulation)	Associated cancers		Validated Gene targets
	Down regulation	Up regulation	
let-7g↓	HCC (Budhu, Jia et al. 2008) OSCC (Kozaki, Imoto et al. 2008) Lung cancer (Kumar, Erkeland et al. 2008) Prostate cancer (Porkka, Pfeiffer et al. 2007)	Colon cancer (Nakajima, Hayashi et al. 2006)	HMGA2 (Kumar, Erkeland et al. 2008)
miR-10b (differentially expressed)	Colorectal cancer (Arndt, Dossey et al. 2009)	Breast cancer (Ma, Teruya-Feldstein et al. 2007) Chronic Lymphocytic Leukemia (CLL) (Calin, Liu et al. 2004; Calin, Pekarsky et al. 2007; Calin, Cimmino et al. 2008) Glioblastoma (Chan, Krichevsky et al. 2005; Ciafre, Galardi et al. 2005; Chen, Liu et al. 2008) Ovarian cancer (Dahiya, Sherman-Baust et al. 2008) Pancreatic cancer (Bloomston, Frankel et al. 2007) Prostate cancer (Volinia, Calin et al. 2006; Prueitt, Yi et al. 2008)	HOXD10 (Ma, Teruya-Feldstein et al. 2007) KLF4 (Tian, Luo et al.)
miR-15a↑	CLL (Calin, Liu et al. 2004; Calin, Pekarsky et al. 2007; Calin, Cimmino et al. 2008) Pituitary adenoma (Bottoni, Piccin et al. 2005) HCC (Budhu, Jia et	Kidney cancer (Chow, Youssef et al. 2009)	BCL2 (Cimmino, Calin et al. 2005; Calin, Pekarsky et al. 2007), CDC25A (Lee, Masyuk et al. 2008), CCND1 (Bandi, Zbinden et al. 2009), CCND2(Bandi, Zbinden et al. 2009),

	al. 2008) Prostate cancer (Bonci, Coppola et al. 2008)		CCNE1(Bandi, Zbinden et al. 2009), WNT3A(Bonci, Coppola et al. 2008), RARS (Calin, Dumitru et al. 2002; Bottoni, Piccin et al. 2005)
miR-16↑	Breast cancer (Iorio, Ferracin et al. 2005) CLL (Calin, Pekarsky et al. 2007) Gastric cancer (Xia, Zhang et al. 2008) Pituitary adenoma (Bottoni, Piccin et al. 2005) Prostate cancer (Porkka, Pfeiffer et al. 2007; Schaefer, Jung et al. 2009)	Lung cancer (Yanaihara, Caplen et al. 2006)	BCL2 (Cimmino, Calin et al. 2005; Xia, Zhang et al. 2008), Caprin-1 (Kaddar, Rouault et al. 2009), CGI-38 (Kiriakidou, Nelson et al. 2004), CCND1 (Liu, Fu et al. 2008; Bandi, Zbinden et al. 2009), CCND3 (Liu, Fu et al. 2008), CCNE1 (Liu, Fu et al. 2008), CDK6 (Liu, Fu et al. 2008), HMGA1 (Kaddar, Rouault et al. 2009), WNT3A (Bonci, Coppola et al. 2008), RARS (Calin, Dumitru et al. 2002)
miR-21↓		Breast cancer (Iorio, Ferracin et al. 2005; Yan, Huang et al. 2008) Cervical cancer (Lui, Pourmand et al. 2007) Cholangiocarcinoma (Meng, Henson et al. 2006) CLL (Marton, Garcia et al. 2008) Colorectal cancer (Volinia, Calin et al. 2006) Diffuse large B cell lymphoma (DLBCL) (Lawrie, Gal et al. 2008) Esophageal cancer (Feber, Xi et al. 2008) Glioblastoma (Chan, Krichevsky et al. 2005; Ciafre, Galardi et al. 2005; Chen, Liu et al. 2008) Head & Neck cancer (Tran, McLean et al. 2007; Chang, Jiang et al. 2008)	BTG2 (Liu, Wu et al. 2009), MARCKS (Li, Li et al. 2009), SERPINB5 (Zhu, Wu et al. 2008), PDCD4 (Asangani, Rasheed et al. 2008; Chen, Liu et al. 2008; Yao, Xu et al. 2009), PTEN (Meng, Henson et al. 2007), TPM1 (Zhu, Si et al. 2007; Zhu, Wu et al. 2008), HNRPK (Papagiannakopoulos, Shapiro et al. 2008), TAp63 (Papagiannakopoulos, Shapiro et al. 2008), LRRFIP1 (Li, Li et al. 2009) JAG1 (Hashimi, Fulcher et al. 2009), WNT1 (Hashimi, Fulcher et al. 2009)

		HCC (Meng, Henson et al. 2007) Kidney cancer (Chow, Youssef et al. 2009) Lung cancer (Yanaihara, Caplen et al. 2006) OSCC (Kozaki, Imoto et al. 2008) Ovarian cancer (Iorio, Visone et al. 2007) Pancreatic cancer (Bloomston, Frankel et al. 2007) Prostate cancer (Volinia, Calin et al. 2006; Prueitt, Yi et al. 2008) Stomach cancer (Volinia, Calin et al. 2006) Uterine leiomyomas (Wang, Zhang et al. 2007)	
miR-27a↓	OSCC (Kozaki, Imoto et al. 2008)	Breast cancer (Mertens-Talcott, Chintharlapalli et al. 2007; Guttilla and White 2009) Kidney cancer (Chow, Youssef et al. 2009) Gastric cancer (Liu, Tang et al. 2009) Prostate cancer (Porkka, Pfeiffer et al. 2007) Uterine leiomyomas (Wang, Zhang et al. 2007)	Myt1 (Mertens-Talcott, Chintharlapalli et al. 2007), MDR1(Zhu, Wu et al. 2008) , FOXO1 (Guttilla and White 2009), PHB (prohibitin) (Schaar, Medina et al. 2009), ZBTB10 (Mertens-Talcott, Chintharlapalli et al. 2007)
miR-365↓		Breast cancer (Yan, Huang et al. 2008)	No previously reported targets
miR-374b	No previously reported association		No previously reported targets
miR-483-5p↑		Adrenocortical cancers (Soon, Tacon et al. 2009)	No previously reported targets
miR-574-3p↓		B cell lymphomas (Malumbres, Sarosiek et al. 2009)	No previously reported targets
miR-575↑	Gastric cancer cell line (Hong-chun Luo 2009) Meningiomas (Saydam, Shen et al. 2009)		No previously reported targets

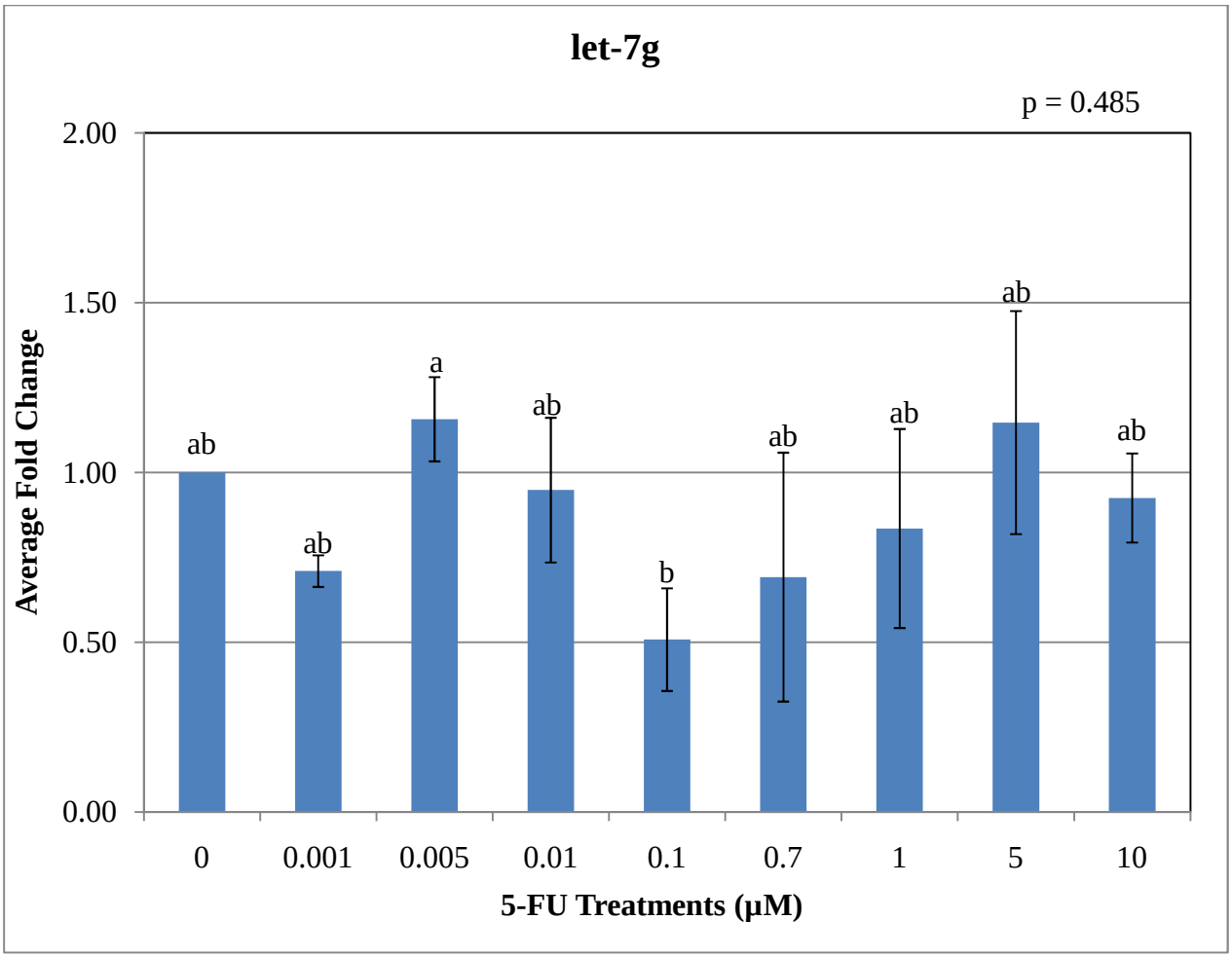

Figure 4.1: Average fold change in expression of let-7g in response to 5-FU concentrations from 0.0001µM to 10µM. The results are represented as mean ± SEM. Statistically significant changes in the expression between concentrations are designated by single letters on error bars ($p \leq 0.05$).

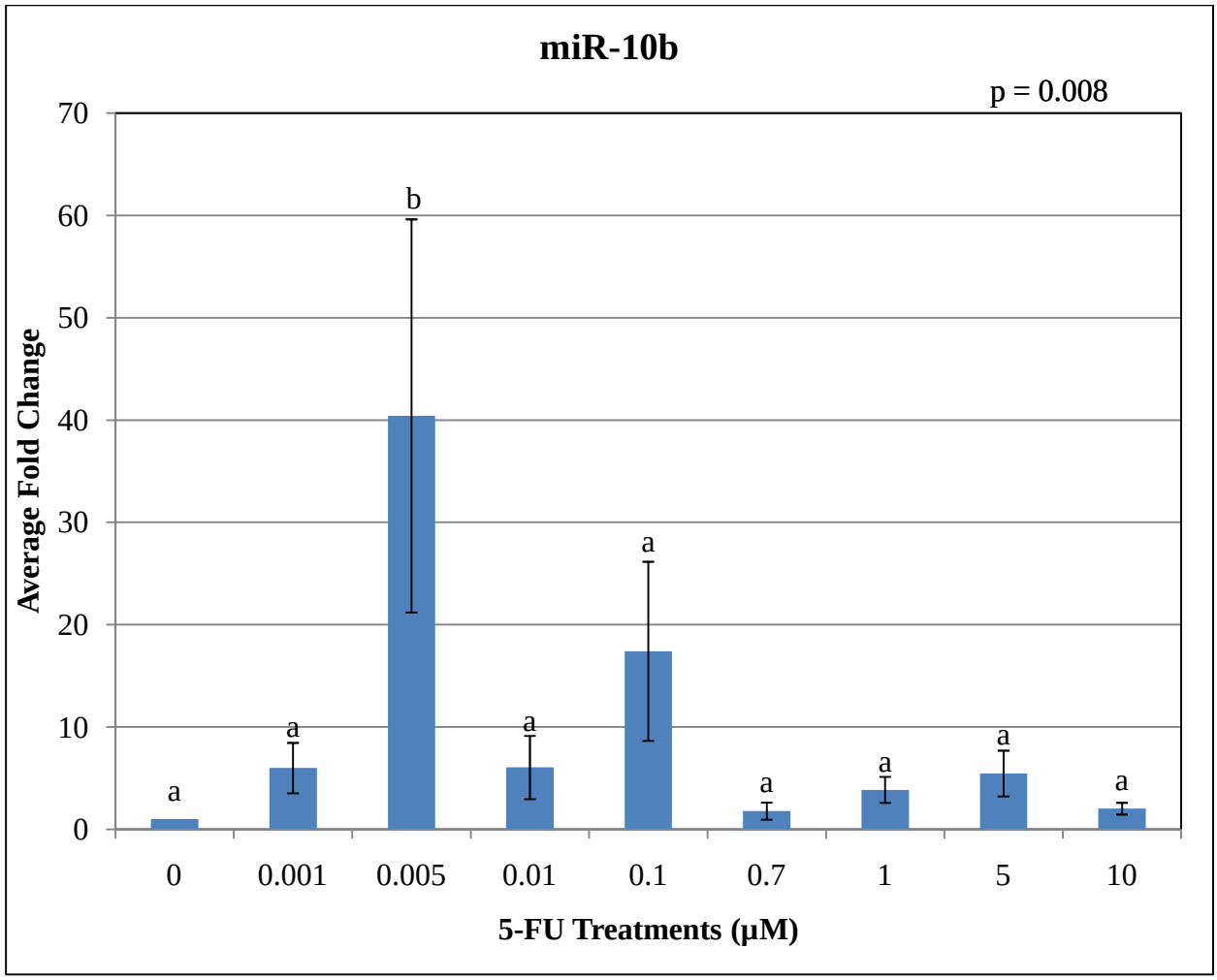

Figure 4.2: Average fold change in expression of miR-10b in response to 5-FU concentrations from 0.0001µM to 10µM. The results are represented as mean ± SEM. Statistically significant changes in the expression between concentrations are designated by single letters on error bars ($p \leq 0.05$).

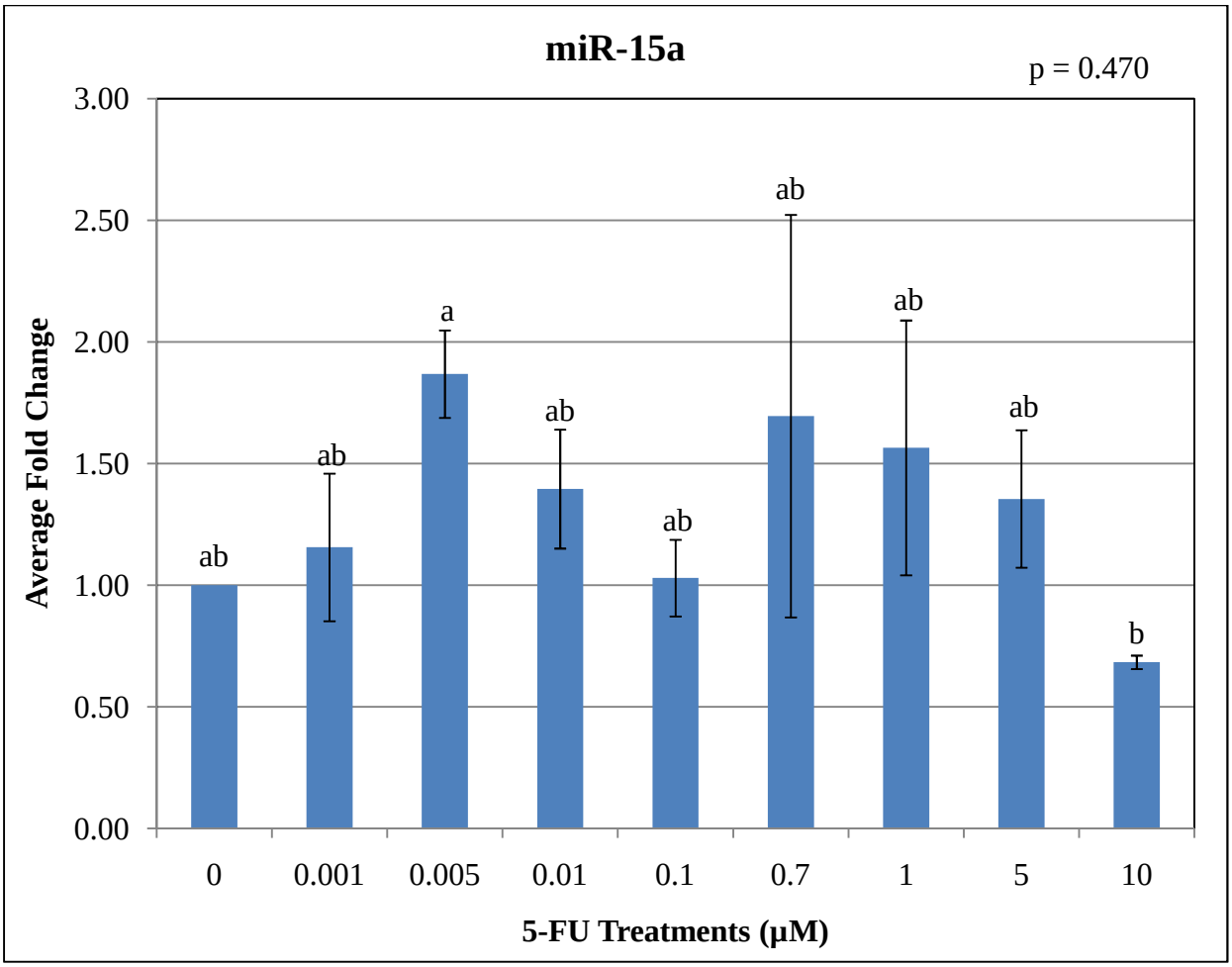

Figure 4.3: Average fold change in expression of miR-15a in response to 5-FU concentrations from 0.0001µM to 10µM. The results are represented as mean ± SEM. Statistically significant changes in the expression between concentrations are designated by single letters on error bars ($p \leq 0.05$).

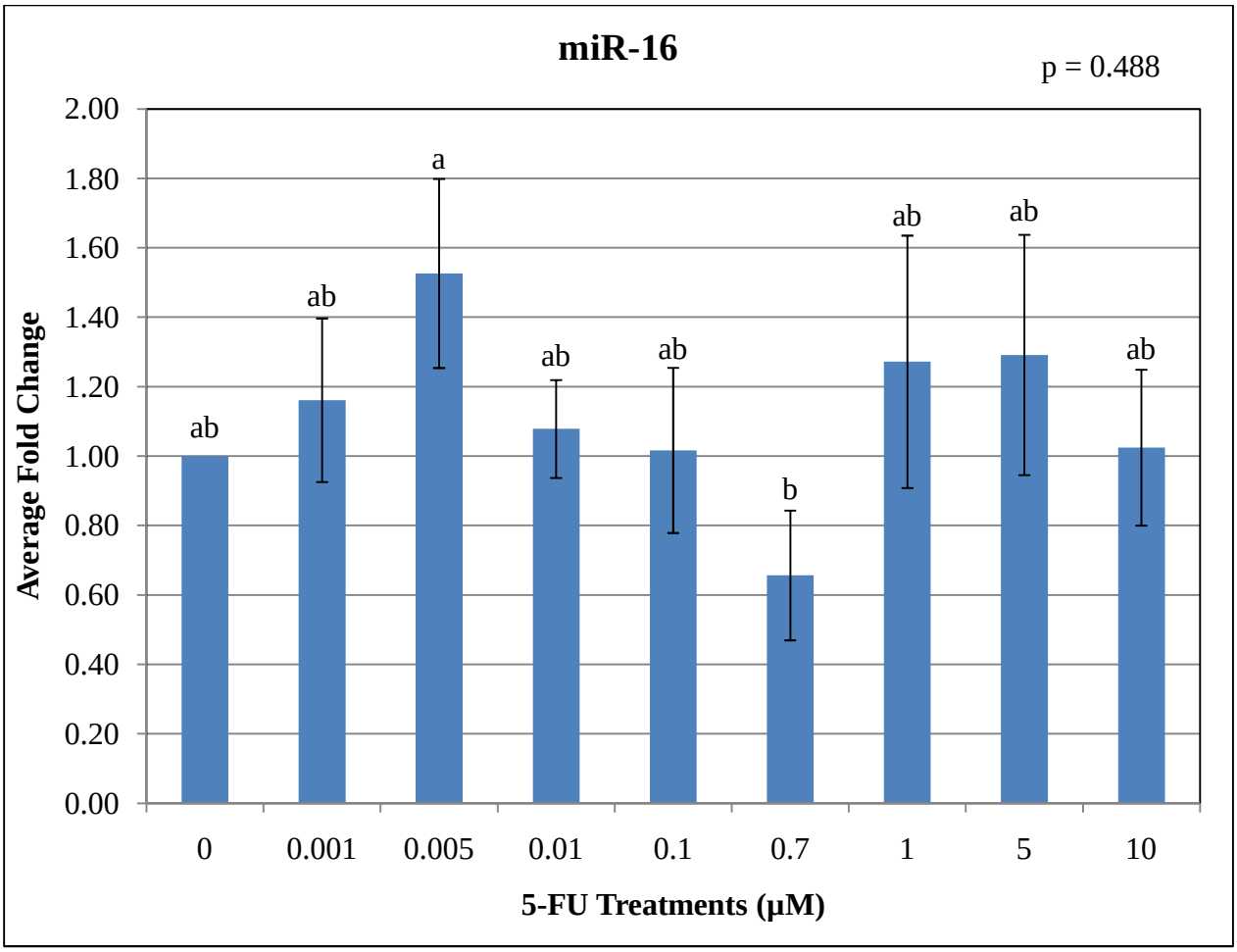

Figure 4.4: Average fold change in expression of miR-16 in response to 5-FU concentrations from 0.0001µM to 10µM. The results are represented as mean ± SEM. Statistically significant changes in the expression between concentrations are designated by single letters on error bars ($p \leq 0.05$).

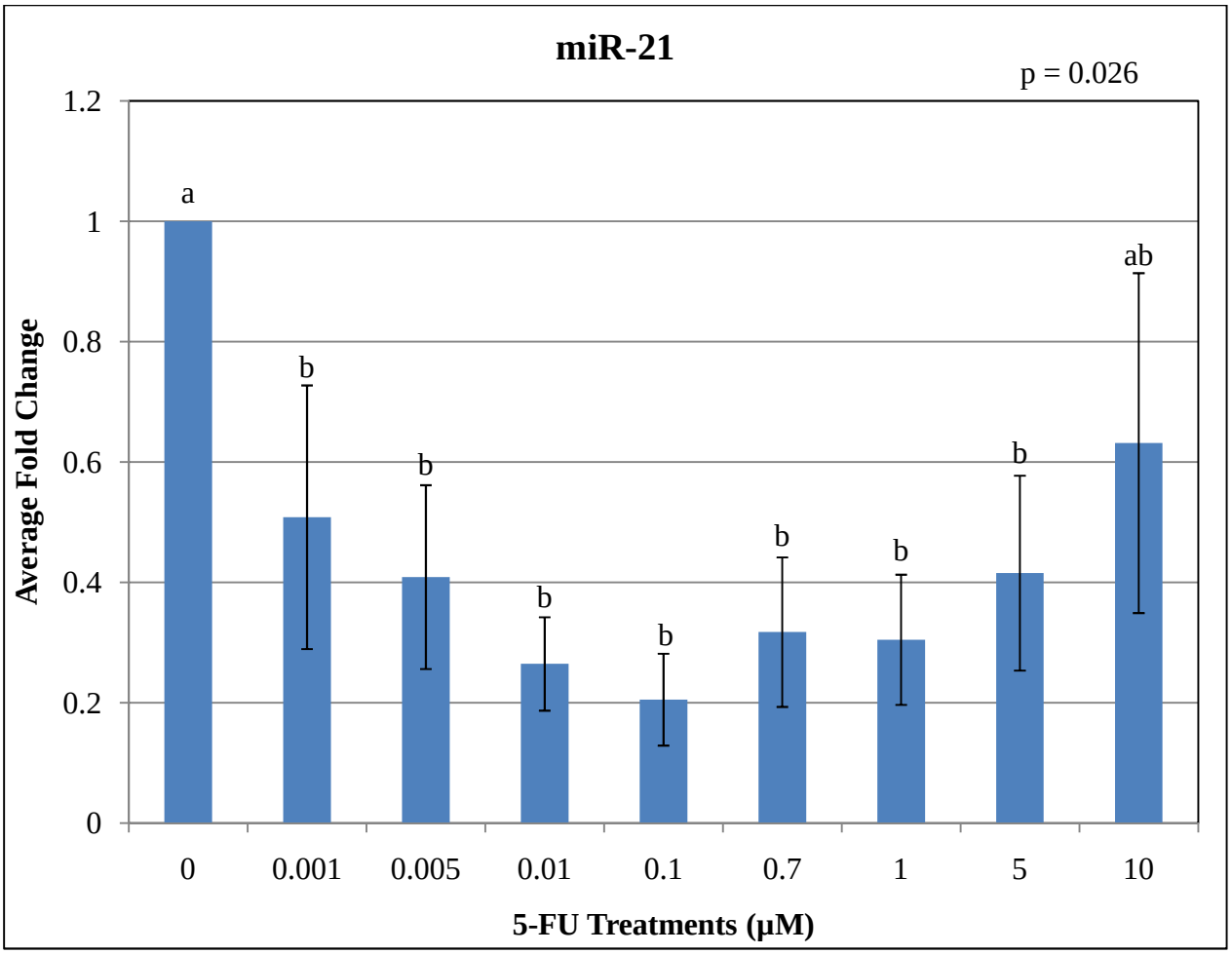

Figure 4.5: Average fold change in expression of miR-21 in response to 5-FU concentrations from 0.0001µM to 10µM. The results are represented as mean ± SEM. Statistically significant changes in the expression between concentrations are designated by single letters on error bars ($p \leq 0.05$).

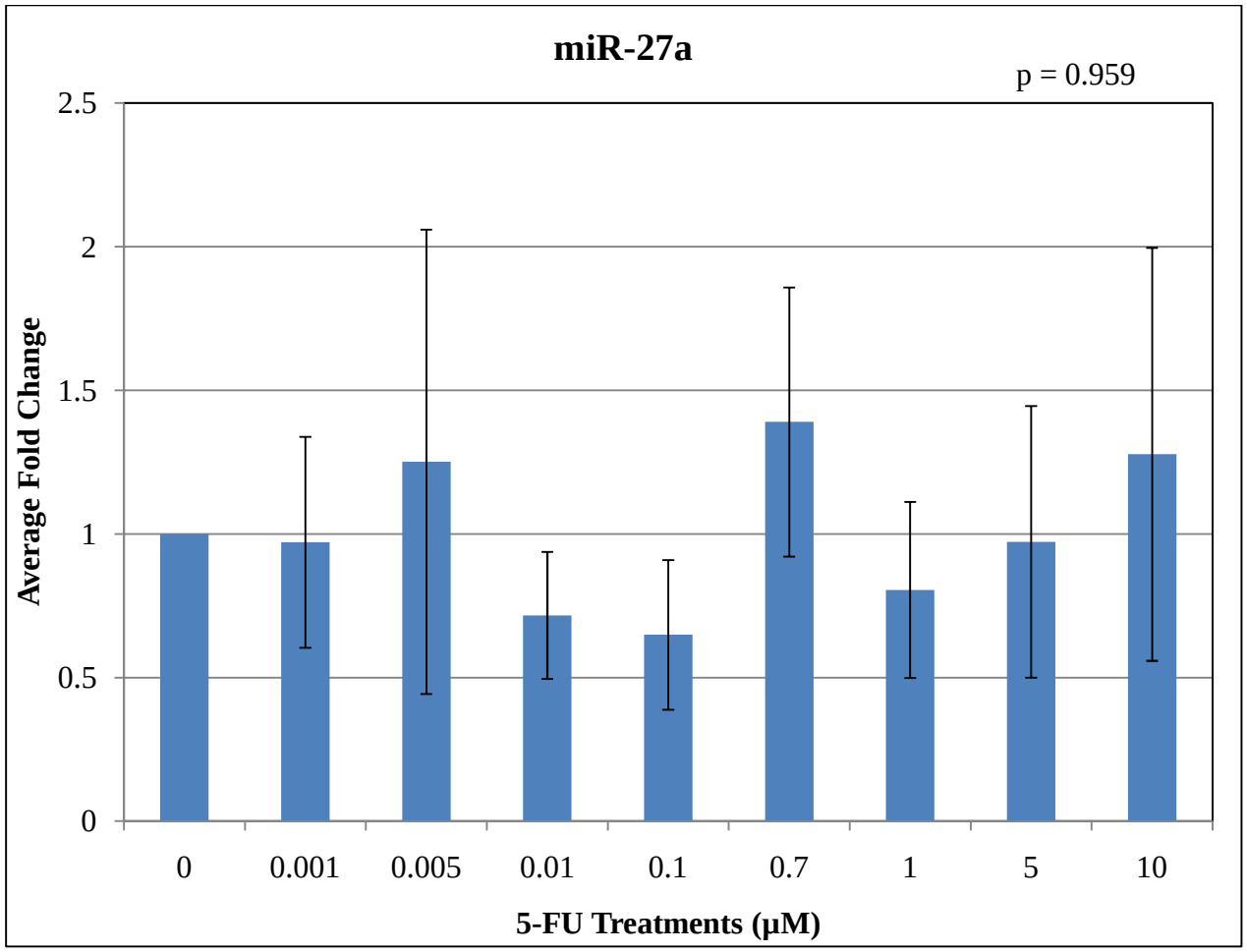

Figure 4.6: Average fold change in expression of miR-27a in response to 5-FU concentrations from 0.0001µM to 10µM. The results are represented as mean ± SEM.

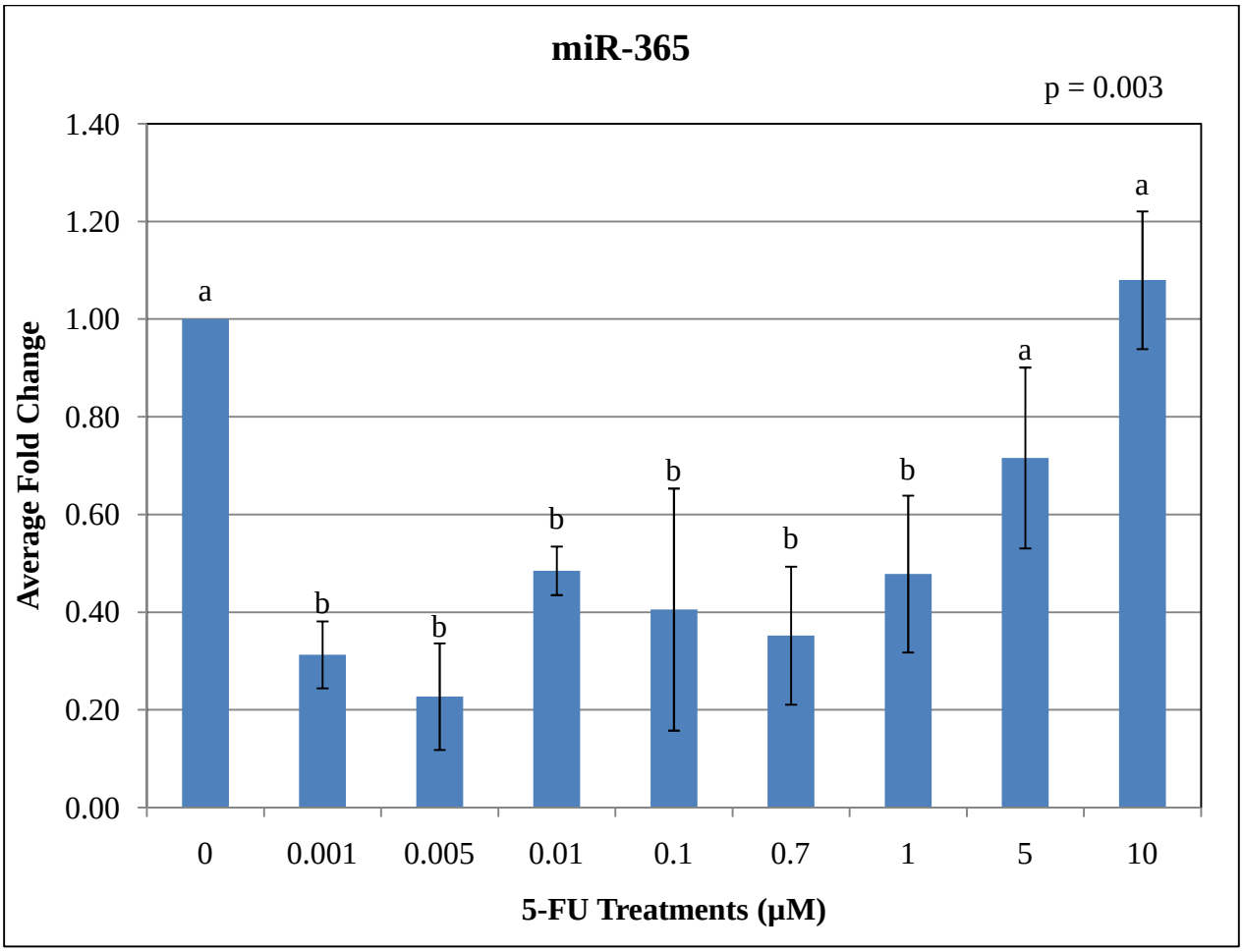

Figure 4.7: Average fold change in expression of miR-365 in response to 5-FU concentrations from 0.0001µM to 10µM. The results are represented as mean ± SEM. Statistically significant changes in the expression between concentrations are designated by single letters on error bars ($p \leq 0.05$).

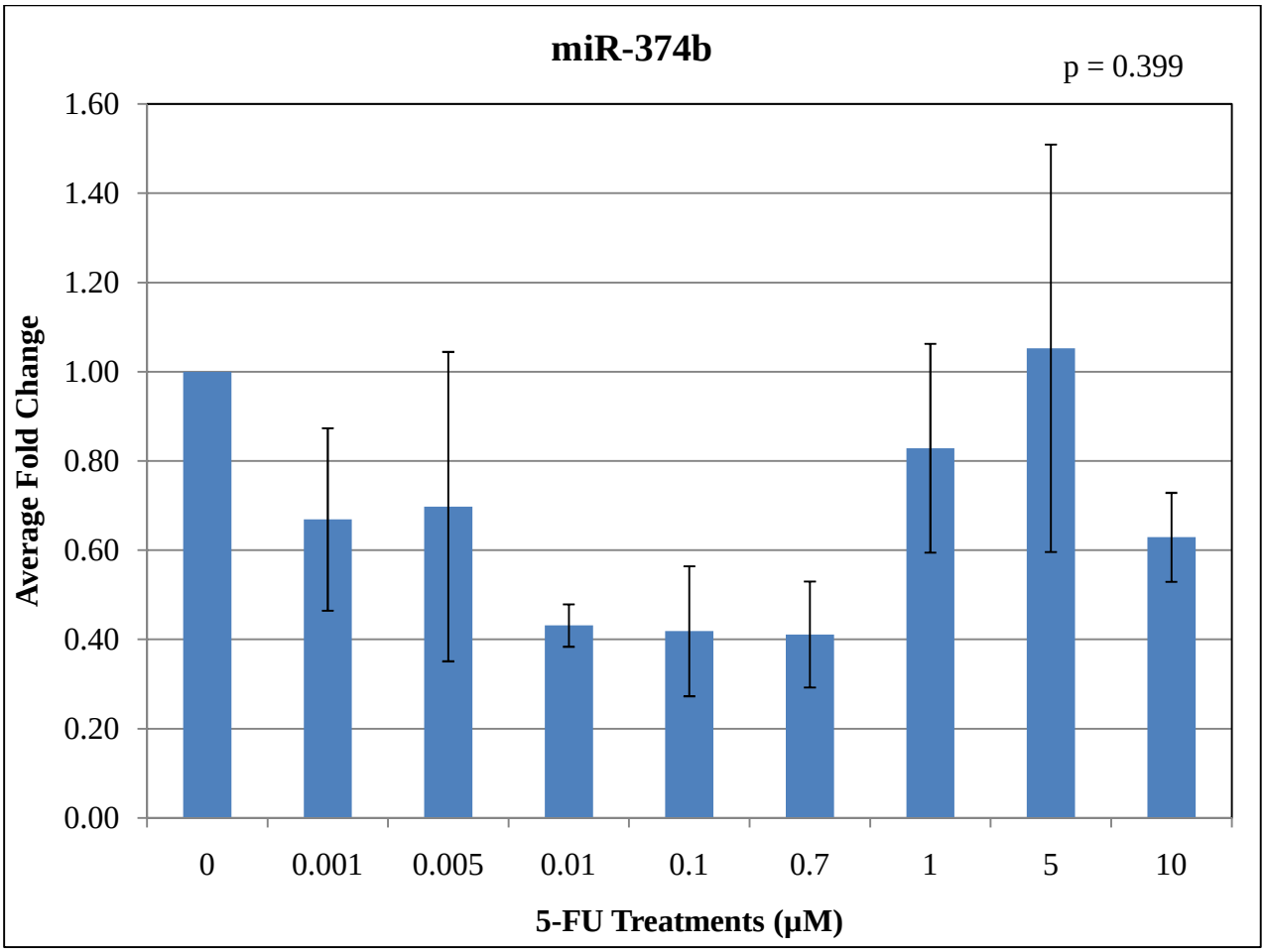

Figure 4.8: Average fold change in expression of miR-374b in response to 5-FU concentrations from 0.0001µM to 10µM. The results are represented as mean ± SEM.

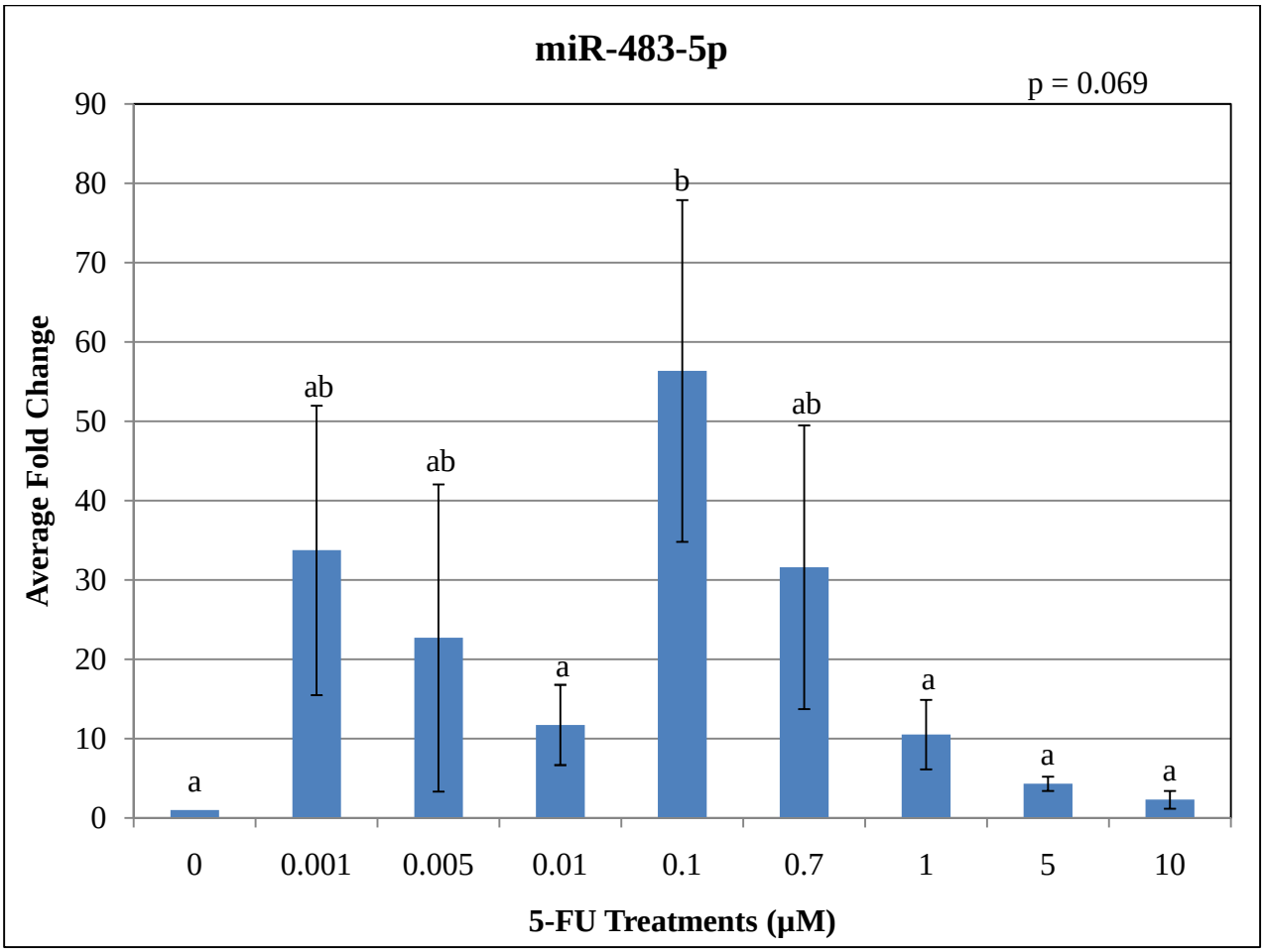

Figure 4.9: Average fold change in expression of miR-483-5p in response to 5-FU concentrations from 0.0001µM to 10µM. The results are represented as mean ± SEM. Statistically significant changes in the expression between concentrations are designated by single letters on error bars ($p \leq 0.05$).

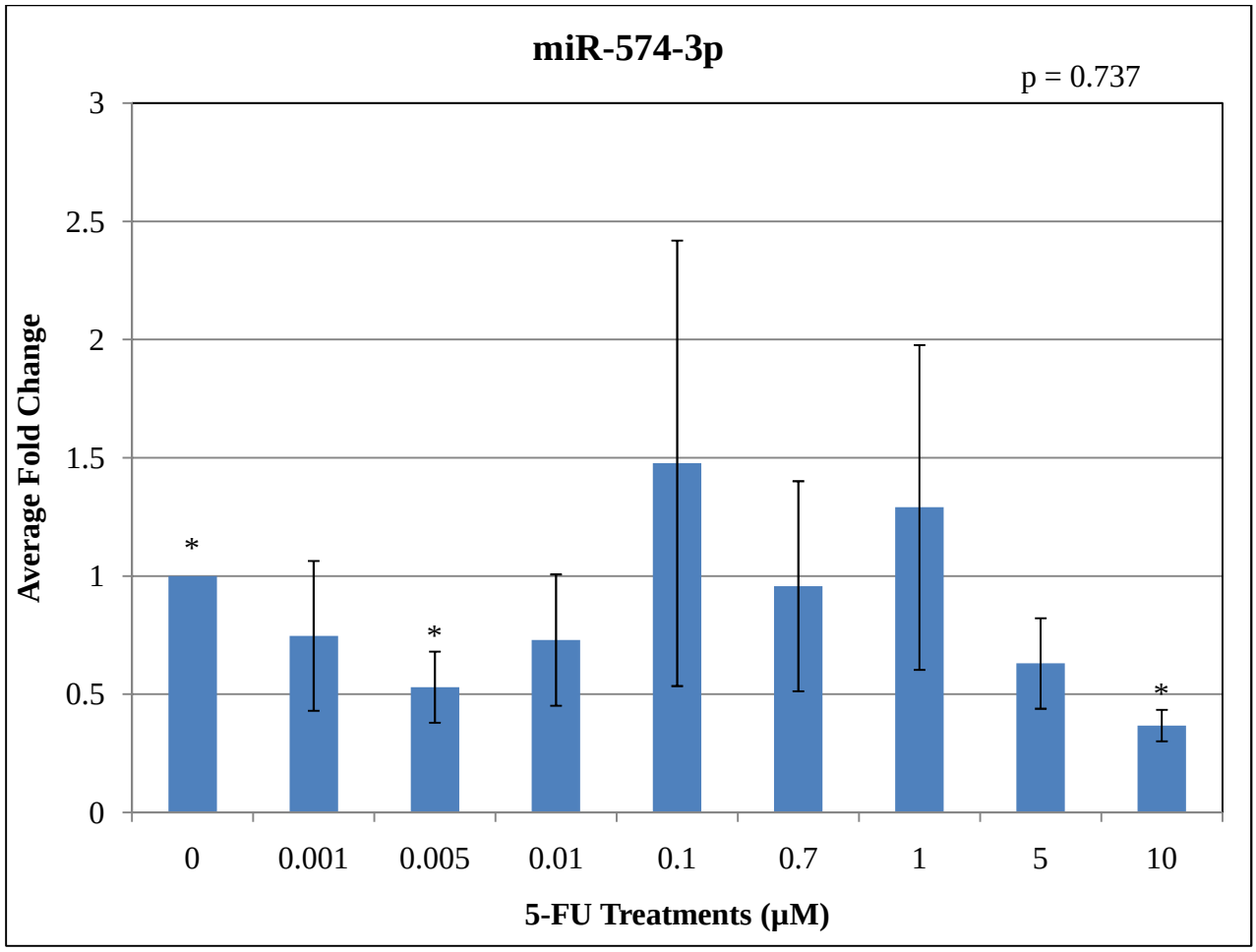

Figure 4.10: Average fold change in expression of miR-574-3p in response to 5-FU concentrations from 0.0001µM to 10µM. The results are represented as mean ± SEM. Statistically significant changes in the expression compared to the control is indicated by asterisks (by Independent samples t-test).

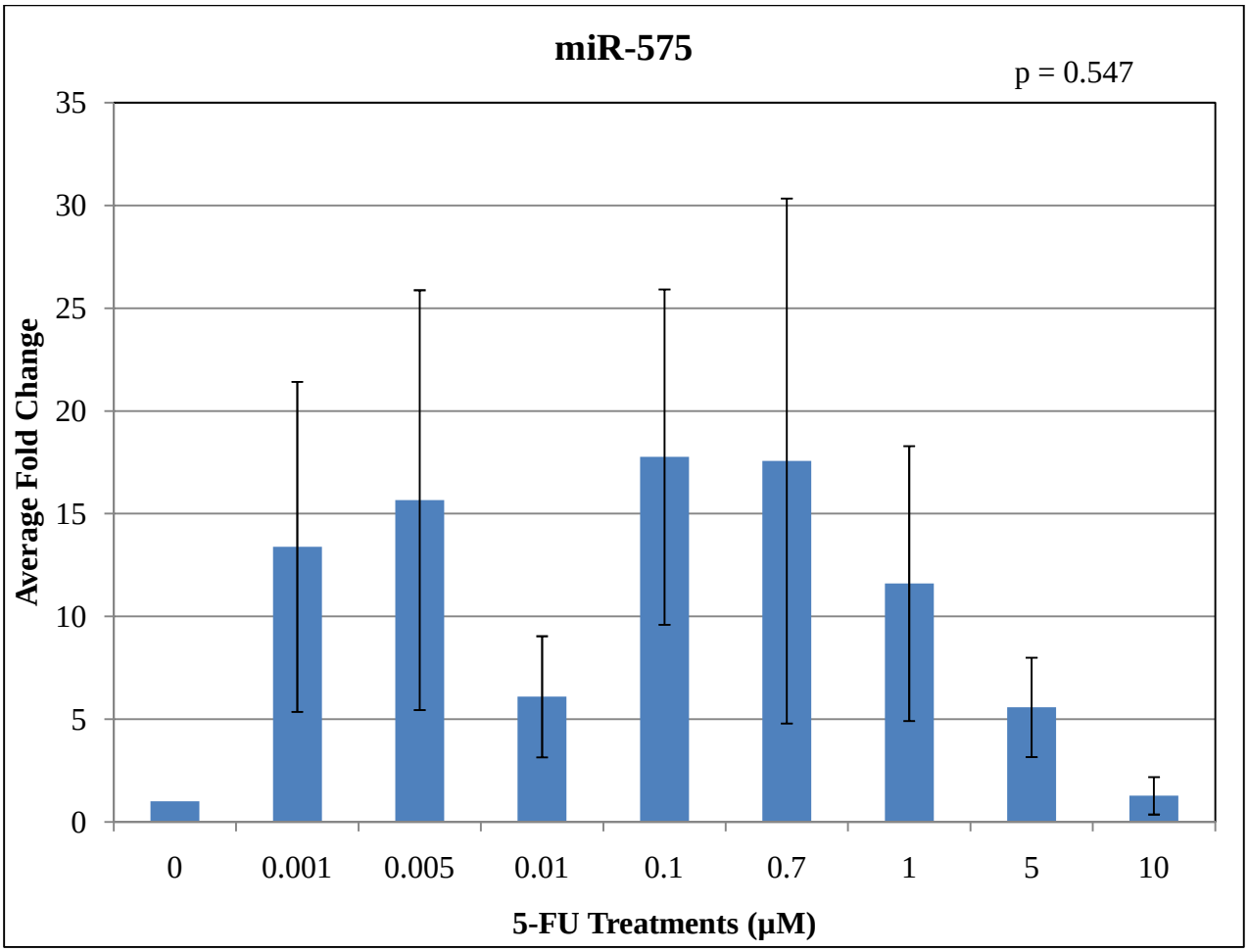

Figure 4.11: Average fold change in expression of miR-575 in response to 5-FU concentrations from 0.0001µM to 10µM. The results are represented as mean ± SEM.

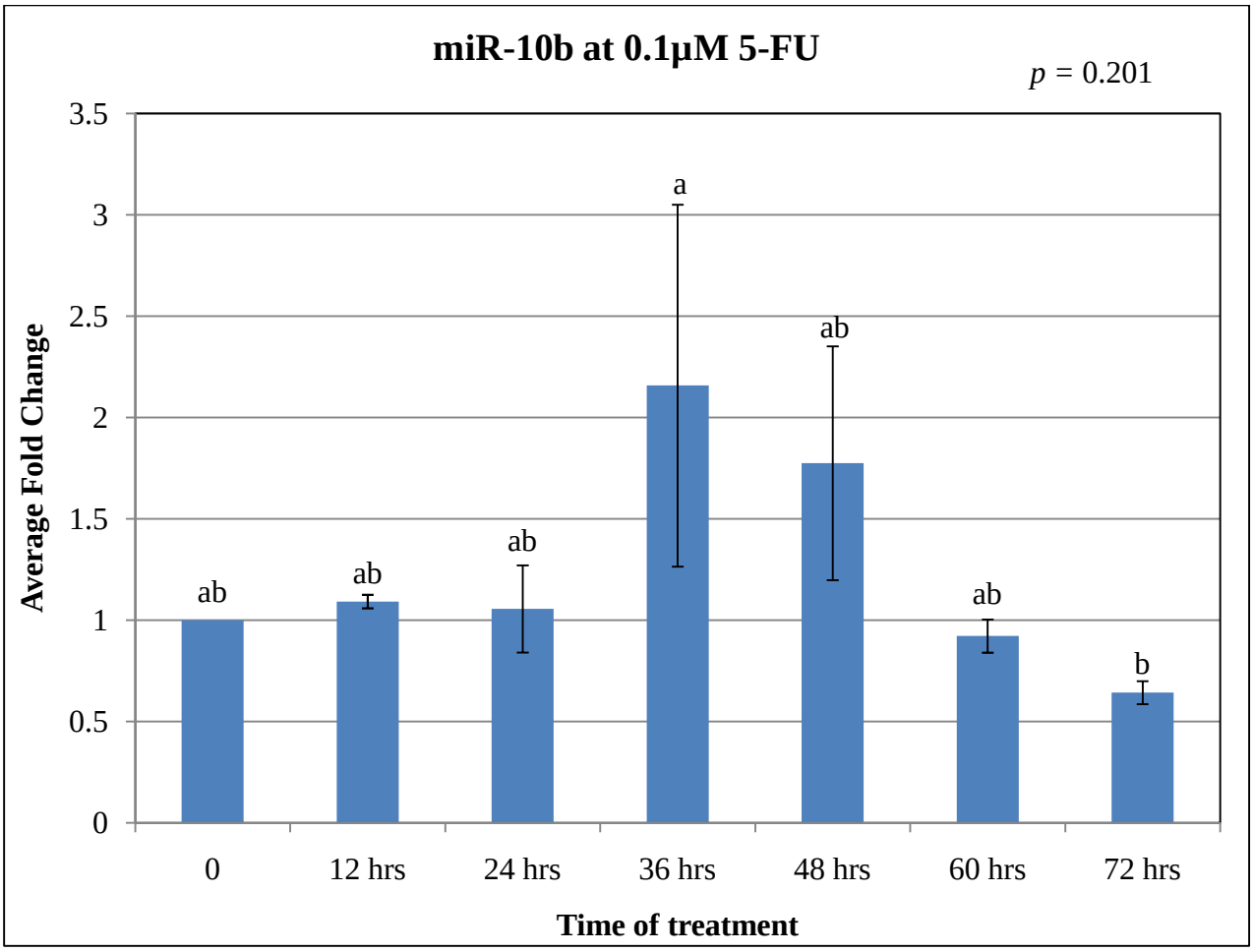

Figure 4.12: Average fold change in expression of miR-10b after treatment with 0.1µM of 5-FU for 12, 24, 36, 48, 60 and 72 hours. The results are represented as mean ± SEM. Statistically significant changes in the expression between concentrations are designated by single letters on error bars ($p \leq 0.05$).

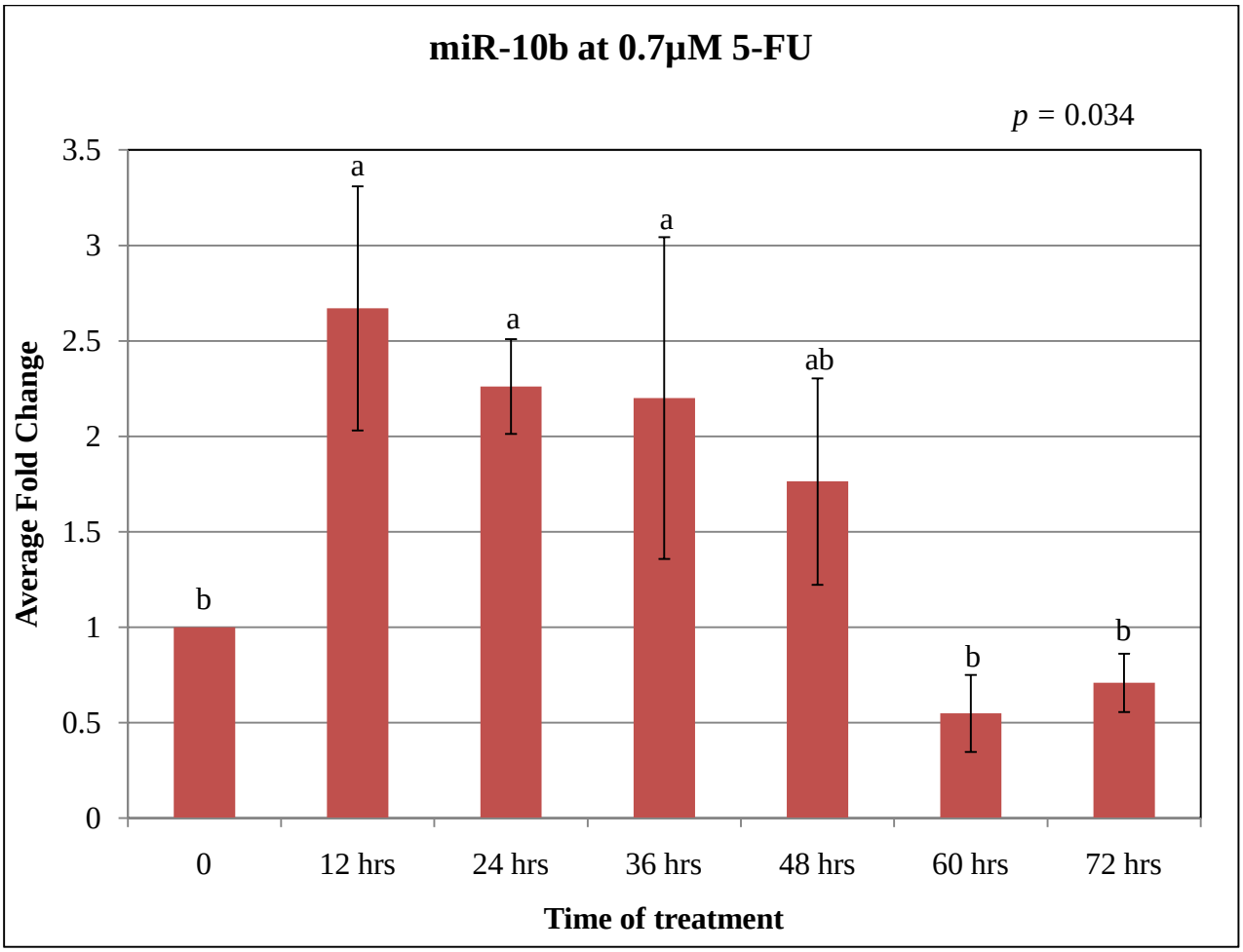

Figure 4.13: Average fold change in expression of miR-10b after treatment with 0.7µM of 5-FU for 12, 24, 36, 48, 60 and 72 hours. The results are represented as mean ± SEM. Statistically significant changes in the expression between concentrations are designated by single letters on error bars ($p \leq 0.05$).

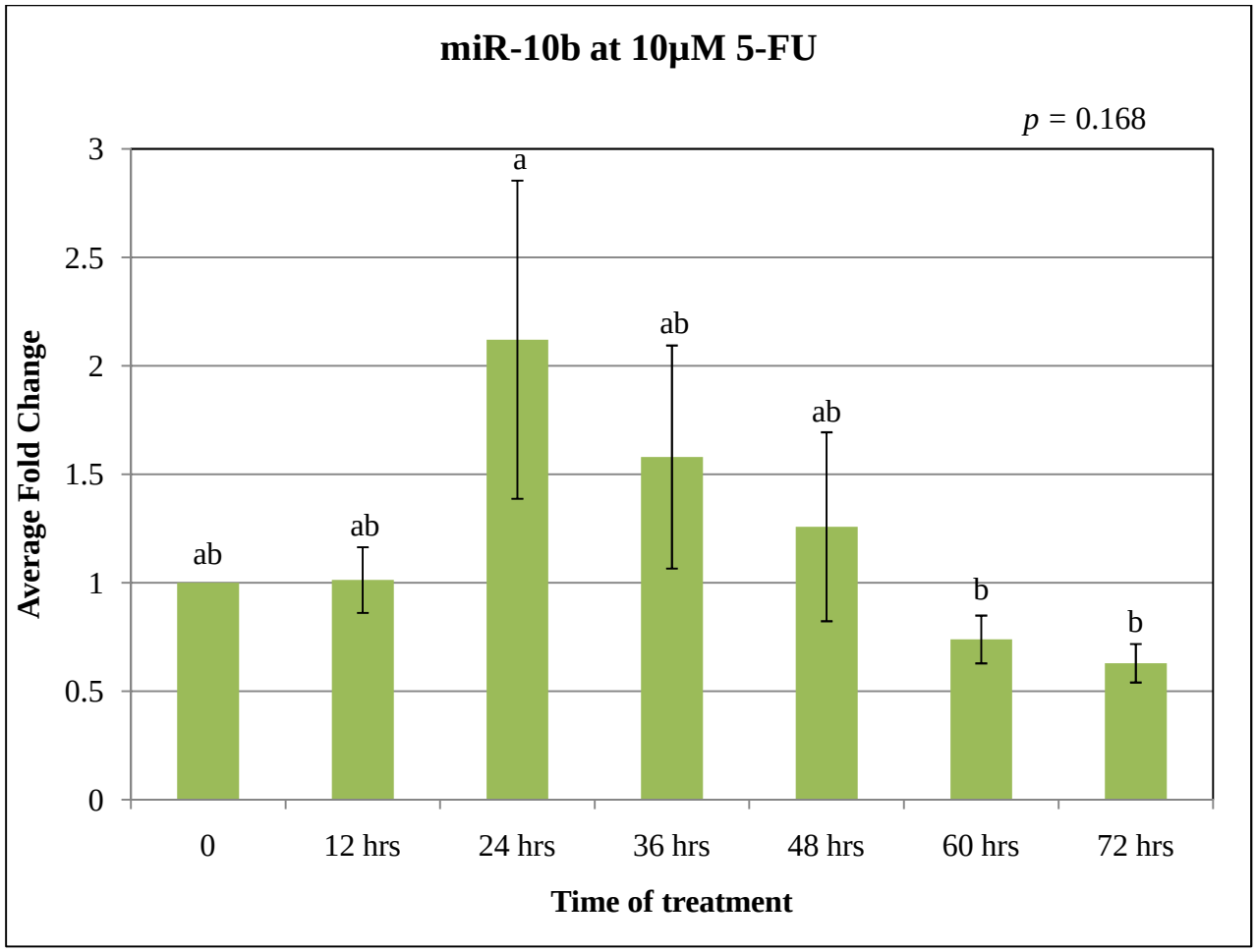

Figure 4.14: Average fold change in expression of miR-10b after treatment with 10µM of 5-FU for 12, 24, 36, 48, 60 and 72 hours. The results are represented as mean ± SEM. Statistically significant changes in the expression between concentrations are designated by single letters on error bars ($p \leq 0.05$).

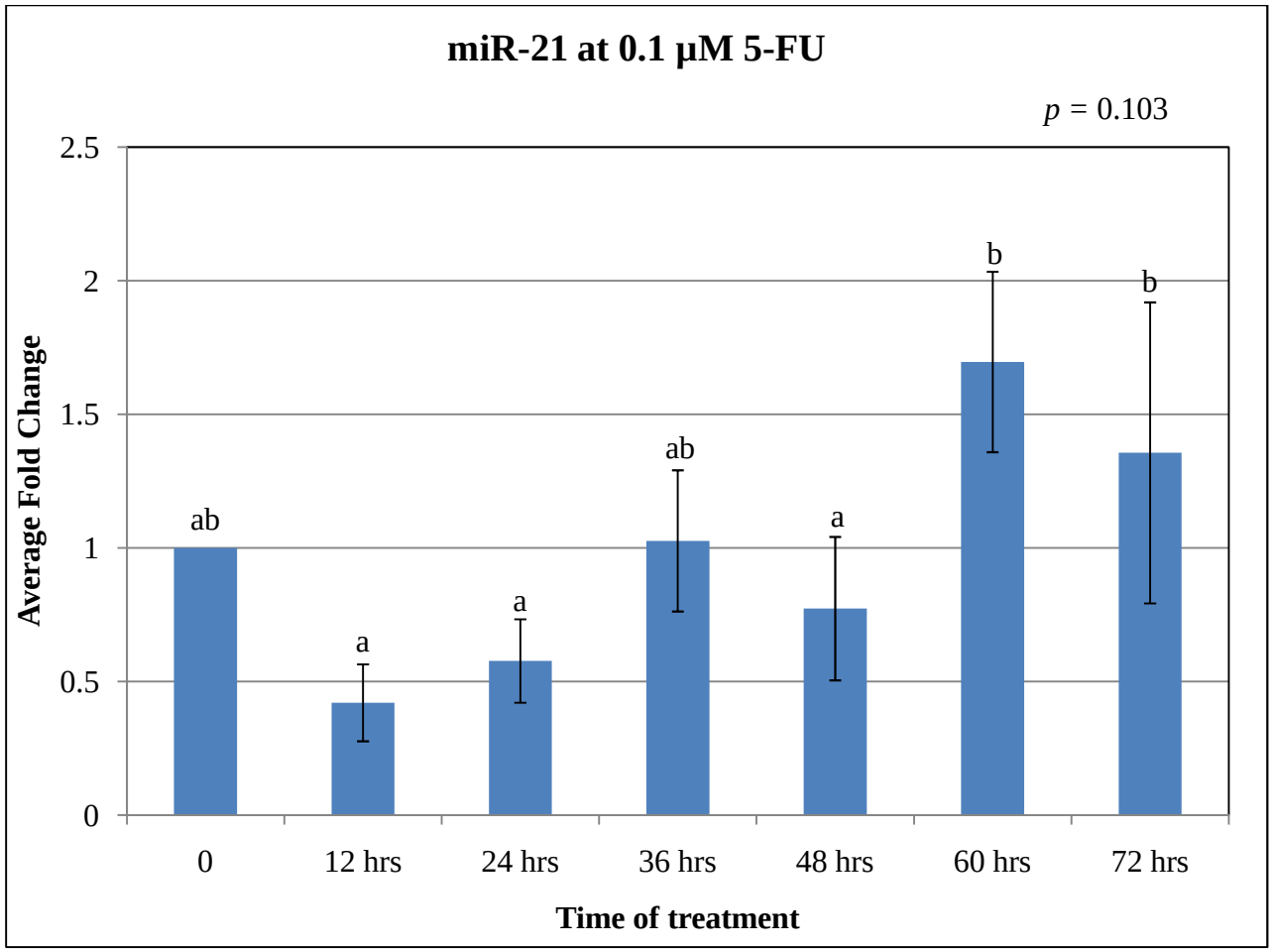

Figure 4.15: Average fold change in expression of miR-21 after treatment with 0.1µM of 5-FU for 12, 24, 36, 48, 60 and 72 hours. The results are represented as mean ± SEM. Statistically significant changes in the expression between concentrations are designated by single letters on error bars ($p \leq 0.05$).

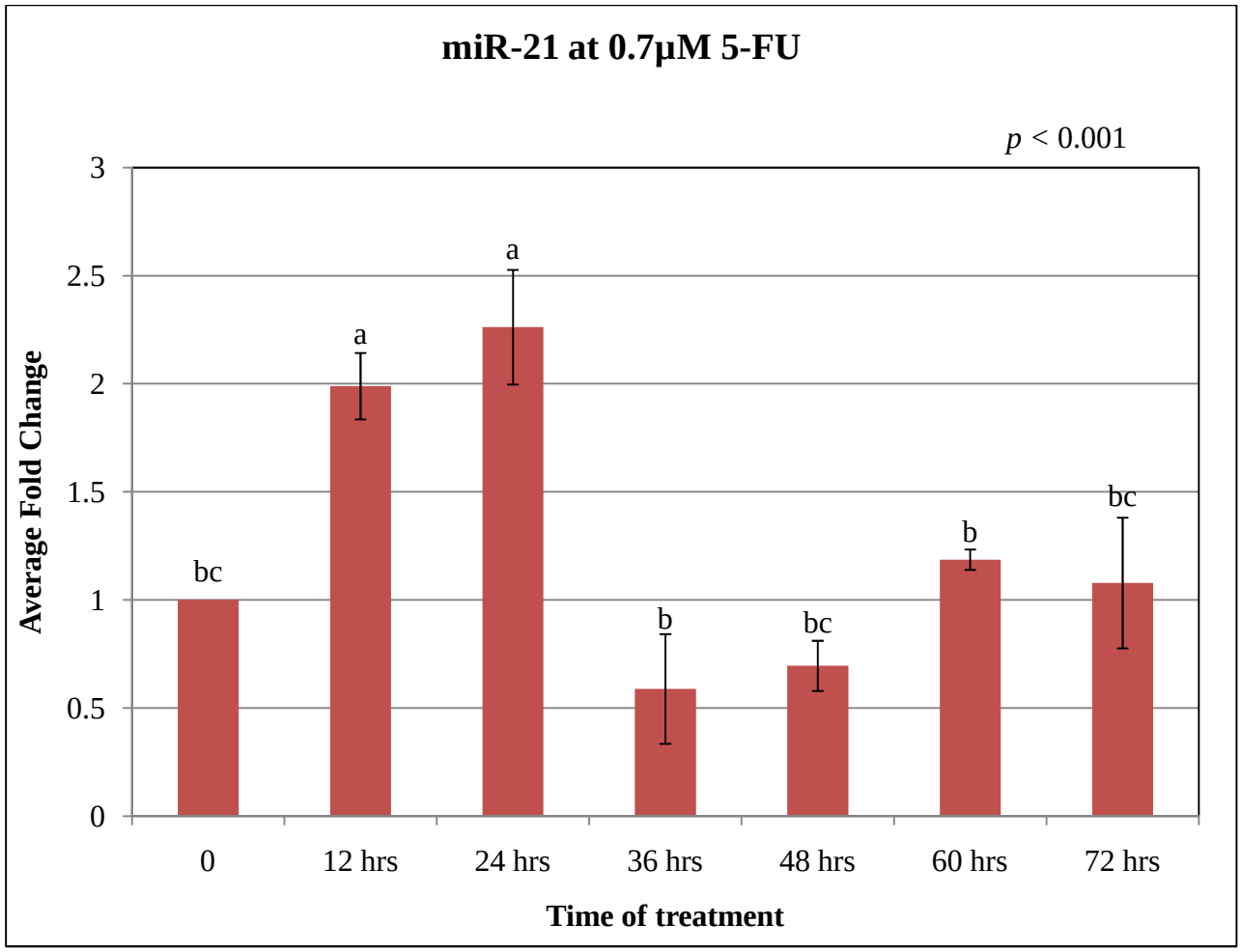

Figure 4.16: Average fold change in expression of miR-21 after treatment with 0.7µM of 5-FU for 12, 24, 36, 48, 60 and 72 hours. The results are represented as mean ± SEM. Statistically significant changes in the expression between concentrations are designated by single letters on error bars ($p \leq 0.05$).

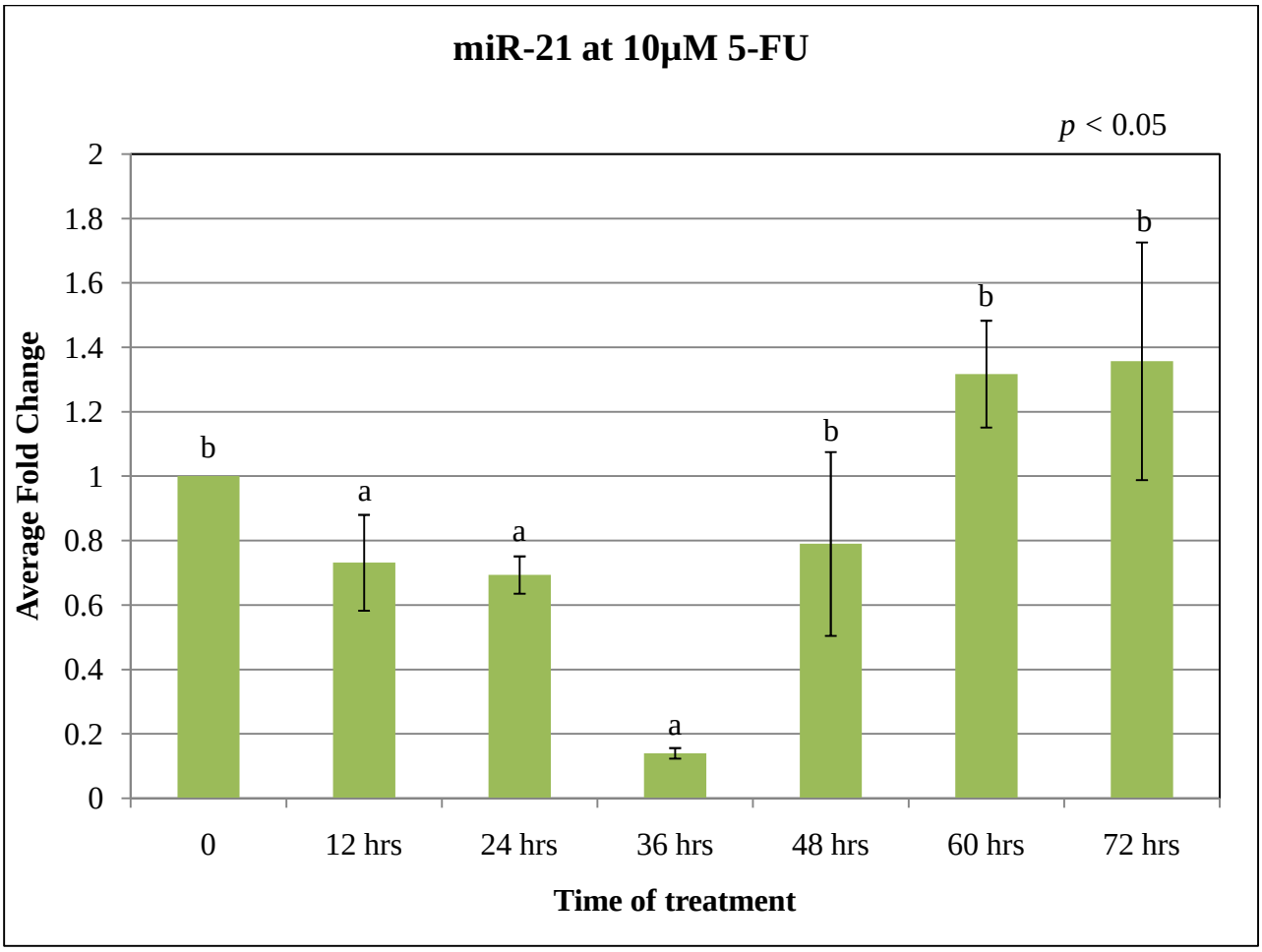

Figure 4.17: Average fold change in expression of miR-21 after treatment with 10µM of 5-FU for 12, 24, 36, 48, 60 and 72 hours. The results are represented as mean ± SEM. Statistically significant changes in the expression between concentrations are designated by single letters on error bars ($p \leq 0.05$).

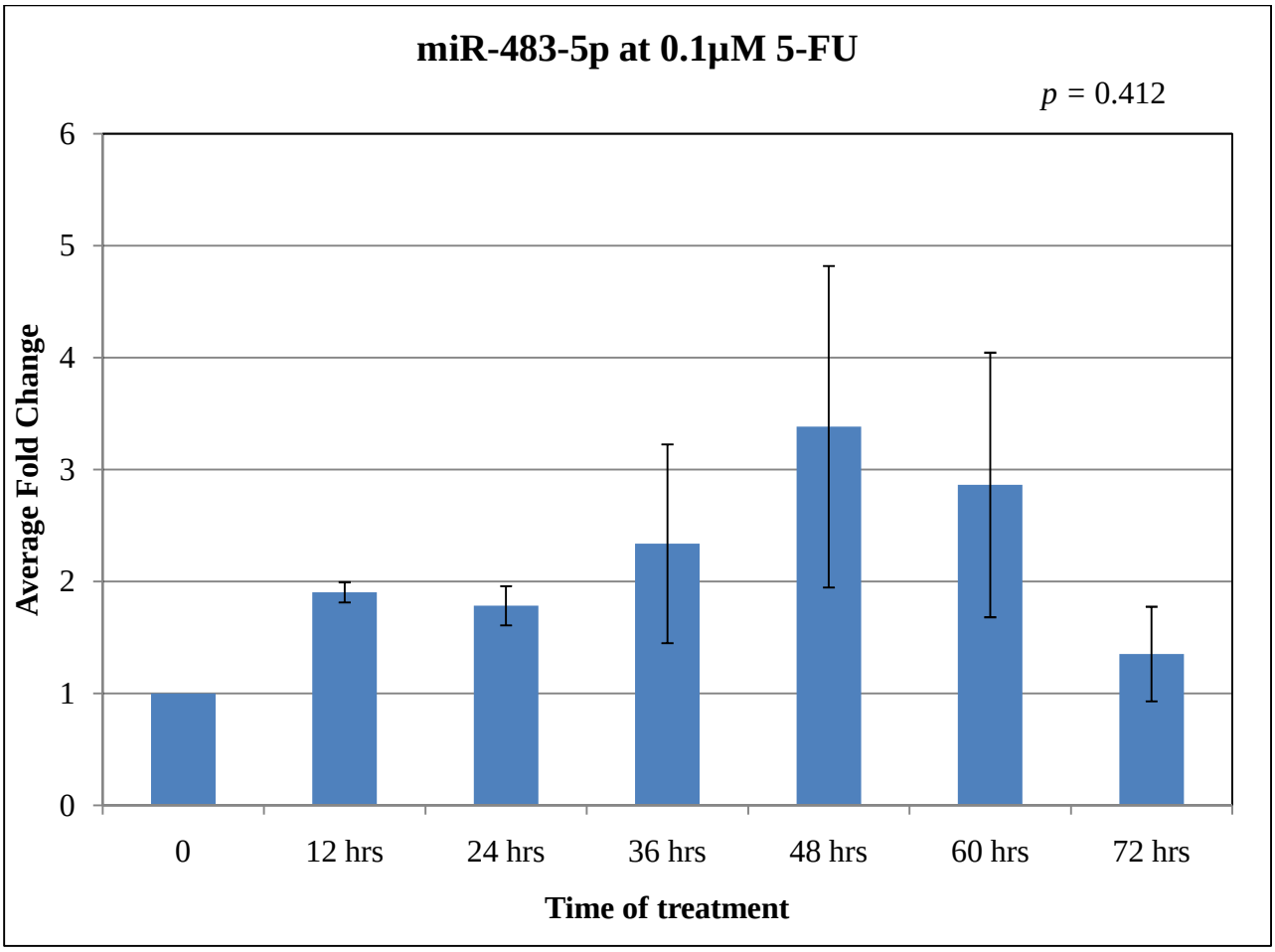

Figure 4.18: Average fold change in expression of miR-483-5p after treatment with 0.1µM of 5-FU for 12, 24, 36, 48, 60 and 72 hours. The results are represented as mean ± SEM.

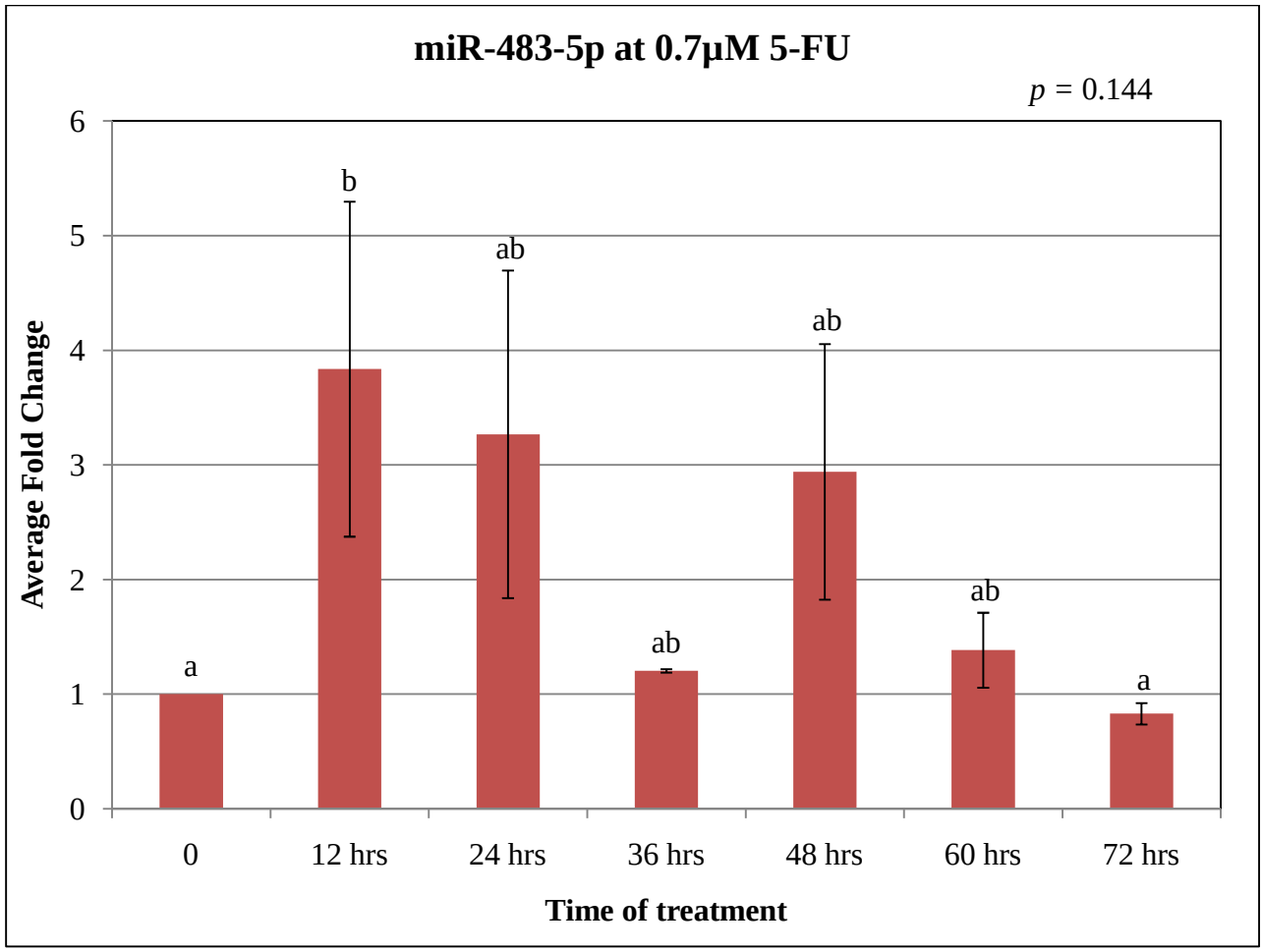

Figure 4.19: Average fold change in expression of miR-483-5p after treatment with 0.7µM of 5-FU for 12, 24, 36, 48, 60 and 72 hours. The results are represented as mean ± SEM. Statistically significant changes in the expression between concentrations are designated by single letters on error bars ($p \leq 0.05$).

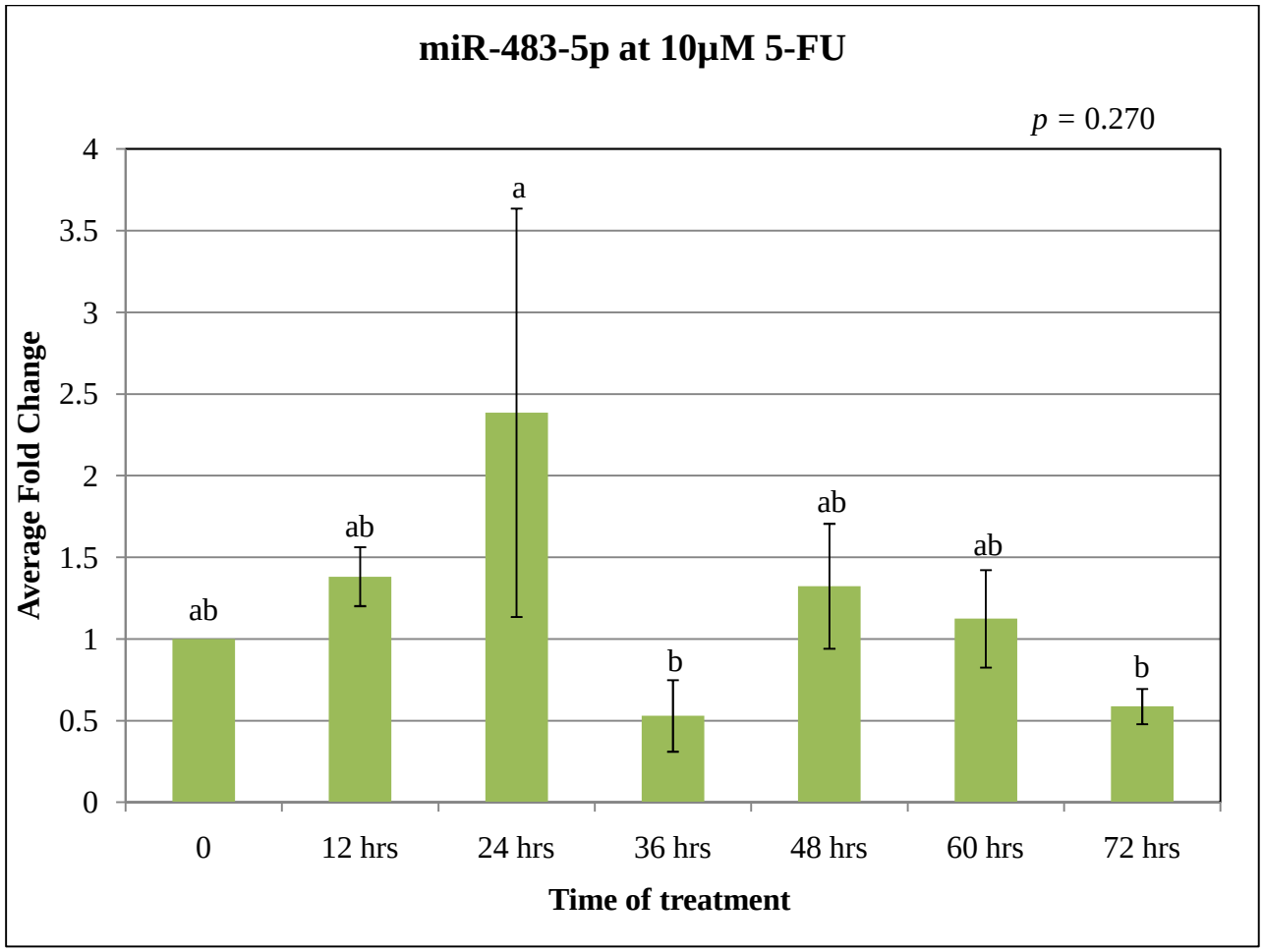

Figure 4.20: Average fold change in expression of miR-483-5p after treatment with 10µM of 5-FU for 12, 24, 36, 48, 60 and 72 hours. The results are represented as mean ± SEM. Statistically significant changes in the expression between concentrations are designated by single letters on error bars ($p \leq 0.05$).

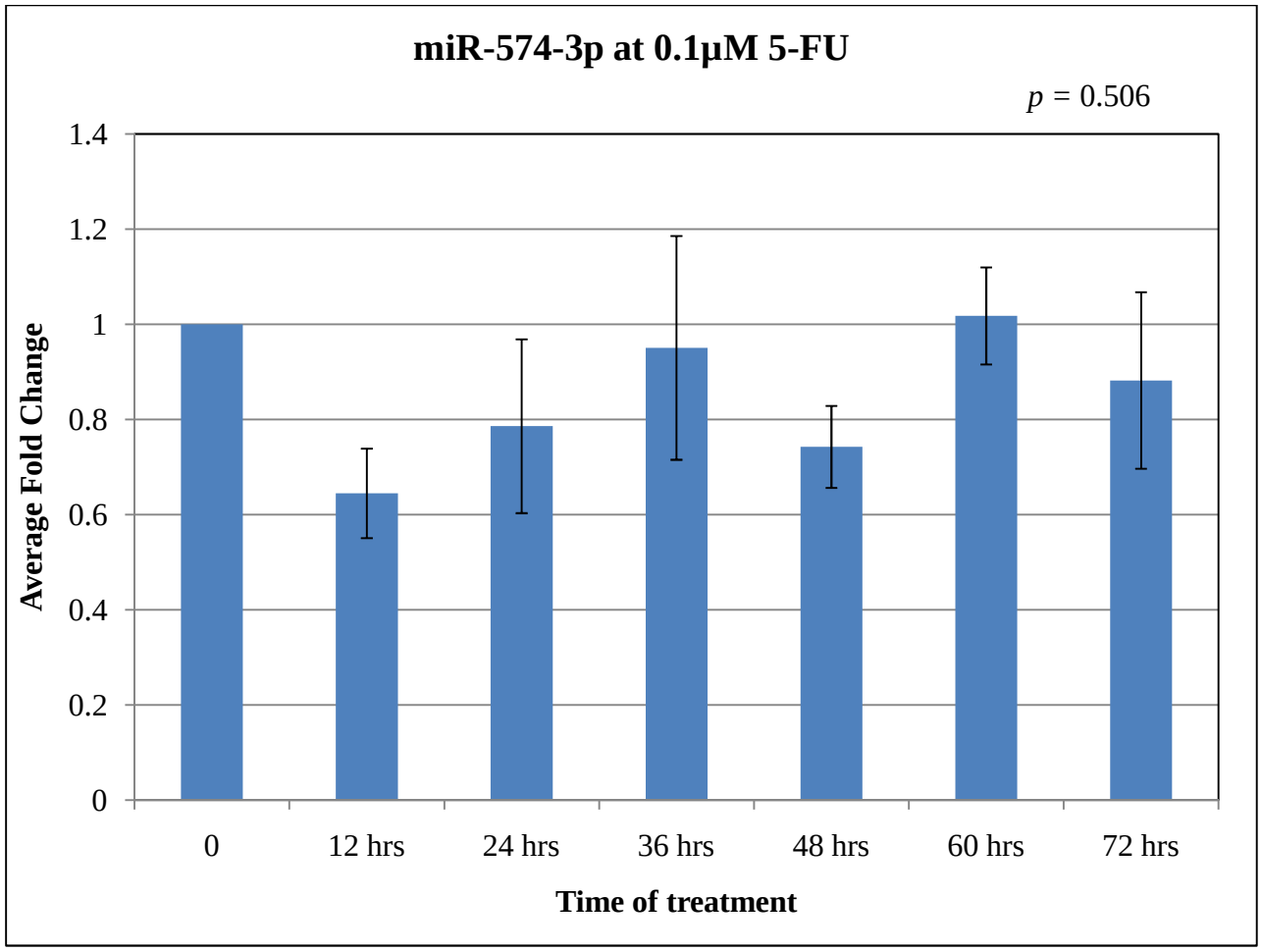

Figure 4.21: Average fold change in expression of miR-574-3p after treatment with 0.1µM of 5-FU for 12, 24, 36, 48, 60 and 72 hours. The results are represented as mean ± SEM.

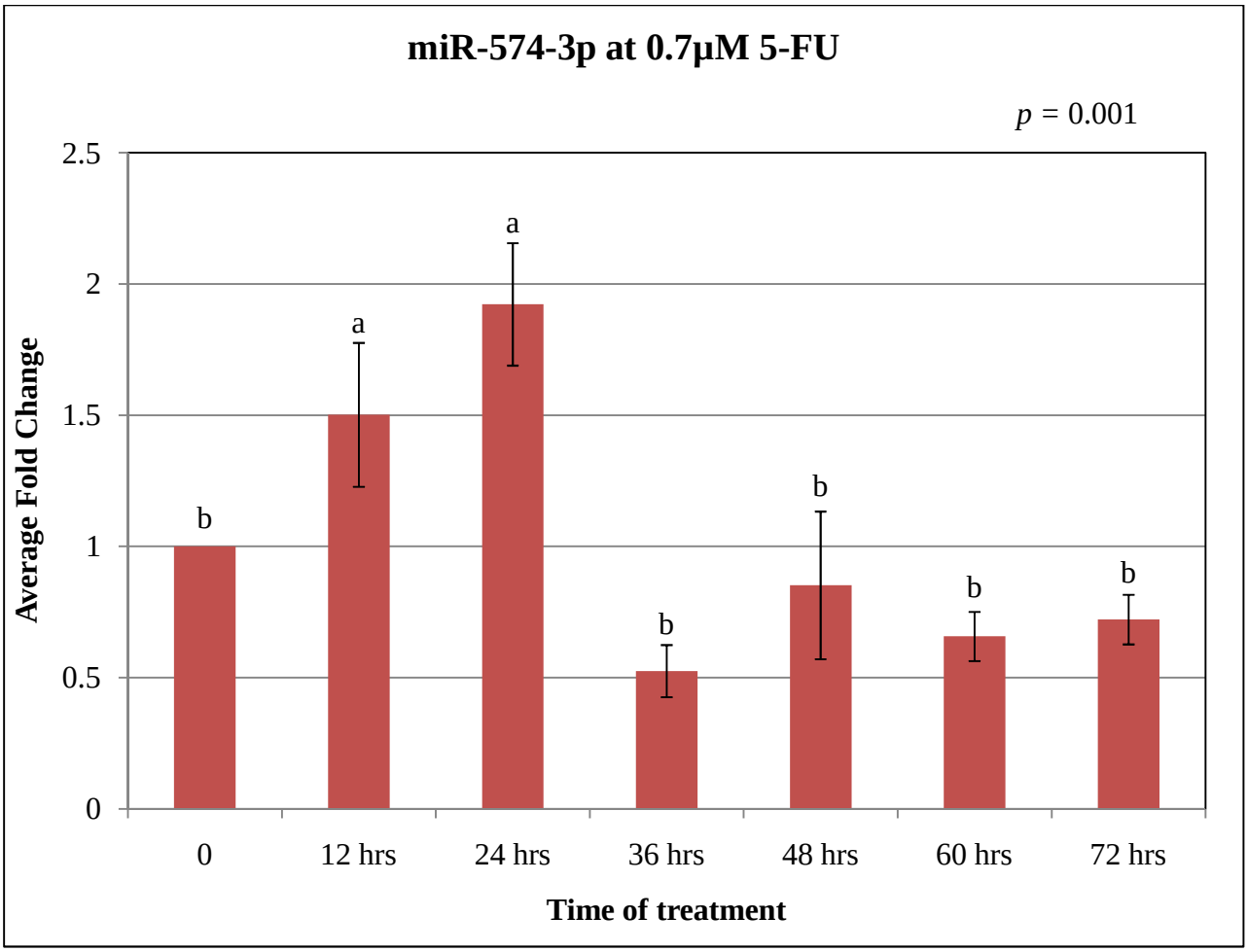

Figure 4.22: Average fold change in expression of miR-574-3p after treatment with 0.7µM of 5-FU for 12, 24, 36, 48, 60 and 72 hours. The results are represented as mean ± SEM. Statistically significant changes in the expression between concentrations are designated by single letters on error bars ($p \leq 0.05$).

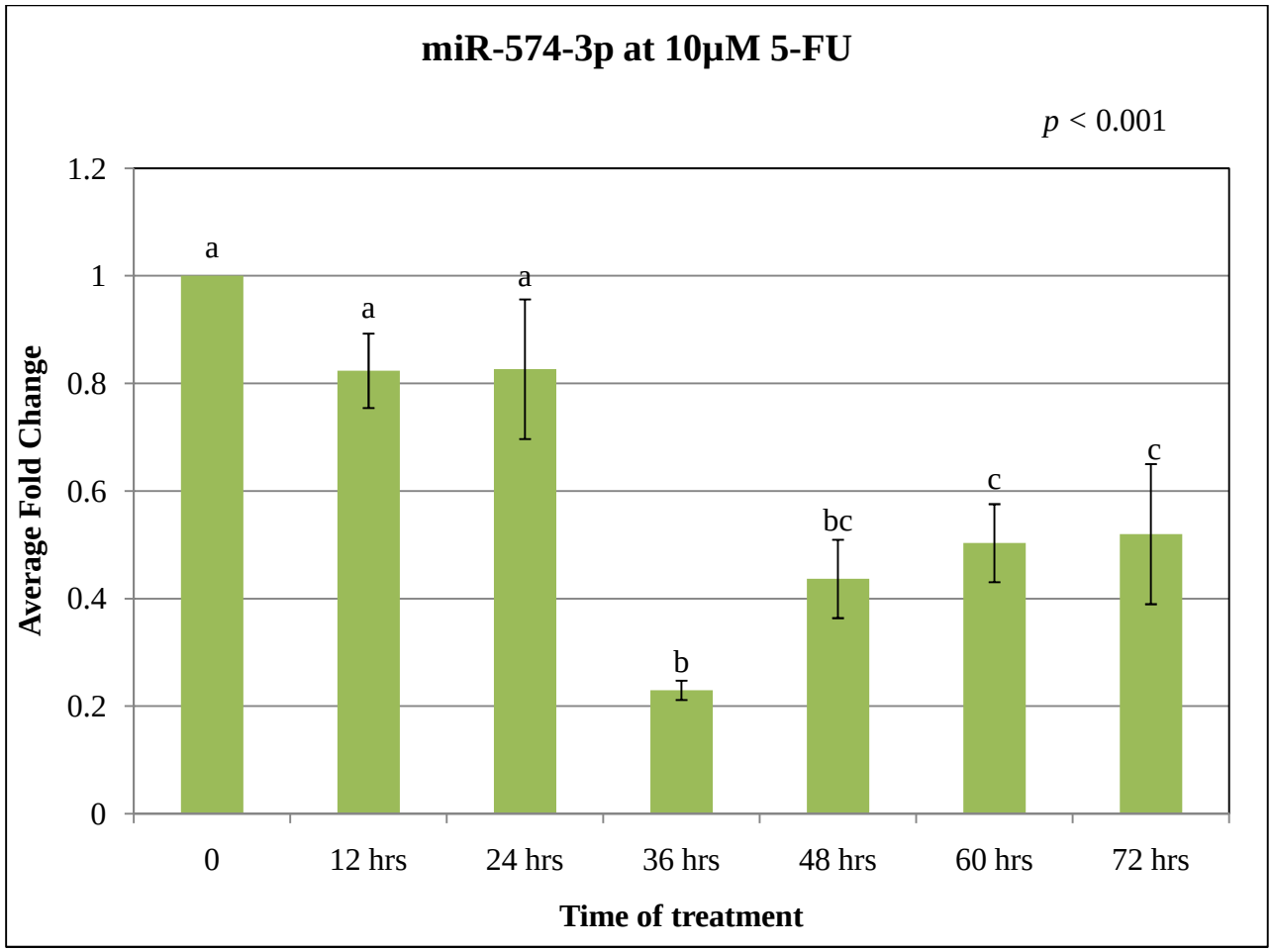

Figure 4.23: Average fold change in expression of miR-574-3p after treatment with 10µM of 5-FU for 12, 24, 36, 48, 60 and 72 hours. The results are represented as mean ± SEM. Statistically significant changes in the expression between concentrations are designated by single letters on error bars ($p \leq 0.05$).

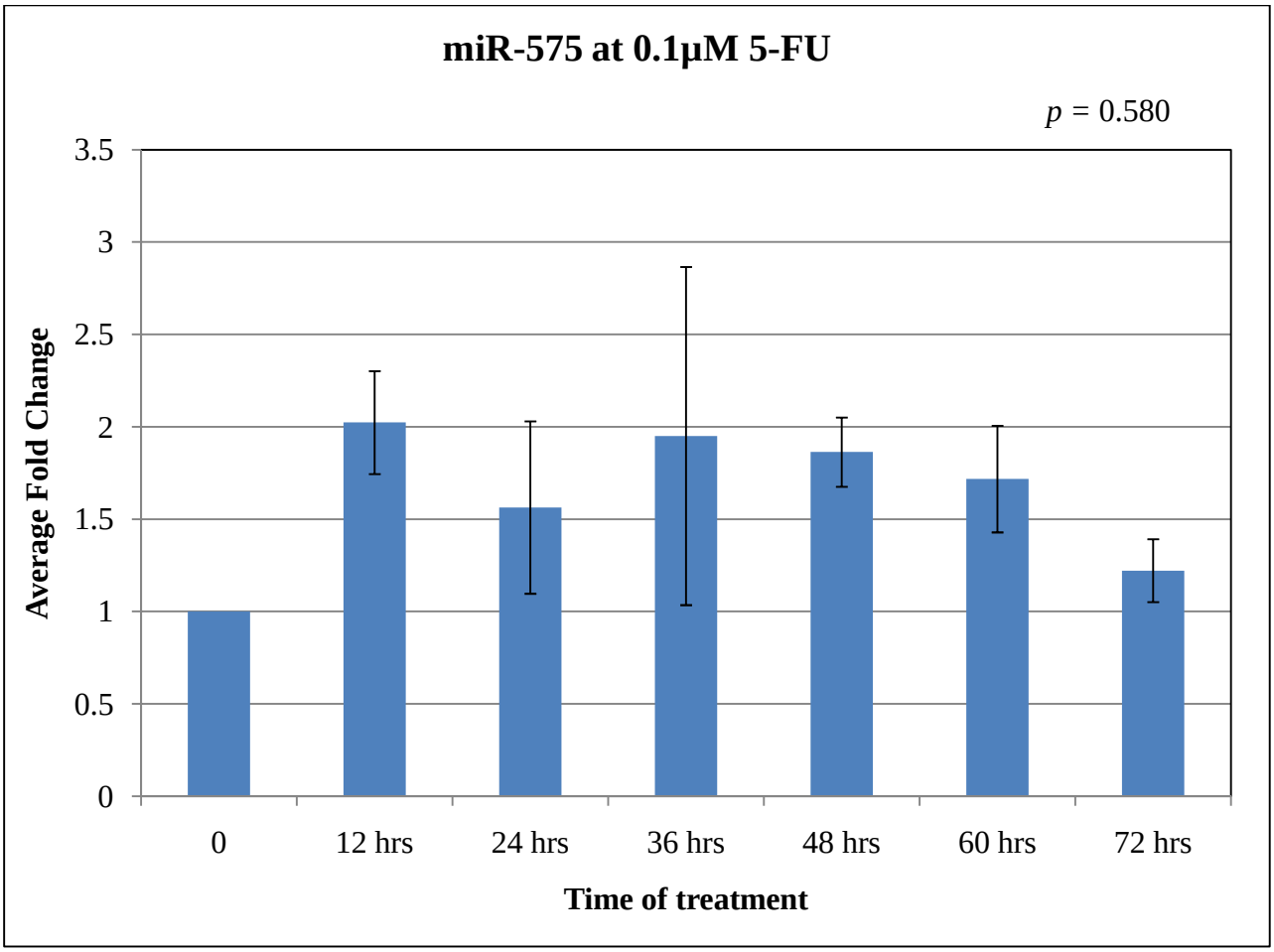

Figure 4.24: Average fold change in expression of miR-575 after treatment with 0.1µM of 5-FU for 12, 24, 36, 48, 60 and 72 hours. The results are represented as mean ± SEM.

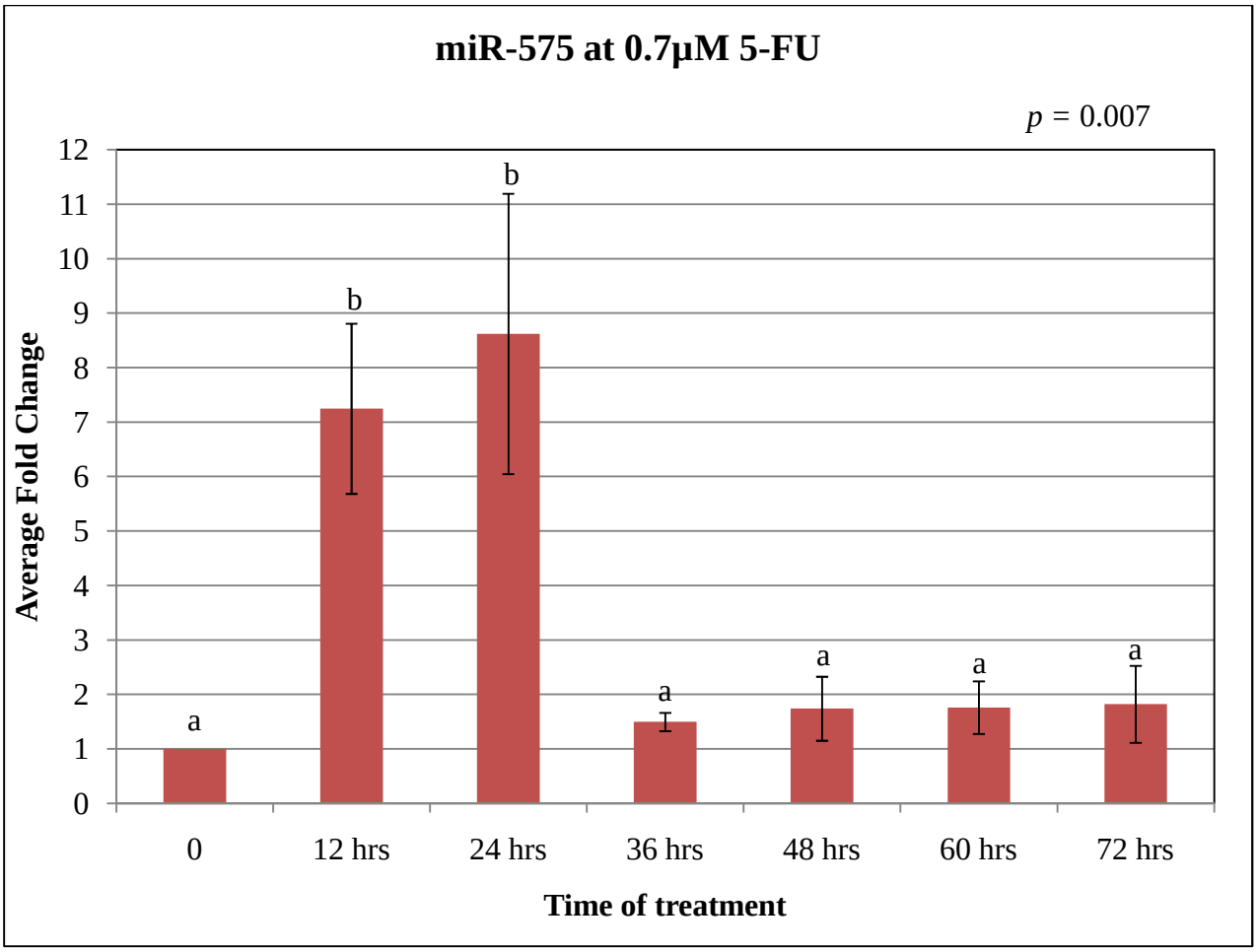

Figure 4.25: Average fold change in expression of miR-575 after treatment with 0.7µM of 5-FU for 12, 24, 36, 48, 60 and 72 hours. The results are represented as mean ± SEM. Statistically significant changes in the expression between concentrations are designated by single letters on error bars ($p \leq 0.05$).

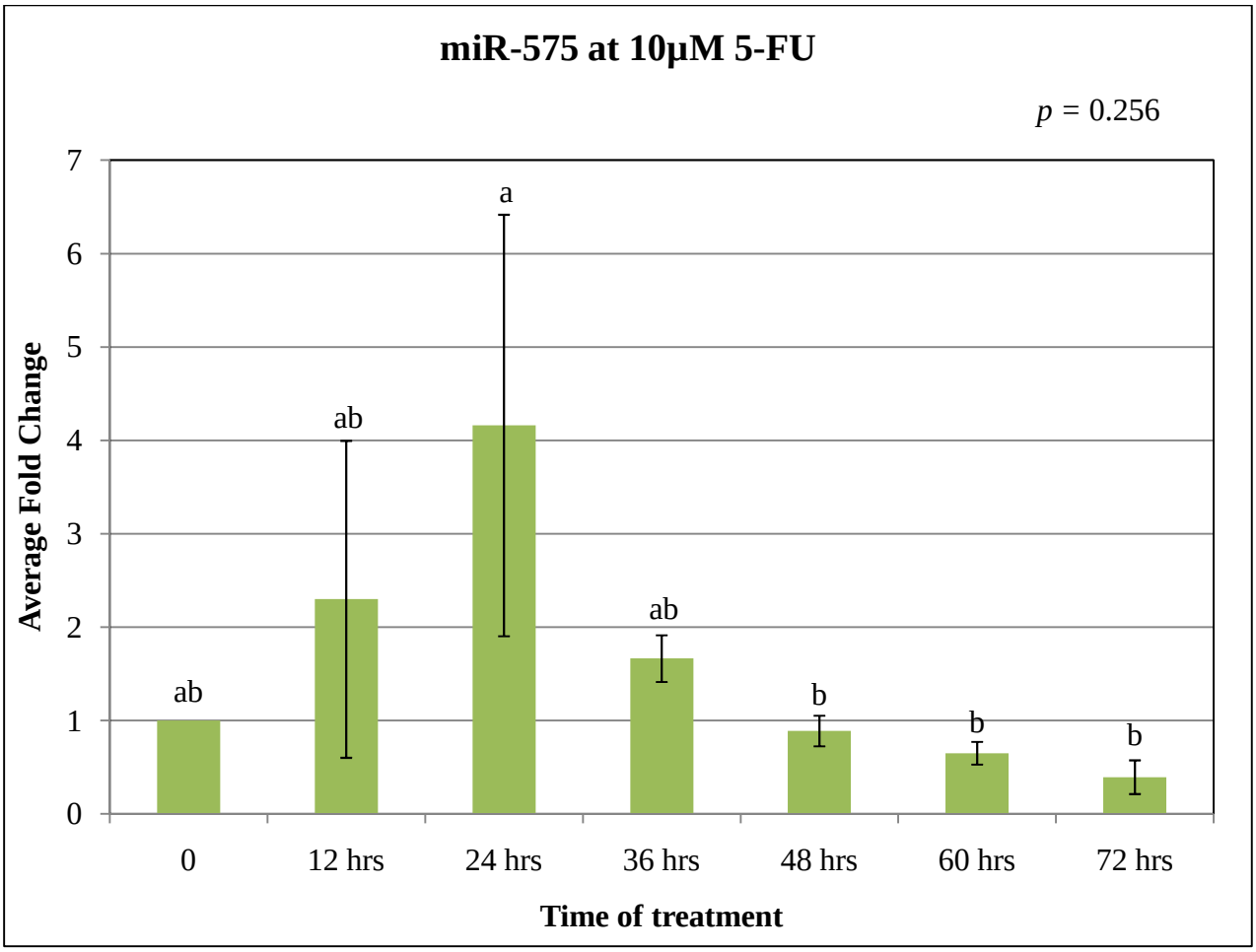

Figure 4.26: Average fold change in expression of miR-575 after treatment with 10µM of 5-FU for 12, 24, 36, 48, 60 and 72 hours. The results are represented as mean ± SEM. Statistically significant changes in the expression between concentrations are designated by single letters on error bars ($p \leq 0.05$).