Abstract

Effect of green tea Polyphenon 60 on microRNA expression in MCF-7 breast cancer cells

by Lindsey Nicole Fix

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Director: Dr. Baohong Zhang

DEPARTMENT OF BIOLOGY

In Western countries, breast cancer is the most common form of cancer and it is the second leading cause of female cancer death in America. The biochemical basis of the observed anti-oncogenic properties of green tea has been linked to the cellular effects of polyphenols found in the *Camellia sinensis* leaf. In this study we obtained dose-response data for the cellular effects of Polyphenon 60 green tea extract on MCF-7 breast cancer cells. Growth inhibition and cell death was observed after 48 hours at concentrations as low as 6 μg/ml (IC10). Results indicate that Polyphenon 60 induces a reduction in cellular metabolism on a similar dose-response starting at a concentration of 10 μg/ml. Cell cycle progression was unaffected by treatment with this tea extract and the method of cell death remained consistent across the treatment concentrations as analyzed by acridine orange/ethidium bromide fluorescence analysis. The specific regulatory mechanism behind how polyphenols affect gene expression and cancer cell survival has yet to be identified but current data suggests that small, non-protein coding microRNAs (miRNAs) may be responsible. In the present study, the effect of Polyphenon 60 green tea extract on MCF-7 breast cancer cell miRNA expression was investigated. Twenty-three miRNAs were identified by microarray analysis with differential expression after a 48 hour treatment of 10 μg/ml Polyphenon 60. This study further investigated the relationship between Polyphenon 60 dosage and miRNA responsiveness using let-7g, let-7e, miR-21, miR-26b,

miR-27a, miR-92a, miR-548m, and miR-720, eight of the previously identified microarray miRNAs of interest. Additionally, miR-10b, miR-15a, miR-16, and miR-200c were investigated based on previous reports of their connection to breast cancer. Several miRNAs showed a change in expression at concentrations as low as 1 μg/ml and did not fluctuate significantly even at the IC80 value of 15 μg/ml. Time-dependent data was also generated for miR-27a, miR-92a, miR-200c, and miR-720 following 24, 48, and 72 hour treatment with low and high dose Polyphenon 60 treatment. At low-doses and short treatment times, miR-720 and miR-200c were up-regulated and directly reversed their typical breast cancer expression. miR-27a, an oncomir, was down-regulated. The subtle changes that low-dose green tea extract can induce in the miRNA expression profile of MCF-7 cells may help substantiate the previous epidemiological claims that green tea can influence breast cancer pathogenesis.

Effect of green tea Polyphenon 60 on microRNA expression in MCF-7 breast cancer cells

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Lindsey Nicole Fix

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EFFECT OF GREEN TEA POLYPHENON 60 ON MICRORNA EXPRESSION IN MCF-7 BREAST CANCER CELLS

by

Lindsey Nicole Fix

APPROVED BY:

DIRECTOR OF THESIS:_____

Baohong Zhang, Ph.D

COMMITTEE MEMBER:_____

Mary Farwell, Ph.D

COMMITTEE MEMBER:_____

Jean-Luc Scemama, Ph. D

COMMITTEE MEMBER:_____

Ping Gong, Ph. D

CHAIR OF THE DEPARTMENT OF BIOLOGY:

_____

Jeff McKinnon, Ph. D

DEAN OF THE GRADUATE SCHOOL:

_____

Paul J. Gemperline, PhD

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4T1	Syngeneic breast cancer model derived from a BALB/c mammary tumor
AKT	v-akt murine thymoma viral oncogene homolog 1
ANOVA	Analysis of Variance
AO	Acridine Orange
BCL2	B-cell CLL/lymphoma 2
BMI1	BMI1 polycomb ring finger oncogene
BRCA1	Breast cancer type 1 susceptibility protein
Cdk	Cyclin Dependent Kinase
cDNA	Complementary DNA
c-fos	V-fos FBJ murine osteosarcoma viral oncogene homolog
C-MYC	V-myc myelocytomatosis viral oncogene homolog
CO_2	Carbon Dioxide
Cy3/5	Cyanine dyes
DA	Dead Apoptotic
DN	Dead Normal
dNTP	Deoxyribonucleotide triphosphate
EB	Ethidium Bromide
EC	Epicatechin
ECG	Epicatechin Gallate
EDTA	Ethylenediaminetetraacetic Acid
EGC	Epigallocatechin
EGCG	Epigallocatechin Gallate
ER	Estrogen Receptor
ERK2	Mitogen-Activated Protein Kinase 1
FAS	Fatty Acid Synthase
FOXO1	Forkhead box O1
G1	Gap 1 of Cell Cycle
G2	Gap 2 of Cell Cycle
GC	Gallocatechin

HER2/neu	Human Epidermal Growth Factor Receptor 2
HNF1α	Hepatocyte Nuclear Factor 1 alpha
HT29	Human colon adenocarcinoma grade II cell line
IC	Inhibitory Concentration
iNOS	Inducible Nitric Oxide Synthase
JNK1	C-Jun N-terminal kinase 1
KRAS	V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
LA	Live Apoptotic
LN	Live Normal
LOWESS	Locally Weighted Scatterplot Smoothing
LSD	Least Significant Difference
MAPK	Mitogen-Activated Protein Kinase
MCF-7	Human Breast Adenocarcinoma cell line
MDA-MB-231	Estrogen-independent, highly metastatic human breast cancer cell line
miRNA	MicroRNA
miRNA*	MicroRNA complementary sequence
MMP-9	Matrix Metalloprotease 9
mRNA	Messenger RNA
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Myt-1	Myelin transcription factor 1
NF-κβ	Nuclear Factor Kappa B
NRAS	Neuroblastoma RAS viral oncogene homolog
p53	Tumor protein 53
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PR	Progesterone Receptor
Pri-miRNA	Primary microRNA
PTEN	Phosphatase and Tensin homolog
qRT-PCR	Quantitative Real Time Polymerase Chain Reaction
RAS	GTPase involved in signal transduction
Rb	Retinoblastoma tumor suppressor protein

RISC	RNA-induced Silencing Complex
RNA	Ribonucleic acid
RNU48	Endogenous reference gene
RPMI	Cell Culture Media
RT	Reverse Transcriptase
S	Synthesis phase of Cell Cycle
UNG	Uracil N-glycosylase
TUBB3	Tubulin beta-3 chain
U937	Human Leukemic Monocyte Lymphoma cell line
UTR	Untranslated Region
VEGFR	Vascular Endothelial Growth Factor Receptor
ZBTB10	Zinc finger and BTB domain containing 10
ZEB1/2	Zinc finger E-box binding homeobox 2

CHAPTER 1: Introduction

MicroRNAs (miRNAs) are small, endogenous, single-stranded RNA molecules that regulate gene expression and have been implicated in a variety of disease states (Calin and Croce 2006). For years, miRNAs were ignored in scientific research due to their non-protein coding nature, but identification of their importance in developmental processes and carcinogenesis has promoted further research into understanding these small gene regulators (Rossi, Bonmassar et al. 2007). Investigation has shown that miRNA expression is tissue-specific and tumor-specific (Blenkiron, Goldstein et al. 2007), giving potential to the identification of miRNAs as a diagnostic tool (Calin and Croce 2006).

In 1993, Victor Ambrose and his research team discovered miRNAs based on their involvement in regulating temporal gene expression during *Caenorhabditis elegans* development (Lee, Feinbaum et al. 1993). The first miRNA identified was *lin-4* which was found to regulate the messenger RNA (mRNA) transcript *lin-14* (Lee, Feinbaum et al. 1993). *lin-4* was shown to possess near-perfect complementarity to a target sequence in the 3' untranslated region of the target mRNA (Johnson, Grosshans et al. 2005). The binding of the miRNA to its target caused a decrease in mRNA translation and promoted the development of the hypothesis that miRNAs could act as gene regulators. Since their discovery, approximately 721 miRNA genes have been identified and located in the human genome, each with the potential for binding several mRNA transcripts (miRBase 14.0, Sanger Institute, 2009).

miRNA biosynthesis involves a variety of molecular processing mechanisms and molecules (Figure 1-1). Transcription of a miRNA gene by RNA polymerase II yields a primary miRNA (pri-miRNA) transcript several hundred nucleotides in length (Lee, Kim et al. 2004). This pri-miRNA forms a hairpin stem-loop structure that associates with a

Drosha-DGCR8/Pasha endonuclease complex that cleaves the stem-loop into a 60-70 nucleotide pre-miRNA (Garzon, Fabbri et al. 2006). The pre-miRNA is then transported from the nucleus to the cytoplasm via the Exportin-5 protein. While in the cytoplasm, the pre-miRNA can be processed by the Dicer complex to form a miRNA:miRNA* double-stranded duplex (Tsuchiya, Okuno et al. 2006). The miRNA:miRNA* complex is further modified by helicases and the mature single stranded miRNA joins with an RNA-induced silencing complex (RISC). The remaining miRNA* is degraded in the cytoplasm by an unknown mechanism and the miRNA-RISC complex can function to regulate gene expression (Gregory, Chendrimada et al. 2005).

miRNAs regulate gene expression by binding to the 3' untranslated region (UTR) of a specific mRNA transcript (Sempere, Christensen et al. 2007). Binding of a miRNA to its target mRNA results in either degradation of the transcript or inhibition of protein translation, both resulting in a decrease in gene expression (Si, Zhu et al. 2007). The regulatory mechanism of degradation versus translational repression depends on the complementarity of the 3' UTR of the transcript to its corresponding miRNA (Calin and Croce 2006). A near-perfect match is understood to promote mRNA cleavage, whereas imperfect binding causes interference of the translational machinery without transcript degradation.

Following further analysis of miRNA gene regulation, it was discovered that miRNAs can act as both oncogenes and tumor suppressors based on the identity of the target mRNA transcript that is down-regulated (Figure 1-2) (Zhang, Pan et al. 2007). A variety of cancer-specific miRNAs have been identified, and their presence or absence in tumor samples may lead to improved diagnostic techniques as the technology expands. In the case of breast cancer, a multitude of miRNAs have been investigated but variations due to the site of origin,

level of malignancy, or drug-resistance have generated very specific data implicating miRNAs in various aspects of carcinogenesis (Blenkiron, Goldstein et al. 2007). Tamoxifen-resistant breast cancer samples have been shown to over-express miR-221, miR-222 and miR-181 and possess decreased expression of miR-21, miR-342 and miR-489 (Miller, Ghoshal et al. 2008). Primary breast carcinomas have displayed a positive correlation between the level of miR-10b expression in cells and classification of metastatic or invasive behavior (Ma, Teruya-Feldstein et al. 2007). Xin and colleagues (2009) have also documented that let-7, miR-212, miR-181, and miR-191 were correlated with breast cancer cases resulting in poor prognoses. Additionally, Xin and colleagues identified consistent down-regulation of several miRNAs (miR-125b, miR-145, miR-21, miR-155, and let-7i) in a multitude of breast cancer samples (Xin, Small et al. 2009). Alternatively, miR-106b, miR-93 and miR-25 were observed as being highly expressed in high-grade advanced breast tumors (Blenkiron, Goldstein et al. 2007). The miR-30 family has been found to be down-regulated in estrogen and progesterone receptor negative cells, and decreases in miR-9-3 have been linked to increased vascular invasion and lymph node metastasis (Iorio, Ferracin et al. 2005). The accumulation of such miRNA data will lead to further understanding of the development of breast cancer and should give insight into the cellular effects of chemotherapeutics and treatments.

The specific miRNA expression profile of MCF-7 breast cancer cells has not currently been standardized but a variety of miRNAs have been shown to be expressed in the cell line. It is observed that miR-21, miR-9 and miR-27b are over-expressed in MCF-7 cells (Tsuchiya, Nakajima et al. 2006; Ma, Teruya-Feldstein et al. 2007; Si, Zhu et al. 2007; Frankel, Christoffersen et al. 2008). Microarray work performed by Sempere et al. also indicates a high expression of miR-200, miR-141, let-7, miR-92 and miR-29 as well as moderate expression of

miR-21, miR-191 and miR-183. Additional work has examined how drug-resistance affects the miRNA expression of MCF-7 cells. Tamoxifen-resistant MCF-7 cells have been shown to over-express miR-221, miR-222, miR-181, miR-375, miR-32, miR-171, miR-213, and miR-203 and consistently under-express miR-21, miR-342, miR-489, miR-24, miR-27, miR-23, and miR-200 (Miller, Ghoshal et al. 2008). Doxorubicin resistance has been shown to cause similar results, such as an increase in miR-206, miR-106a, miR-214 and a decrease in miR-10, miR-21, miR-155, and miR-200c (Kovalchuk 2008). Fulvestrant resistance has also been analyzed and down-regulation of let-7i, miR-181a, miR-191, miR-199b, miR-204, miR-211, miR-212, miR-216, miR-328, miR-346, miR-424, miR-638, and miR-768-3p was observed, along with up-regulation of miR-222 and miR-221 (Xin, Small et al. 2009).

The National Cancer Institute predicted that during the 2009 calendar year, 192,370 women in the United States were diagnosed with breast cancer, and 40,170 women succumbed to the disease. In Western countries, breast cancer is the most common form of cancer and the second leading cause of female cancer death in America. In Eastern countries, such as Japan and China, the incidence and aggressiveness of breast cancer is much lower. In 1998, it was said that 1 in 10 women in the United States would develop breast cancer during their lifetimes, but only 1 in 40 women in Japan would develop this disease (Nakachi, Suemasu et al. 1998). Numerous epidemiological studies have been performed in an attempt to discover what may be the cause of this unequal distribution. Several lifestyle differences have been identified as potential causes for this phenomenon, in particular, the consumption of green tea. It has been estimated that 98% of the Japanese population drinks green tea regularly (Sun CL 2006). Green tea is known to be high in anti-oxidants and other pharmacologically active substances commonly used in homeopathic or alternative medicine (Figure 1-3). On average, polyphenols have been attributed with most of

the anti-cancer abilities that are credited to green tea, but the cellular mechanisms behind why these compounds are so beneficial have yet to be determined.

As developments are being made in the field of miRNA research, an increasing amount of large scale molecular effects are being attributed to miRNA regulation. miRNAs have already been shown to act as tumor suppressors by negatively regulating gene expression of oncogenic factors. Alternatively, miRNAs that down-regulate expression of tumor suppressor genes have been named oncomirs, and have been identified in a variety of cancer types (Zhang, Pan et al. 2007). Through analysis of miRNA expression, it can be shown that gene regulation is a highly sensitive procedure and the slightest change in the expression of these small non-coding RNAs can disrupt critical cellular functioning.

Green tea has been shown to decrease breast cancer cell estrogen receptor expression, increase cellular sensitivity to specific chemotherapies and initiate natural cell death (Farabegoli, Barbi et al. 2007). Both estrogen receptor down-regulation and chemotherapy sensitization are of interest, as most cancer patients will develop resistance to at least one therapeutic drug during their course of treatment. The ability to initiate apoptosis in cancer cells with green tea leads to many questions regarding cellular mechanism. If the cytotoxic effect of green tea can be linked to a specific miRNA, it is conceivable that treatments targeting the over-expressed miRNA can be developed. It has already been shown *in vitro*, that transfection of cancer cells with specific over-expressing miRNA plasmids can cause specific cellular responses without the widespread systemic effects caused by chemotherapy. The potential impact of these miRNA specific therapies makes miRNA research applicable to a wide variety of medical fields, not limited to cancer research. As more data becomes available, and new procedures and techniques are

developed, miRNA should lead to new and exciting advances in diagnostic medicine and disease treatment.

Previous studies demonstrate that green tea affects cancer cell growth and influences protein-coding gene expression (Komori, 1993; Valcic, 1996; Yeh, 2003; Farabegoli, 2007). However, the regulatory mechanism governing this gene expression is largely unknown. Recently discovered miRNAs are an essential regulatory component in many biological and metabolic processes, including response to chemical exposure. Here, I hypothesize that ***the green tea extract, Polyphenon 60, induces the aberrant expression of a set of miRNAs which regulate MCF-7 cellular response to chemical treatment***.

To test this hypothesis, this study focuses on explaining the effects that green tea polyphenols have on MCF-7 breast cancer cell miRNA expression with the following specific objectives:

1. **The effect of green tea Polyphenon 60 treatment on MCF-7 breast cancer cells (Chapter 2)**

In this specific objective, I have determined an accurate dose-response cell viability curve for Polyphenon 60 treatment of MCF-7 breast cancer cells and investigated the effect of Polyphenon 60 treatment on cell cycle progression.

2. **Identification of miRNAs that are differentially expressed between Polyphenon 60 treated and untreated cells (Chapter 3)**

To accomplish this objective, a miRNA microarray experiment was performed to analyze differential miRNA expression between Polyphenon 60 treated and untreated MCF-7 cells. The

results of the microarray experiment were verified by qRT-PCR analysis utilizing probes designed for the most significantly up- or down-regulated miRNAs identified by the array.

3. **Dose-response and time-dependence of the miRNA expression response to Polyphenon 60 treatment (Chapter 4)**

In this study, the effect of Polyphenon 60 on selected miRNAs was investigated in a time- and dose-dependent manner.

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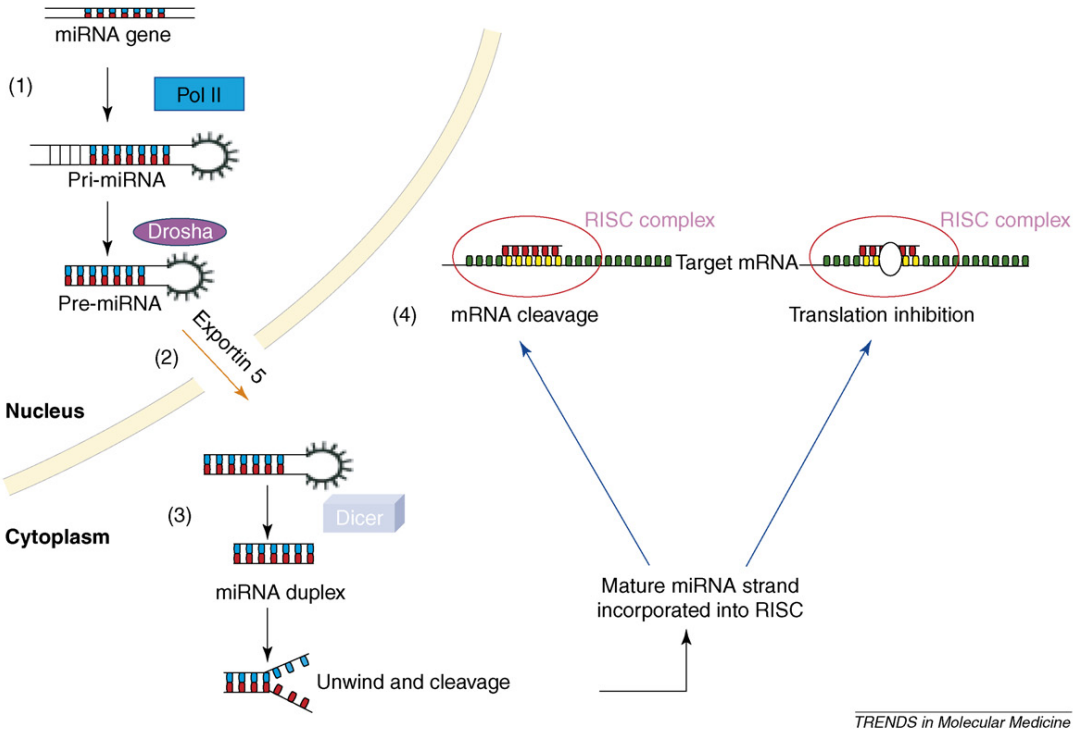

Figure 1-1: miRNA Biosynthetic Pathway (Garzon, Fabbri et al. 2006)

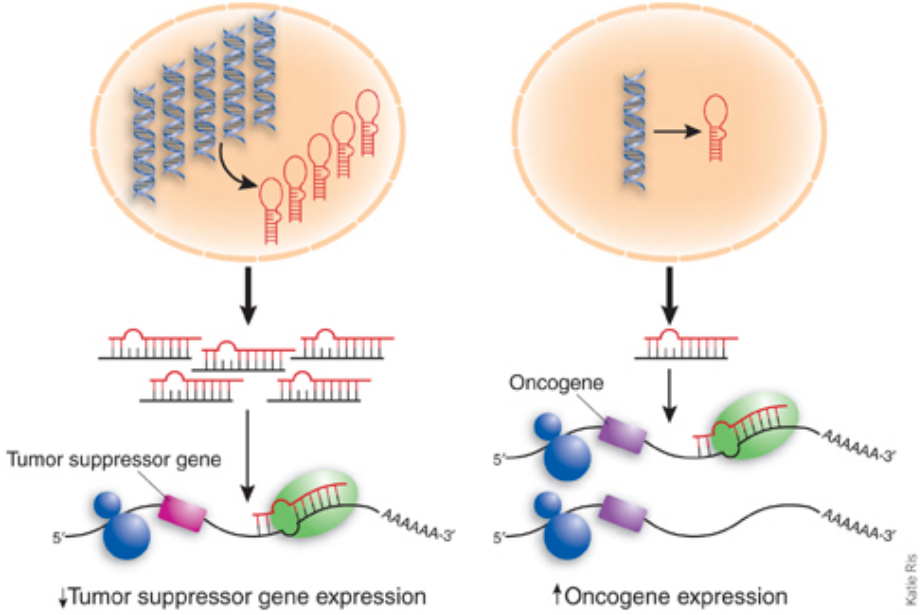

Figure 1-2: miRNA regulation of oncogenes and tumor suppressor genes (Caldas C 2005)

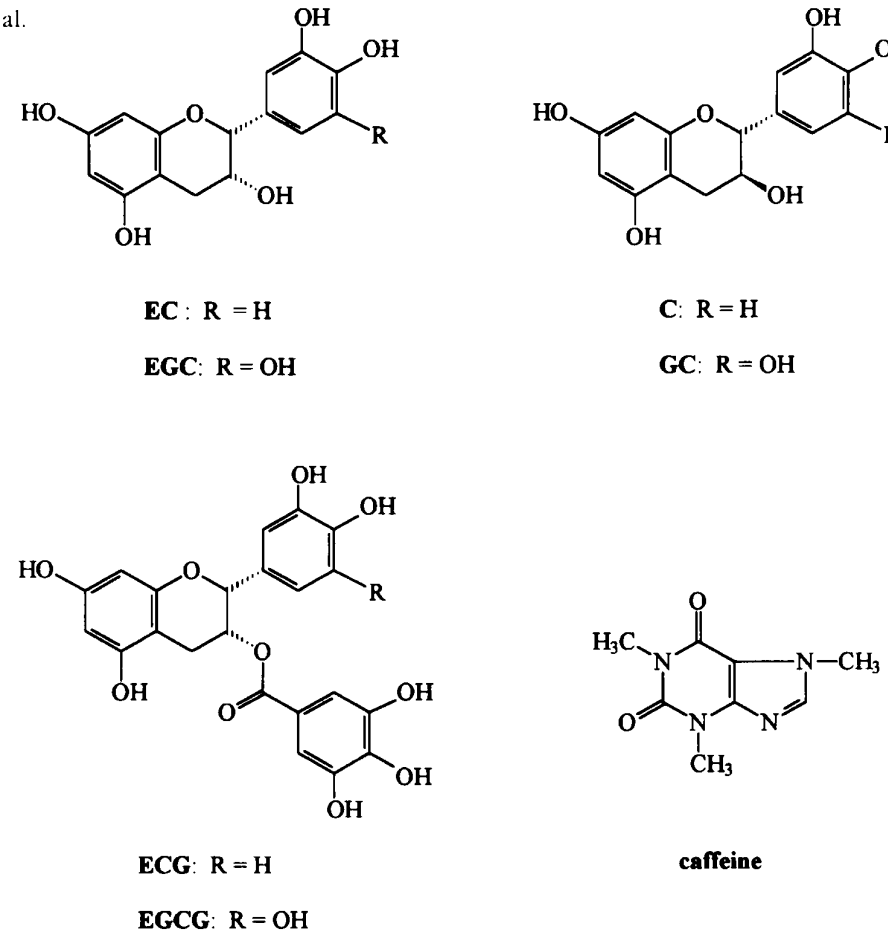

Figure 1-3: Chemical structure of green tea catechins (Valcic S 1996)

CHAPTER 2: Effect of Green tea Polyphenon 60 on the Growth of the MCF-7 Breast Cancer Cell Line

Abstract:

The beneficial effects of green tea polyphenols on limiting the progression of breast cancer development leads to the potential for the development of an all-natural therapeutic option. Here we present dose-response data for the cellular effects of Polyphenon 60 green tea extract on MCF-7 breast cancer cells. Growth inhibition and cell death was observed after 48 hours at concentrations as low as 6 μg/ml (IC10). Results indicate that Polyphenon 60 induces a reduction in cellular metabolism on a similar dose-response starting at a concentration of 10 μg/ml. Cell cycle progression was unaffected by treatment with this representative tea extract and the mechanism of cell death was consistently apoptosis as analyzed by acridine orange/ethidium bromide fluorescence analysis. These findings suggest a method of toxicity unrelated to disruptions of natural cell death, which may be of increased importance specifically in breast cancers lacking normal apoptotic pathways.

Keywords: Green Tea, Polyphenols, MCF-7, miRNA, Breast Cancer, Cell Cycle, MTT, Ethidium Bromide/Acridine Orange

Introduction:

Previous epidemiological studies have established a strong correlation between green tea consumption and a decreased risk of developing breast cancer. The polyphenols found in the leaves of the green tea plant, *Camellia sinensis*, are thought to be the biochemical compounds that contain the observed anti-oxidant and anti-oncogenic properties. The major polyphenols found in green tea are epigallocatechin gallate (EGCG), epicatechin gallate (ECG),

epigallocatechin (EGC), epicatechin (EC), gallocatechin (GC) and catechin (Figure 1-3) (Valcic S 1996). The aromatic phenol rings present in these molecules cause them to behave as antioxidants by reducing free radicals and chelating metals in the body (Vergote D 2002). EGCG has been found to be the most abundant and most biologically active of the catechins present in green tea. Studies have shown that EGCG can induce apoptosis, cell cycle arrest, inhibition of angiogenesis and down-regulation of telomerase expression in various cancer cells both *in vitro* and *in vivo* (Sun, Estrov et al. 2008). EGCG-induced apoptosis has been linked to production of hydrogen peroxide and inhibition of the NF-κβ transcription factor from binding to the inducible nitric oxide synthase (iNOS) promoter (Liang YC 1999; Vergote D 2002).

Research surrounding the effects of green tea on breast cancer has focused primarily on large scale cellular effects. MCF-7 breast adenocarcinoma cells have been utilized as a standard estrogen-dependent breast cancer model for *in vitro* studies. Green tea extracts and EGCG have both been shown to inhibit MCF-7 cell growth (Komori 1993; Valcic S 1996; Liang YC 1999; Vergote D 2002). The potency for the various catechins has been ranked from greatest growth inhibitor to weakest as follows: EGCG > GC > EGC > ECG > EC > catechin (Valcic S 1996). EGCG has been implicated in down-regulating the estrogen receptor alpha (ERα) pathway and interfering with estrogen binding to its receptor, which acts to inhibit the growth signal (Komori 1993; Farabegoli, Barbi et al. 2007).

EGCG has also been identified as inhibiting proper progression of MCF-7 cells through the cell cycle by blocking cells at the G1 phase (Liang YC 1999). This inhibition was linked to hypophosphorylation of the retinoblastoma tumor suppressor protein (Rb), inhibition of cyclin-dependent kinase 2 (Cdk2) and 4 (Cdk4), and induction of the Cdk inhibitors p21 and p27 (Liang YC 1999). EGC, on the other hand, inhibits cell growth without interfering with the cell

cycle, independent of the p53 pathway (Vergote D 2002). Epicatechin has been shown to possess no ability to inhibit cell growth (Vergote D 2002). EGCG has also been shown to suppress expression of fatty acid synthase (FAS) which causes a decrease in incorporation of 14C-acetyl-coA into triglycerides, fatty acids and cholesterol necessary for cell survival (Yeh 2003).

EGCG has also been shown to bind the cell surface receptor Fas as an alternative ligand, capable of initiating the caspase cascade responsible for inducing apoptosis (Hayakawa S. 2001). Additionally, EGCG can regulate key molecules in the mitogen-activated protein kinase (MAPK) pathway to prevent cell proliferation. Gupta et al. exhibited that EGCG targets nuclear factor kappa B (NF-κβ) for caspase cleavage (Gupta S 2004). Green tea polyphenols have also been shown to increase transcription of *c-jun and c-fos,* components of the ERK2 and JNK1 classes of the MAPK family (Yu, Yao et al. 2007).

The ability of a natural extract to limit cancer cell division and tissue invasion is of great interest in the field of oncology. In this study, Polyphenon 60 was used in place of a specific polyphenol compound to more closely simulate the effects of green tea obtained from the diet. Polyphenon 60 contains sixty percent of the catechins found in green tea, preserving their natural abundance and concentration proportions. The data presented here should help direct further investigations using Polyphenon 60 in cell culture and clinically.

Materials and Methods

Cell lines

The human MCF-7 breast adenocarcinoma cell line was obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). The cells were grown in RPMI 1640 media

supplemented with 10% Fetal Bovine Serum, 0.1% gentamycin, and 0.1% bovine insulin (Gibco, Grand Island, NY) and sterilized using a 0.22 µm polyethersulfone filter (Corning Inc., Corning, NY). Cells were grown at 37°C in a 5% CO_2 humidified incubator.

Preparation of Polyphenon 60 treatment

Polyphenon 60 was purchased from Sigma (St. Louis, MO, USA) and dissolved in supplemented RPMI 1640 media prior to each cell treatment. The Polyphenon 60 solution was sterilized using a 0.22 µm polyethersulfone filter. Appropriate dilutions were made from an initial stock concentration of 1000 µg/ml.

Cell dose-response treatment

Cells were plated in 12-well plates at a seeding density of 10,000 cells/cm^2 to a total volume of 1 ml per well. Treatments were made from dilutions of a 1000 mg/L stock made fresh prior to each treatment. The stock was filtered through a 0.22 µm polyethersulfone filter prior to additional dilution. After seeding for 24 hours, cells were drained of media and treated with 1 ml of RPMI 1640 media with various Polyphenon 60 concentrations. After 48 hours, the treated plates were drained of media, washed with 2.5 ml cold Phosphate Buffered Saline (PBS) then incubated at 37°C for 5 minutes in 750 µl of 2X concentrated Trypsin/EDTA. The cells were manually agitated and detached from the well using 1 ml of RPMI complete cell media. The cells were centrifuged for 5 minutes at 3000 rpm and the pellet was resuspended in 100 µl of RPMI media. 50 µl of the cell solution was added to 50 µl of 0.4% trypan blue dye. A 10 µl sample was removed and live cells were counted using a Hausser Scientific Hy lite 0.1 mm Hemacytometer. Cells that stained blue based on uptake of trypan dye were presumed to be dead and were not included in the live cell counts. Dose-response effects on metabolism were calculated using

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) analysis (see below "Analysis of Cellular Metabolism"). The dose-response curve for cell viability was generated and the inhibitory concentrations (ICx) were calculated. Each treatment was repeated six times.

Method of cell death analysis

The method of cell death was analyzed following 48 hour treatment with Polyphenon 60 using the Acridine Orange/Ethidium Bromide (AO/EB) assay. For the AO/EB assay, 50 µl of a 1 µg/ml mixture of acridine orange and ethidium bromide in PBS was added to 50 µl of trypsinized and detached cells grown in a specific concentration of treatment. At least 100 cells from the sample were then counted using a fluorescence microscope. Live cells appeared green, dead cells had orange nuclei, and non-nucleated cell blebs were unstained or had a faint orange color. Cells were classified as live normal, live apoptotic, dead normal and dead apoptotic. All samples were done in triplicate.

Analysis of Cellular Metabolism

For the MTT cellular metabolism assay, cells were plated at 5000 cells per well in a 96 well plate for a total volume of 100 µl per well. Cells were allowed to attach for 24 hours then media was removed and 100 µl of Polyphenon 60 treatment was added. Plates were treated for 48 hours at 37°C. The wells were then drained of treatment and 20 µl of 5 mg/ml MTT in PBS was added along with 100 µl of RPMI complete media without phenol red. Cells were returned to 37ºC for 3 hours. Following incubation, the MTT was removed and 100 µl of dimethyl sulfoxide (DMSO) was added to each well and shaken for 15 minutes at room temperature. The plates were read at 540 nm using a ThermoScientific multiscan MCC/340 plate reader with

accompanying Ascent Software version 2.6. Treatments were done in 4-8 replicate wells per plate. Percent viability was calculated as follows:

% metabolism = (mean absorbance of the sample / mean absorbance of the control) X 100

Cell cycle progression

For cell cycle analysis, cells were synchronized by growth factor deprivation for 5 days then treated for 48 hours with various Polyphenon 60 concentrations and analyzed using a FACScan Flow Cytometer. A 75 cm^2 flask of confluent cells was trypsinized and centrifuged at 200 x g for 5 minutes. The pellet was washed with 1 ml of PBS and resuspended in 1 ml of RPMI complete media. The cells were fixed by injection through a 23 gauge needle into cold 70% ethanol. Cells were incubated at -20ºC for 30 minutes or until all samples were collected. The cells were centrifuged and washed with 1 ml PBS. 10 µl of 1 mg/ml RNase was added and the cells were incubated at 37ºC for 30 minutes. Following incubation, the cells were resuspended in 1 ml of PBS with 10 µl of 1 mg/ml propidium iodide and left in the dark at room temperature for 30 minutes. The cells were stored at -20ºC prior to propidium iodide staining until analysis using the flow cytometer could be performed.

Results

MCF-7 Dose-response to Polyphenon 60 green tea extract

Following trypan blue dye exclusion analysis, a dose response curve was generated for inhibition of MCF-7 breast cancer cells with Polyphenon 60 green tea extract (Figure 2-1) Approximately linear responsiveness was seen for concentrations between 5 and 20 µg/ml with an insignificant increase in inhibition prior to 5 µg/ml and a plateau following 20 µg/ml. The

inhibitory concentration values were calculated as follows: IC80 = 15 μg/ml, IC50 = 10 μg/ml, IC20 = 7 μg/ml and IC10 = 6 μg/ml.

Effect of Polyphenon 60 on MCF-7 Apoptosis

To compare the rate of apoptosis between various treatment samples, acridine orange/ethidium bromide analysis was used. The results revealed no obvious differences in the mechanism of cell death between the 0, 10 and 20 μg/ml treatments (Figure 2-2). The 30 μg/ml treatment displayed a larger amount of the representative cell population as dead apoptotic but this would be expected at a concentration higher than IC90.

Dose Response of Polyphenon 60 on Cellular Metabolism

The activity of cellular metabolism following Polyphenon 60 treatment was analyzed using the MTT assay. The dose-response curve for metabolic activity did not directly correspond with the trypan blue cell viability assay. The treated MCF-7 cells retained normal metabolic function up to a 10 μg/ml treatment concentration. From 10 to 35 μg/ml a linear response curve was observed (Figure 2-3). Even at high Polyphenon 60 concentrations, exceeding the cell viability IC80, cellular metabolism failed to reach 100% inhibition. All percentages were calculated by comparing treatment values to the control untreated samples.

Cell Cycle Progression following Polyphenon 60 treatment

Flow cytometry analysis of cell cycle progression under concentrations of 0, 5, 10, 15, and 20 μg/ml Polyphenon 60 treatment showed no significant differences between the five concentrations (Figure 2-4). Consistently, about 60% of the pre-synchronized, treated cell samples were in G1, 30% were in S and the remaining 10% were in G2.

Discussion

MCF-7 dose-response to Polyphenon 60 treatment.

Previous studies have focused on investigating the biochemical effects of specific green tea catechins, such as EGCG, on a variety of cancer models. Using high concentrations of isolated compounds does not adequately simulate achievable plasma levels or indicate natural biodegradation of the treatment product. Tea polyphenols have been shown to have very low bioavailability, in part due to the abundance of hydroxyl groups participating in hydrogen bonding and partially due to the high molecular weight of catechins (Lambert JD 2003). Previous studies show that an oral intake of 20 mg of green tea per kilogram of body weight leads to maximum plasma concentrations of 223 μg/L of EGC, 124 μg/L of EC and 77.9 μg/L of EGCG (Lee 2002). Such finding suggest the need for a purified extract, like Polyphenon 60, to be used for medicinal purposes instead of relying on the polyphenol content obtained from brewed green tea. It is estimated that one cup of green tea can only contain up to 200 mg of EGCG, whereas extracts can be concentrated to possess the required dosages for therapeutic efficiency.

Reports indicate that 77% of ingested EGCG stays in a non-biotransformed form, whereas most other catechins are rapidly methylated, glucuronidated, or sulfated (Lee 2002). This may explain why EGCG is commonly identified as the most bioreactive catechin in green tea, as it is able to resist rapid biodegradation and inactivation. However, it has been shown that EGCG has increased anti-oxidant activity when combined with the other plant polyphenols, suggesting that combinatorial extracts possess greater effects than isolated compounds (Bode 2009). Such results support the need for further research using biologically relevant extracts that

possess a representative sample of green tea catechins, in place of the previously used purified polyphenols.

One study showed that as little as 1 μg/ml of EGCG or the extract, Poly E, for 96 hours inhibited the growth of a variety of colon cancer cell lines but had no effect on normal colon cell cultures (Shimizu M 2005). Numerous biological mechanisms have been implicated in the ability of green tea to restrict cancer cell growth. EGCG has been shown to inhibit the reverse transcriptase, telomerase, limiting the amount of replications a cell can undergo in U937 or HT29 cell lines with an IC50 as low as 1 μM (Naasani I. 1998). Thangapazham and colleagues (2007) showed that green tea polyphenols can inhibit the AKT pathway and decrease MMP-9 expression, leading to apoptosis and decreased invasiveness in the MDA-MB-231 invasive breast cancer cell line (Thangapazham 2007). The extract presented here, Polyphenon 60, has previously been shown to down-regulate MMP-9 expression and inhibit cell shedding from murine mammacarcinoma cell line 4T1 tumor spheroids, limiting metastasis and invasiveness *in vivo* (Günther 2007).

In MCF-7 cells, several EGCG specific studies have been performed analyzing inhibition of cell growth. EGCG was shown to decrease cell migration and invasiveness after 24 hours at a concentration of 50 μg/ml (Zhang Y 2009). Another study saw a decrease in cell viability at the same dosage and time incubation but also indicated rapid growth inhibition at 30 μg/ml after 48 hours (Liang YC 1999). Although results vary, these reports support the relative range of the dose-response curve generated by trypan blue dye exclusion. The previous data was based on pure EGCG, and based on the concept that other catechins further enhance the effects of EGCG it can be reasoned that the dose response seen for Polyphenon 60 below 20 μg/ml can be expected.

Cellular analysis using MTT and AO/EB assays

Previous reports of MTT analysis of MCF-7 cells using polyphenols, give a wide variety of results. A study done by Farabegoli et al. showed a gradual decrease in cellular metabolism after 24 hours of EGCG concentrations ranging from 20 to 160 μg/ml (Farabegoli, Barbi et al. 2007). A separate study gave an EGCG and green tea extract IC50 value of 420 μg/ml (Komori 1993). Alterations in media and cell culture treatment conditions may be responsible for the variations in data, however, dose-response curves were evident in all reported data, including the results presented here. Induction of apoptosis by green tea polyphenols has been greatly documented but no dose-response changes to the apoptotic mechanism have been identified. This seems to support the findings that the method of cell death is apoptosis and is consistent across the dosages analyzed using acridine orange/ethidium bromide staining.

The results from the MTT and AO/EB assays do not contradict one another even though no changes in apoptosis were seen at the higher treatment concentrations. The MTT assay is a spectrophotometric color-based assay that shows metabolic activity throught the cells ability to reduce 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to formazan crystals which are soluble in DMSO and give a purple color. Therefore, it does not specifically differentiate between alive and dying cells. Although there is an obvious response curve shown, it merely identifies the effect of Polyphenon 60 treatment on general cell metabolism. The AO/EB assay looked more specifically at what stages of cell death adherent cells were in following Polyphenon 60 treatment. By only analyzing adherent cells, the data fails to incorporate cells that have already died or were damaged enough to detach. The AO/EB data that is presented here is only intended to show the effects of Polyphenon 60 on adherent cell progression to apoptosis.

Cell cycle analysis using flow cytometry.

Flow Cytometry was used to analyze the effects of Polyphenon 60 treatment on the progression of MCF-7 cells through the cell cycle. The results presented here suggest that minimal cell cycle interference is seen at concentrations lower than 20 μg/ml. Previous studies using green tea polyphenol compounds have presented similar data. Vergote et al. reported that 24 hour treatment of MCF-7 cells with 100 μM epigallocatechin (EGC) yielded no significant alterations in cell cycle (Vergote D 2002). Similar to reported results, approximately 60% of the treated and untreated cells were in G0/G1 phase. On average 35% of the cells were in S phase and 5% were in G2/M phase. This support may come from the fact that EGC is one of the many catechins that make up the Polyphenon 60 extract. Farabegoli et al. also presented supporting data indicating that MCF-7 cells treated with EGCG for 24 hours also lacked statistically significant differences in cell cycle distribution (Farabegoli, Barbi et al. 2007). Kavanagh et al. indirectly showed that EGCG potentially induces p27 expression which can promote G1/S arrest, however supporting data was not presented (Kavanagh 2001). Similar reports show that MDA-MB-231 breast cancer cells can be arrested at G1 by green tea polyphenol blends and by EGCG specifically (Thangapazham 2007). The reports seem to vary based on the cell line and concentration of treatment used. Further studies will have to be performed to adequately assess the role of cell cycle inhibition in the mechanism that green tea uses to prevent cancer cell growth.

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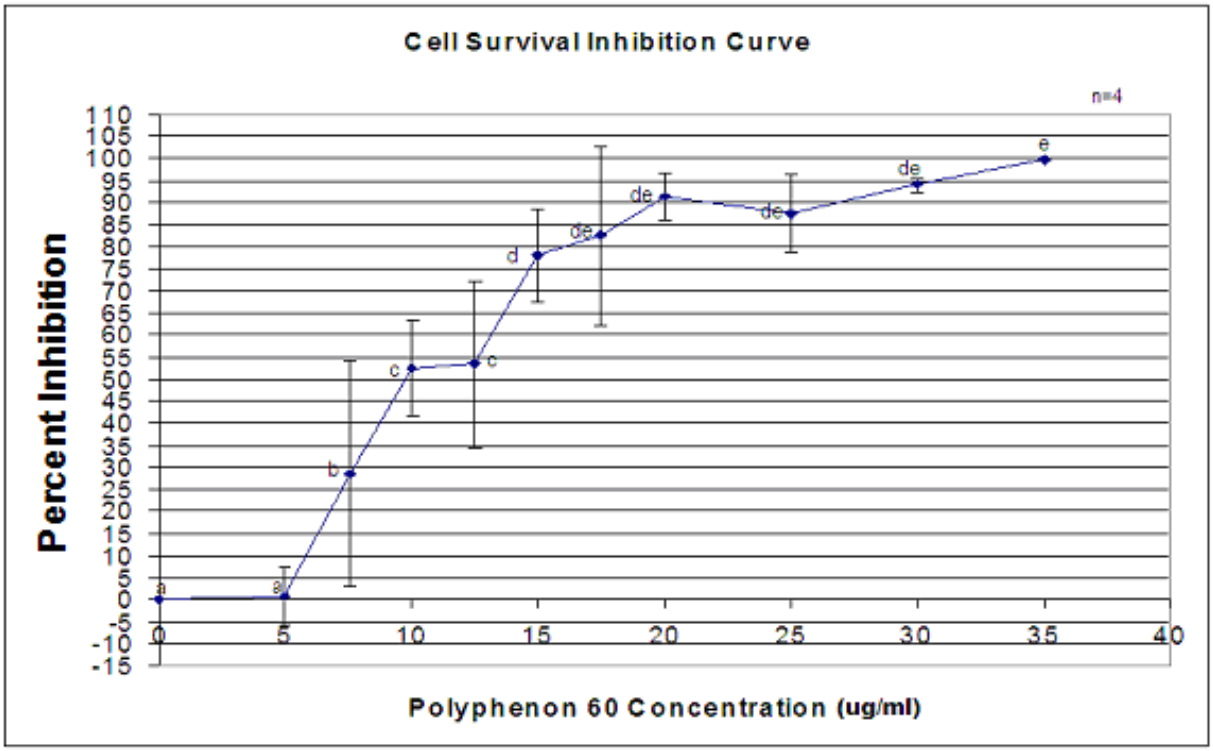

Figure 2-1: Dose-response curve for Polyphenon 60 treatment of MCF-7 breast cancer cells. Percent inhibition was calculated by comparing manual counts of viable cells using the trypan blue dye exclusion assay between control and treated samples. Statistically significant differences ($p < 0.05$) are indicated by single letters beside the standard error bars.

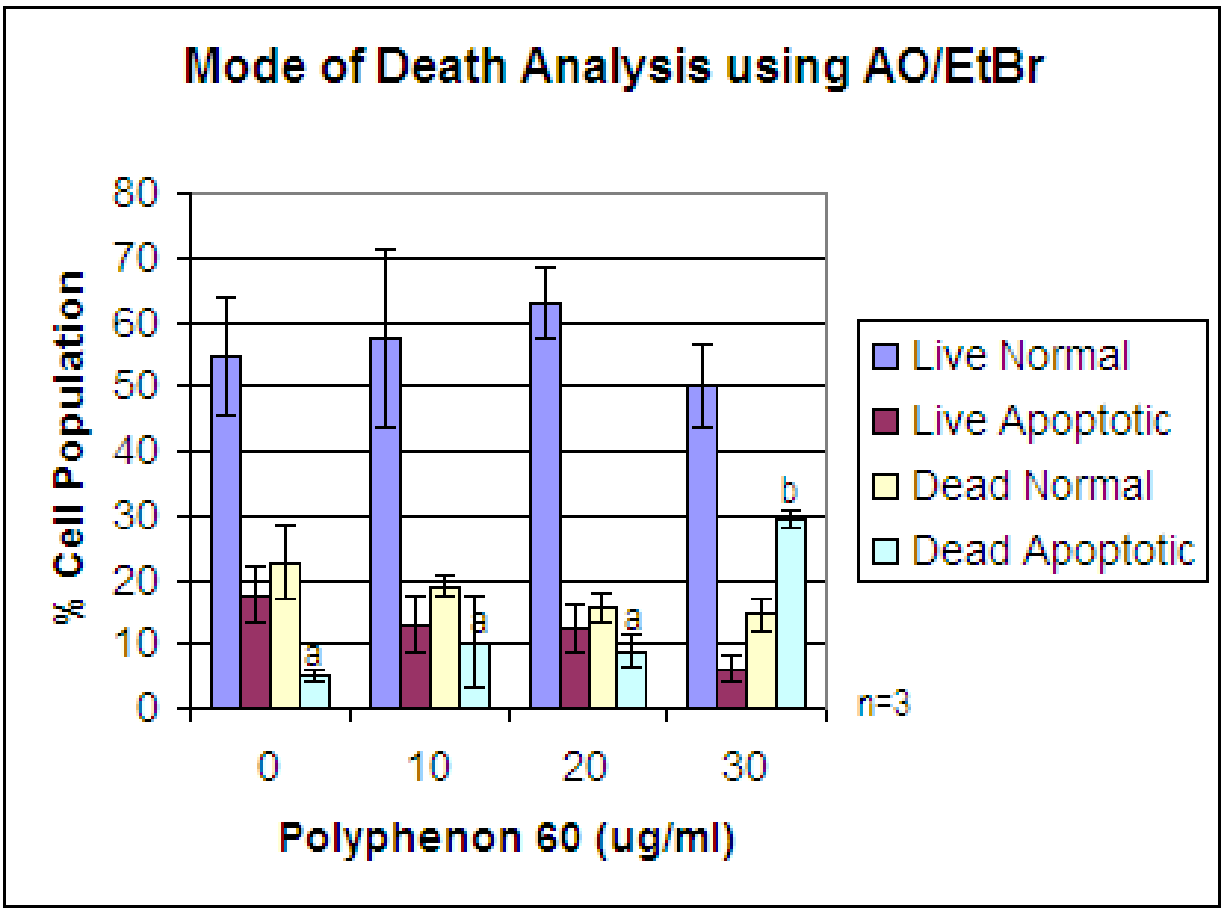

Figure 2-2: Acridine Orange/Ethidium Bromide Apoptosis analysis of MCF-7 cells treated with Polyphenon 60 for 48 hours. LN= live normal, LA= live apoptotic, DN= dead normal, DA= dead apoptotic. Standard error bars show statistically significant differences between the concentrations with single letter designations ($p < 0.05$).

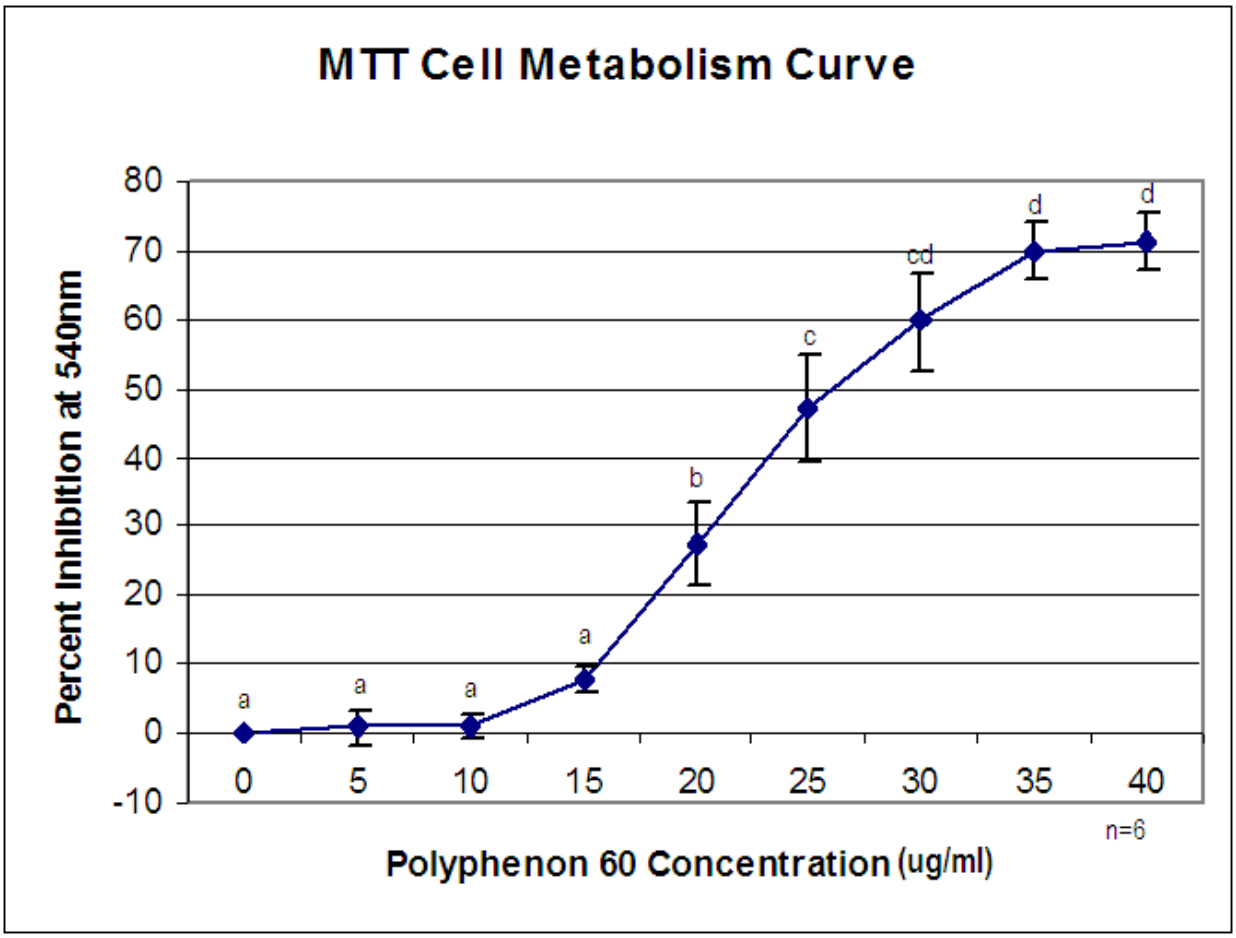

Figure 2-3: Analysis of MCF-7 metabolism following 48 hours of Polyphenon 60 treatment using the MTT assay. Error bars are shown with single letter designations showing statistically significant differences in percent inhibition of metabolism ($p < 0.05$).

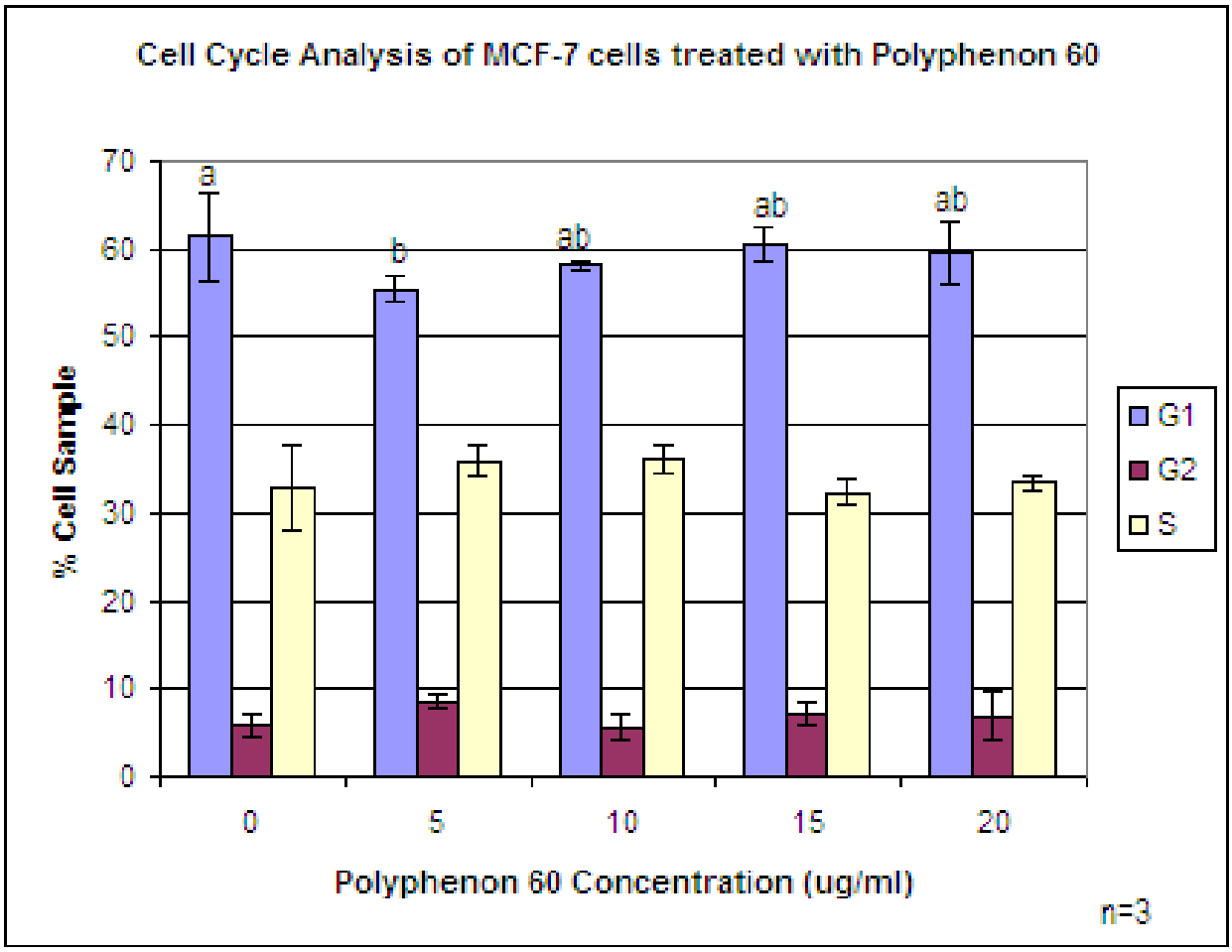

Figure 2-4: Cell Cycle analysis of MCF-7 cells treated with Polyphenon 60 for 48 hours following 5 day growth factor depravation synchronization. Analysis was performed on a FACScan Flow Cytometer. Error Bars are shown with single letter designations indicating statistically significant differences across the concentrations ($p < 0.05$).

CHAPTER 3: MicroRNA expression profile of MCF-7 human breast cancer cells and the effect of green tea Polyphenon 60

Abstract:

In Western countries, breast cancer is the most common form of cancer and it is the second leading cause of female cancer death in America. Previous epidemiological studies have established a strong correlation between green tea consumption and a decreased risk of developing breast cancer. The biochemical basis of the observed anti-cancer properties of green tea has been linked to the cellular effects of the polyphenols or catechins found in the *Camellia sinensis* leaf. The specific regulatory mechanism behind how polyphenols affect gene expression and further affect cancer cell survival has yet to be identified but current data suggests that small, non-protein coding microRNAs (miRNAs) may be responsible. However, there has been no report on the systematic analysis of a global miRNA expression profile for MCF-7 breast cancer cells and no investigation has been reported on the effect of green tea on miRNA expression. In the present study, MCF-7 breast adenocarcinoma cells were treated with Polyphenon 60, a green tea extract containing 60% catechins, and the effect of green tea polyphenols on the miRNA expression profile was investigated. Although hundreds of miRNAs have been identified in humans, only a small proportion (25.6%) of miRNAs were expressed in the MCF-7 cell line. Low concentration treatment with Polyphenon 60 altered the miRNA expression profile of MCF-7 cells. Twenty-three miRNAs have been identified with differential expression following a 48 hour treatment with 10 μg/ml of Polyphenon 60. These miRNAs include miR-21 and miR-27 that were found to be down-regulated following treatment with green tea. These two miRNAs have previously been identified as being over-expressed in MCF-7 breast cancer cells, with miR-21 specifically implicated in down-regulating the tumor suppressor gene,

tropomyosin-1. This data supports the hypothesis that polyphenol-induced modification of the breast cancer miRNA expression profile contributes to the efficacy of green tea treatment. The resulting decrease in carcinogenesis is further supported by the altered miRNA regulation of potential oncogenes and tumor-suppressor genes. This is the first report on the miRNA expression profile of the MCF-7 breast cancer cell line.

Keywords: Green tea; microRNA; microarray; gene expression; Polyphenon 60; breast cancer; MCF-7

Introduction:

Breast cancer is the second leading cause of female death in America (Catzavelos, Bhattacharya et al. 1997). Breast cancer samples are typically classified as positive or negative for the expression of specific proteins including estrogen receptor (ER), progesterone receptor (PR), vascular endothelial growth factor receptor (VEGFR) and human epidermal growth factor receptor 2 (HER2/*neu*). Gene amplification or reduction of these receptors significantly alters the biological characteristics of the breast cancer cells and modifies the therapeutic agents that can be used in their treatment. Estrogen receptors are found in approximately 65% of breast cancer cases (Teixeira, Reed et al. 1995). Receptor association with estradiol stimulates cell growth and up-regulates epidermal growth factor receptors such as HER2/*neu* (Teixeira, Reed et al. 1995). It has been experimentally shown that ER-positive tumors express the oncogene *BCL2*, whereas ER-negative tumors do not (Wang and Phang 1995). BCL2 is an anti-apoptotic protein that has been shown to assist in tumor cell resistance to chemotherapeutic agents. Treatment of ER-positive breast cancers involves endocrine agents such as tamoxifen, a receptor antagonist

for the ER receptor, and accompanies a better long-term prognosis than ER-negative patients (Frasor, Weaver et al. 2009).

A similar situation involves the human epidermal growth factor receptor 2 (HER2/*neu*). HER2/*neu* is a receptor tyrosine kinase that has been shown to be over-expressed in invasive breast carcinoma (Eccles 2001). Over-expression of the *HER2* gene has been experimentally shown to be associated with shorter disease-free survival, increased tumor size, advanced tumor growth and development of drug resistance (Hicks and Kulkarni 2008). One therapeutic drug that has been designed to target HER2/*neu* expression is trastuzumab, marketed as Herceptin®, which is a monoclonal antibody that targets the extracellular domain of the HER2/*neu* receptor. Herceptin® is able to block cell proliferation and induce cellular toxicity, leading to apoptosis (Lebeau, Deimling et al. 2001).

Analysis of protein markers such as HER2/*neu* and ER assists in predicting clinical response to therapy, invasiveness and malignancy in breast cancer patients. Several genes are also investigated in a similar matter, specifically up-regulated oncogenes such as *RAS*, *C-MYC*, and *BCL-2*, and down-regulated tumor suppressor genes like *BRCA1*, *BRCA2*, *p53*, and *PTEN*. The *BRCA* genes are involved in repairing DNA damage and inherited mutations in *BRCA1/BRCA2* have been linked to a significantly higher risk of developing breast cancer (King, Marks et al. 2003). The oncogenes implicated in breast cancer are also associated with a wide variety of other cancer types, based on their involvement in stimulating cell cycle progression and cell proliferation. Therapies that can target over-expressed oncogenes are critical for patients that lack the cell surface targets like estrogen or epidermal growth factor receptors. However, very few targeted chemotherapeutics currently exist. When receptor antagonists or antibodies cannot be used, therapies are used that function to eliminate rapidly dividing cells

nonspecifically. A commonly used chemotherapeutic, Paclitaxel (Taxol) stabilizes microtubule formation and inhibits their disassembly, directly inhibiting cell division (Rowinsky, Eisenhauer et al. 1993). Other chemotherapies use similar methods to disrupt cell proliferation. Further investigation into intracellular targets and non-chemical treatments must be performed to improve the cellular specificity and effectiveness of cancer therapies.

MicroRNAs (miRNAs) are small endogenous non-coding single-stranded RNA molecules which, in animals, typically bind to the 3' untranslated region (UTR) of specific messenger RNA (mRNA) transcripts. Binding of a miRNA to a target mRNA results in either degradation of the transcript or inhibition of translation, both of which result in a decrease in gene expression. The regulatory mechanism of degradation versus translational interference depends on the complementarity of the miRNA sequence to the 3' UTR target region of the transcript. A near-perfect match typically promotes mRNA cleavage, whereas imperfect binding causes interference of the translational machinery without transcript degradation. It has been predicted that the human genome encodes thousands of these miRNA genes, each with the potential for binding several target mRNA transcripts (Blenkiron, Goldstein et al. 2007). This target multiplicity further amplifies the potential regulatory presence of miRNAs in developmental and disease processes.

Depending on the identity of the target that is regulated, miRNAs may act with oncogenic or tumor suppressor capabilities (Zhang, Pan et al. 2007). One example present in breast cancer is miR-21, which is identified as an oncomir due to its ability to down-regulate a variety of tumor suppressor proteins. Among the targets of miR-21 are BCL-2, PTEN and tropomyosin 1, which are proteins that are implicated in proper cell cycle progression and regulation of apoptosis (Si, Zhu et al. 2007; Frankel, Christoffersen et al. 2008). It has also been shown that specific cancer

types express particular miRNA expression profiles. The tumor specific patterns of miRNA expression give very useful diagnostic information in determining how various chemicals are effective in the treatment or prevention of cancer.

For years, green tea consumption has been linked to a decreased incidence of breast cancer. Several studies point to polyphenols, such as epigallocatechin gallate (EGCG), as the molecular components behind the anti-carcinogenic effects of green tea. Such studies indicate that polyphenols induce apoptosis, cell cycle arrest, inhibition of angiogenesis, and down-regulation of telomerase expression (Sun, Estrov et al. 2008). Green tea has also been found to possess antihypotensive, antihepatotoxic, and antimutagenic properties (Zhu, Si et al. 2007). Although broad cellular impact has been investigated, the molecular mechanisms behind such effects remain unclear. The current study demonstrates that aberration of specific cancer related miRNAs may be the cause for the beneficial properties of green tea polyphenols on breast cancer.

Materials and Methods

Cell lines

The human MCF-7 breast adenocarcinoma cell line was obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). The cells were grown in RPMI 1640 media supplemented with 10% Fetal Bovine Serum, 0.1% gentamycin, and 0.1% bovine insulin (Gibco, Grand Island, NY) and sterilized using a 0.22 µm polyethersulfone filter (Corning Inc., Corning, NY). Cells were grown at 37°C in a 5% CO_2 humidified incubator.

Preparation of Polyphenon 60 treatment

Polyphenon 60 was purchased from Sigma (St. Louis, MO, USA) and dissolved in supplemented RPMI 1640 media prior to each cell treatment. The Polyphenon 60 solution was sterilized using a 0.22 µm polyethersulfone filter. Appropriate dilutions were made from an initial stock concentration of 1000 µg/ml.

Cell treatment

Cells were plated in 12-well plates at a seeding density of 10,000 cells/cm^2 to a total volume of 1 ml per well. Treatments were made from dilutions of a 1000 mg/L stock made fresh prior to each treatment. The stock was filtered through a 0.22 µm polyethersulfone filter prior to additional dilution. After seeding for 24 hours, cells were drained of media and treated with 1 ml of RPMI 1640 media with various Polyphenon 60 concentrations. After 48 hours of incubation, the cells were detached and counted using trypan blue dye-exclusion. Dose-response was verified using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) analysis. The dose-response curve of Polyphenon 60 was generated and the inhibitory concentrations (ICx) were calculated. Each treatment was repeated six times.

Total RNA isolation

Total RNA was extracted from MCF-7 cells treated with 0 (control) and 10 µg/ml of Polyphenon 60 using the mirVana™ miRNA Isolation Kit (Ambion) according to manufacturer's instructions. The treatment of 10 µg/ml was chosen because it was calculated to be the IC50 concentration for cell viability. Briefly, about 10^6-10^7 cells were collected from each of six biological replicate treatments and pelleted by centrifuging at 1000 rpm. The pellet was resuspended in 1 ml of phosphate buffered saline (PBS) and repelleted at 1000 rpm. The cells were then placed on ice. The excess PBS was then drained and 500 µl of Lysis/Binding Solution

was added and vortexed with the cells at 1000 rpm. 50 µl of miRNA Homogenate Additive was added to the cells and mixed by vortexing. The mixture was then placed on ice for 10 minutes, 500 µl of Acid-Phenol:Chloroform was added and the cells were vortexed for 60 seconds. The cells were centrifuged for 5 minutes at maximum speed. The aqueous phase was removed and transferred to a new tube and its volume was recorded. 1.25 times the aqueous phase volume of room temperature 100% ethanol was added to the aqueous phase and mixed. Each sample was transferred to its own filter cartridge collection tube and centrifuged for 15 seconds at 10,000 rpm. The flow-through was discarded and the sample was centrifuged until all of the lysate/ethanol mixture had passed through the filter. miRNA Wash Solution 1 (700 µl) was then added to the filter cartridge and centrifuged for 10 seconds and the flow-through was discarded. Wash Solution 2/3 (500 µl) was then added and centrifuged through the filter as previously performed. This was completed twice, with each flow-through discarded. The cartridge filter was centrifuged a final time for 1 minute at 10,000 rpm. The total RNA was eluted from the cartridge using 100 µl of 95°C pre-heated nuclease-free water. The quality and quantity of total RNA was measured using a NanoDrop 1000 (NanoDrop Technologies, Wilmington, DE).

miRNA microarray and data analysis

miRNA microarray analysis was performed by LC Sciences (Houston, TX) using a µParaFlo® microfluidics chip, analyzing the expression of the 871 human miRNAs (and functional miRNA*s) present in Sanger miRBase Release 13.0. Briefly, approximately 10 µg of each RNA sample was size fractionated and small RNA sequences were length-extended with the addition of a Poly(A) tail. Control and treatment samples were loaded on to the µParaFlo® microfluidics chip containing 871 miRNA detection probes and 50 positive and negative control probes. All probes contained a nucleotide sequence complementary to the detected miRNA and a

polyethylene glycol spacer sequence to extend the detection probe away from the substrate. Following RNA hybridization, control samples were labeled with Cy3 fluorescent dye and treatment samples were labeled with Cy5 fluorescent dye. All detection probes were performed in 4 replicates and the control probes were performed anywhere from 4 to 16 times.

The miRNA microarray data was processed by subtracting the background signal from the samples and normalizing based on cyclic locally weighted regression (LOWESS), as previously described (Bolstad, Irizarry et al. 2003). Detectable miRNAs possessed a signal intensity greater than three times the background standard deviation with a product of the standard deviation and signal intensity less than 0.5. For replicate probes, detectable signal had to be present in at least 50% of the samples for miRNAs to be considered "detectable". miRNAs that are reported as differentially expressed possessed a t-test p-value less than 0.01. Background subtraction, Cy3/Cy5 normalization, directivity determination and p-value calculation was determined by LC Sciences in-house developed computer programs. Cluster Plots were created using TIGR MeV software from The Institute of Genomic Research.

RT-PCR and qRT-PCR

miRNAs with differential expression patterns in microarray analysis were selected and then validated using qRT-PCR on an Applied Biosystems 7300 system (Applied Biosystems, Foster City, CA). Applied Biosystems TaqMan microRNA Assays were employed to detect and quantify miRNAs in human breast cancer cells exposed to 0 (control) and 10 μg/ml of Polyphenon 60 according to the manufacturer's instructions. This reaction included two steps: reverse transcription and real time PCR (Chen, Ridzon et al. 2005). Briefly, a mature miRNA was first reverse-transcribed to a longer single-stranded cDNA sequence from 500 ng of the total

RNA sample using the Applied Biosystems TaqMan microRNA Reverse Transcription Kit and a miRNA-specific stem-looped RT primer. Reverse transcription was reacted in 15 μL of solution that contained 500 ng of total RNAs, 1 mM each of dNTPs, 1 μL of MultiScribe Reverse Transcriptase (50 U/μL), 1.5 μL of 10X RT Buffer, 0.188 μL of RNase Inhibitor, and 3 μL of 5X Taqman microRNA RT primer for a specific miRNA. The RT reaction was performed with a Eppendorf Mastercycler Personal PCR (Westbury, NY) with the following temperature program: initial 16°C for 30 minutes followed by 42°C for 30 minutes; then the reaction was held for 5 minutes at 85°C; and finally the reaction was held at 4°C until next analysis or stored at -20°C.

Quantitative real time PCR (qRT-PCR) was performed with miRNA-specific primers provided by the Applied Biosystems Taqman MicroRNA Assay kit. Each reaction was performed in 20 μL of solution that contained 2 μL of RT PCR product (10-fold dilution from RT PCR reaction), 10 μL of Taqman 2X Universal PCR Master Mix (No AmpErase UNG), and 1 μL of Taqman MicroRNA Assays 20X Real Time Primers. Nuclease free water was used to adjust the final volume to 20 μL. The temperature program for qRT-PCR reactions was 95°C for 10 minutes followed by 45 cycles of 95°C for 15 seconds (to denature DNA) and 60°C for 60 seconds (for primer annealing and extending). Each reaction had three replicates. Each treatment had 5 replicates. In qRT-PCR, small RNA RNU48 was employed as an endogenous reference gene for normalizing qRT-PCR results. Relative miRNA expression data were analyzed using the $\Delta\Delta C_T$ method.

Results

MCF-7 miRNA expression profile

Although the human MCF-7 breast cancer cell line is important for modeling ER-positive breast cancer cells, and several miRNAs have been identified with differential expression in this cell line, the global miRNA expression profile of MCF-7 cells has not previously been documented. After miRNA microarray analysis of a total of 871 human miRNAs (and functional miRNA*s) deposited in the miRNA database miRBase, we found that the human MCF-7 breast cancer cell line possesses a unique pattern of miRNA expression (Figure 3-1A). Of the 871 human miRNAs, 25.6% (223) miRNAs were detected and a majority of miRNAs (74.4% or 648) were not detected in MCF-7 cells (Figure 3-1B and Table 3-1). This result suggests that only a small part of the miRNA population is expressed in human MCF-7 breast cancer cells. Increasing evidence demonstrates that many miRNAs are expressed in a species- or tissue-specific manner and only a small proportion of miRNAs are expressed in a specific tissue at a specific time. Our result is consistent with this conclusion.

Not only was there a small number of miRNAs (223 miRNAs) expressed in MCF-7 cells, but their expression profile and abundance were significantly different among the detected miRNAs (Figure 3-1 and Table 3-1). Based on their signal intensity during microarray analysis, the signal intensities varied widely with a range of 24 to 57600. This suggests that the expression level of miRNAs vary within the sample (Figure 3-1 and Table 3-1). Some miRNAs are expressed in many copies while others are only expressed with a few copies in MCF-7 cells. Among the 223 detected miRNAs, the expression levels were very low with weak signal intensity of less than 500 for a majority of miRNAs (148 or 66.37%). However, 11 miRNAs

were highly expressed in human MCF-7 breast cancer cells with signal intensities greater than 10,000; these 11 miRNAs were 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 had the highest expression with a signal intensity of 57,600. miR-21 was followed in intensity by let-7f and let-7a, which were expressed with intensities of 21,201 and 20,206, respectively.

Although hundreds of miRNA genes have been identified in the human genome, only a few have been reported in breast cancer cells. It is important to investigate the global expression profile of all miRNAs and their abundance in breast cancer. Based on our current microarray analysis, 223 miRNAs are expressed in human MCF-7 breast cancer cells with different abundance for each miRNA. A majority of these miRNAs have not been reported or investigated in breast cancer cells. Expression of one gene is always associated with its function. Investigating miRNA expression and factors affected by those miRNAs will help to elucidate the function of miRNAs in breast cancer pathogenesis and treatment. Table 3-2 lists the validated targets for the 11 most abundant miRNAs in MCF-7 cells (Table 3-2). From this list, it clearly shows that a majority of the previously confirmed targets are involved in DNA binding, and several are known transcription factors. One potential target of miR-21 is the tumor suppressor *PTEN*, which is consistent with the conceptual understanding of miR-21 reducing the expression of *PTEN* in MCF-7 breast cancer cells. However, let-7a is also highly expressed in MCF-7 cells and has two oncogenes as potential targets; *KRAS* and *NRAS*. The expression of these two oncogenes is decreased in MCF-7 cells, and may contribute to the early-stage, moderately aggressive characteristics of this cell line.

Polyphenon 60 induces differential miRNA expression

To investigate the effect of Polyphenon 60 on the global miRNA expression profile of human MCF-7 breast cancer cells, we first performed a dose-response study to investigate the effect of Polyphenon 60 on MCF-7 cell growth. Our results demonstrated that Polyphenon 60 significantly inhibited human MCF-7 breast cancer cell growth (data not shown). To avoid the potential effect of cell death on miRNA gene expression, in this study, we choose a moderate inhibitory concentration (10 μg/ml) to investigate the effect of Polyphenon 60 on the global miRNA expression profile in human MCF-7 breast cancer cells.

The miRNA expression pattern of MCF-7 breast cancer cells was distinctly different between non-treatment and treatment with 10 μg/ml Polyphenon 60 green tea extract for 48 hours (Figures 3-2 and 3-3). First, Polyphenon 60 treatment induced more miRNA expression in the human MCF-7 breast cancer cell line. Of the 871 investigated human miRNAs, 221 miRNAs were detected in MCF-7 cells exposed to 10 μg/ml Polyphenon 60 for 48 hours whereas only 155 miRNAs were detected in the corresponding untreated control samples (Figures 3-2 and 3-3). Although there were fewer miRNAs expressed in untreated samples, not all of the miRNAs expressed in the control sample were expressed in the Polyphenon 60-treated sample. There were 7 miRNAs (miR-30b*, miR-29a, miR-221, miR-936, miR-1249, miR-200a, and miR-424) detected in untreated MCF-7 cells but not detected in cells exposed to Polyphenon 60. In contrast, there were 73 miRNAs detected in treated samples but not in control samples. However, these differentially expressed miRNAs for the most part were expressed at a low level and the signal intensity ranged between 0 to 500.

Polyphenon 60 treatment not only altered the total number of miRNAs expressed in MCF-7 cells but also altered the expression level for miRNAs expressed in treated MCF-7 cells. Based on the miRNA microarray analysis, there were 23 miRNAs identified with differential

expression after 48 hours of treatment with 10 μg/ml Polyphenon 60 (Table 3-3). Of the 23 miRNAs with differential expression, seven were shown to be up-regulated after Polyphenon 60 treatment for 48 hours (let-7a, miR-107, miR-548m, miR-720, miR-1826, miR-1978, and miR-1979). In contrast, sixteen miRNAs were shown to be down-regulated after Polyphenon 60 treatment for 48 hours and these 16 miRNAs were let-7c, let-7e, let-7g, miR-21, miR-25, miR-26b, miR-27a, miR-27b, miR-92a, miR-125a-5p, miR-200b, miR-203, miR-342-3p, miR-454, miR-1469, and miR-1977. With the exception of miRNAs let-7a and miR-107, no function has been attributed to the remaining five up-regulated miRNAs. In particular, miR-1826, miR-1978 and miR-1979 are recently identified new miRNAs. Several questions can be asked: Why are these miRNAs so sensitive to Polyphenon 60 green tea extract? Do these miRNAs specifically respond to Polyphenon 60 treatment? Elucidating these questions will allow us to better understand the function of these miRNAs and their regulatory mechanism in breast cancer treatment.

The fold change for each miRNA varied in MCF-7 cells following Polyphenon 60 treatment (Table 3-4; Figure 3-4). With a low Polyphenon 60 concentration treatment, the miRNA fold change ranged from 2.14 fold down-regulation to 3.80 fold up-regulation. The three most up-regulated miRNAs were miR-548m, miR-720 and miR-1979 and all of these three miRNAs had at least three fold changes in expression after Polyphenon 60 treatment. Based on the microarray data, the signal intensity of miR-1826 was 16833 for control and 23230 for 10 μg/ml Polyphenon 60 treatment. Although miR-1826 had a 1.38 fold change, its expression level was dramatically increased after Polyphenon 60 treatment because of the high expression level of this miRNA.

Validation of miRNA expression using qRT-PCR

Verification of the miRNA microarray data was performed for the top two up-regulated miRNAs (miR-548m and miR-720) and the top down-regulated miRNA (miR-27a). The qRT-PCR analyzed fold-changes for these three miRNA validated the fold-changes calculated from the microarray signal data. miR-548m was shown to have a 3.8 fold increase based on the microarray data, and a 2.18 fold increase based on the qRT-PCR data. The microarray data for miR-720 gave a 2.59 fold increase, and the qRT-PCR data gave a 2.25 fold increase. miR-27a had a 2.14 fold decrease in the microarray data and 1 fold change in the qRT-PCR data. This reduction in fold values was believed to originate from the 6 month storage of the RNA samples at -80°C. The overall trend of this data confirms that the values provided by the miRNA microarray data seem to be accurate for the sample and can be extrapolated to include the remaining miRNAs identified by the microarray as being aberrantly expressed.

Discussion:

miRNAs responding to drug treatment

Several recent investigations demonstrate that chemicals induce differential expression of miRNAs in a variety of animals and also in animal cell lines (Marsit, Eddy et al. 2006; Saito, Liang et al. 2006; Moffat, Boutros et al. 2007; Pogribny, Tryndyak et al. 2007; Rossi, Bonmassar et al. 2007; Blower, Chung et al. 2008; Sun, Estrov et al. 2008; Zhang and Pan 2009). These chemicals include anti-cancer drugs as well as environmental toxicants. However, no study has been reported on the effect of natural products on miRNA expression in any organism *in vivo* or *in vitro*. In this study, we report, for the first time, the global expression profile of miRNAs in human MCF-7 breast cancer cells and the effect on the miRNA expression profile after 48 hours of treatment with a moderate dose of Polyphenon 60 green tea extract (10 μg/ml). Based on our

microarray analysis with 871 currently known human miRNAs, 23 miRNAs were identified as having differential expression after Polyphenon 60 treatment. Although some of the differentially expressed miRNAs, such as miRNA let-7, have been reported previously to have differential expression in response to chemical treatment in human cell lines or model animals, a majority of the Polyphenon-regulated miRNAs have not been reported in previous studies. Therefore, it seems that these miRNAs uniquely respond to Polyphenon 60 treatment. In another investigation in our laboratory, we used a well-studied chemotherapy drug, 5-fluorouracil, to treat and investigate its effects on the miRNA expression profile of the MCF-7 cell line. We found a completely different expression profile of miRNAs for 5-fluorouracil treatment compared with green tea Polyphenon 60 treatment (Shah et al., unpublished data). The results of our investigations and others suggest that the spectrum of miRNAs that respond to drug treatment is unique for specific groups of drugs. Investigating the molecular regulatory mechanism of the common and drug-specific miRNAs will allow us to better understand the mechanism of action for different drugs as well as provide new insight on screening new drugs for cancer treatment.

Polyphenol-regulated miRNAs are involved in cancer pathogenesis

The majority of miRNAs regulated by Polyphenon 60 were associated with the pathogenesis and development of cancer (Tables 5 and 6). miR-548m was the most up-regulated miRNA in Polyphenon 60- treated MCF-7 cells. Over-expression of this miRNA has been previously observed in colorectal cancer samples (Piriyapongsa, Marino-Ramirez et al. 2007). Four of the other up-regulated miRNAs were current additions to the miRNA database without further information on the function of these miRNAs. miR-107, which showed a 1.24 fold increase in expression upon Polyphenon 60 treatment, has been linked to potentiating the disease states of Alzheimer's disease and pancreatic cancer along with a variety of others (Wang, Rajeev

et al. 2008) (Volinia, Calin et al. 2006). By contrast, a decrease in miR-107 has been identified in numerous diseases such as head, neck and oral cancer as well as HNF1α-postive hepatocellular tumors (Wilfred, Wang et al. 2007) (Liu, Yeh et al. 2009). The increase in miR-107 upon Polyphenon 60 treatment in MCF-7 cells has to be further exploited for its therapeutic potential in miR-107-deficient diseases. Another up-regulated miRNA in treated MCF-7 cells was let-7a, which is a known tumor suppressor miRNA and has been shown to be down-regulated in several cancers including lung and breast cancer (Wang, Weng et al. 2007) (Yu, Yao et al. 2007). Let-7a was one of the most highly expressed miRNAs in the MCF-7 expression profile data, showing that green tea Polyphenon 60 treatment caused a significant increase in let-7a expression even at existing high expression levels in untreated cells.

Among the miRNAs down-regulated by Polyphenon 60 treatment, miR-27a was the most dramatic. Breast cancer and gastric cancer have been reported to reveal increased amounts of miR-27a (Guttilla and White 2009) (Liu, Tang et al. 2009). The ability of Polyphenon 60 to reverse miR-27a expression further supports the therapeutic potential of green tea. miR-27b, which was one of the most down-regulated miRNAs has previously been found at very low expression levels in colorectal cancer and reinforcement of the aberrant miR-27b expression may raise concerns when using green tea for treatment purposes (Xi, Shalgi et al. 2006). Similar concern could be raised about the let-7 family members, let-7c, let-7e, and let-7g that were down-regulated upon treatment. These miRNAs are normally down-regulated in breast cancer cells and a variety of other cancer types (Yu, Yao et al. 2007). The let-7 family is also down-regulated in lung cancer (Lee and Dutta 2007) (Wang, Weng et al. 2007) (Johnson, Esquela-Kerscher et al. 2007). However, let-7a seems to be a specific miRNA that was most

abundantly expressed and was found to be up-regulated following Polyphenon 60 treatment, suggesting fluctuation in other let-7 variants may be less important.

Among the down-regulated miRNAs, miR-26b seems to be highly up-regulated in bladder cancer, showing a marked decrease in expression with increasing diagnostic tumor-node-metastasis staging (Gottardo F 2007). A correlation between schizophrenia and decreased expression of miR-26b was also reported (Perkins, Jeffries et al. 2007). In this respect, further investigations on green tea's ability to further decrease the expression of miR-26b in these two diseases might provide interesting results. miR-200b expression was also decreased following Polyphenon 60 treatment. Over-expression of miR-200b has previously been identified in cholangiocarcinoma and ovarian cancer (Bendoraite, Knouf et al.) (Meng, Henson et al. 2006). The murine homologue of miR-200b, along with miR-203 and miR-342-3p, are over-expressed in mice infected with scrapie, a prion disease similar to Creutzfeldt-Jakob disease in humans (Saba, Goodman et al. 2008). Over-expression of miR-203 was also observed in colorectal and pancreatic cancer and psoriatic plaques (Sonkoly 2007) (Greither, Grochola et al.) (Schetter, Leung et al. 2008). In addition to the scrapie model, miR-342-3p is also over-expressed in multiple myeloma (Adamia, AvetLoiseau et al. 2008).

Of the remaining polyphenol- induced down-regulated miRNAs, miR-25 is increased in gastric cancers and multiple myeloma but decreased in colorectal cancer and glioma (Kim, Choi et al. 2009) (Pichiorri, Suh et al. 2008) (Xi, Shalgi et al. 2006) (Malzkorn, Wolter et al. 2009). miR-92a is increased in hepatocellular carcinoma and medulloblastoma (Connolly, Tchaikovskaya et al. 2007) (Northcott, Fernandez-L et al. 2009). miR-21, which is commonly implicated in disease states, is up-regulated in breast, pancreatic and cervical cancers, as well as glioblastoma, head neck and oral cancer, cholangiocarcinoma and dermatological disorders such

as psoriasis and atopic eczema, just to name a few (Meng, Henson et al. 2006; Lui, Pourmand et al. 2007; Si, Zhu et al. 2007; Sonkoly 2007; Chang, Jiang et al. 2008; Papagiannakopoulos, Shapiro et al. 2008; Cervigne, Reis et al. 2009; Park, Lee et al. 2009). All miRNAs over-expressed in these diseases and disorders were down-regulated in MCF-7 cells by Polyphenon 60 treatment. Whether green tea also exerts potential benefits in the mentioned diseases deserves further investigation.

The National Cancer Institute estimated that during the 2009 calendar year, 192,370 women in the United States were diagnosed with breast cancer, and 40,170 women died from the disease. In Western countries, breast cancer is the most common form of cancer and the second leading cause of female cancer death in America. In Eastern countries, such as Japan and China, the incidence and aggressiveness of breast cancer is much lower. In 1998, it was estimated that 1 in 10 women in the United States would develop breast cancer during their lifetimes, but only 1 in 40 women in Japan would develop the disease (Nakachi, Suemasu et al. 1998). Numerous epidemiological studies have been performed in an attempt to discover what may be the cause of this unequal distribution. Several lifestyle differences have been identified as potential causes for this phenomenon, in particular, the consumption of green tea. It has been estimated that 98% of the Japanese population drinks green tea regularly (Sun, Estrov et al. 2008). Green tea is known to be high in anti-oxidants and other pharmacologically active substances commonly used in homeopathic or alternative medicine. On average, polyphenols have been attributed with most of the anti-cancer abilities that are credited to green tea, but the cellular mechanisms behind why these compounds are so beneficial have yet to be determined.

As developments are being made in the field of miRNA research and profiling, an increasing amount of large scale molecular effects are being attributed to miRNA regulation.

miRNAs have already been shown to act as tumor suppressors by negatively regulating gene expression of oncogenic factors. Alternatively, miRNAs which down-regulate expression of tumor suppressor genes have been named oncomirs, and have been identified in a variety of cancer types (Zhang, Pan et al. 2007). Through analysis of miRNA expression, it can be seen that gene regulation is a highly sensitive procedure and the slightest change in the expression of these small non-coding RNAs can disrupt critical cellular functioning.

Green tea has been shown to decrease breast cancer cell estrogen receptor expression, increase cell sensitivity to specific chemotherapies and initiate natural cell death (Farabegoli, Barbi et al. 2007). Both estrogen receptor down-regulation and chemotherapy sensitization have obvious merit, as most cancer patients develop resistance to at least one therapeutic drug during their course of treatment. The ability to initiate apoptosis in cancer cells with green tea leads to many questions regarding cellular mechanism. If the cytotoxic effect of green tea can be linked to a specific miRNA, it is conceivable that treatments targeting the over-expressed miRNA can be developed. It has already been shown that transfection of cancer cells in culture with specific over-expressing miRNA plasmids can cause specific cellular responses without the widespread systemic effects caused by chemotherapy. The potential impact of these miRNA-specific therapies makes miRNA research applicable to a wide variety of medical fields, not limited to cancer research. As more data becomes available, and new procedures and techniques are developed, miRNAs should lead to new and exciting advances in diagnostic medicine and disease treatment.

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Table 3-1. Microarray miRNA expression for MCF-7 breast cancer cells.

Microarray Signal Intensity	**Number**	**miRNAs**
>20,000	3	miR-21, let-7f, let-7a
10,000-20,000	8	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
500-1,000	16	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
0-500	70	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. Validated targets for most abundant miRNAs in human MCF-7 breast cancer cells

miRNA	Validated targets	Function of target gene	References
miR-21	Tropomyosin 1 (TPM1) (luciferase/western)	Actin-binding protein	(Zhu, Si et al. 2007)
	TPM1 (v.5, v.1) (luciferase)	Actin-binding protein	(Zhu, Si et al. 2007)
	SERPINB5 (luciferase/western)	Serine or Cysteine Proteinase inhibitor	(Zhu, Si et al. 2007)
	PTEN(luciferase)	Tumor Suppressor: Phosphatase	(Meng, Henson et al. 2006)
	PDCD4(luciferase)	neoplastic transformation inhibitor	(Zhu, Si et al. 2007; Asangani, Rasheed et al. 2008; Chen, Liu et al. 2008; Frankel, Christoffersen et al. 2008; Lu, Liu et al. 2008)
	CDK6 (luciferase/western)	cyclin dependent kinase	(Frankel, Christoffersen et al. 2008)
	TIMP3 (western)	metallopeptidase inhibitor	(Krichevsky and Gabriely 2009)
	NFIB (western/luciferase)	Nuclear Factor	(Fujita, Ito et al. 2008)
	CDKN1A (qRT-PCR)	Cyclin-Dependent Kinase Inhibitor	(Frankel, Christoffersen et al. 2008)
	BTG2 (luciferase)	BTG/Tob family member	(Frankel, Christoffersen et al. 2008)
	BMPR2 (luciferase)	Bone Morphogenic protein receptor/ serine threonine kinase	(Frankel, Christoffersen et al. 2008)
	IL6R (luciferase)	Interleukin receptor	(Frankel, Christoffersen et al. 2008)
	SOCS5 (luciferase)	suppressor of cytokine signaling	(Frankel, Christoffersen et al. 2008)
	CFL2 (western blot)	intracellular protein regulates actin-filament dynamics	(Frankel, Christoffersen et al. 2008)
	RECK (qRT-PCR)	Extracellular protein with protease inhibitor-like domains	(Krichevsky and Gabriely 2009)
	MTAP (Luciferase)	Methylthioadenosine phosphorylase	(Chen, Liu et al. 2008)
	SOX5 (luciferase)	transcription factor: Sex determining region Y box-5	(Chen, Liu et al. 2008)
let-7f	N/A		N/A
let-7a	Trim71 (Real-time RT-PCR)	mRNA Repression	(Lin, Hsieh et al. 2007)
	NF2 (luciferase)	neurofibromin 2	(Meng, Henson et al. 2007)

	RAVER2(luciferase)	Ribonucleoprotein	(Beitzinger, Peters et al. 2007)
	LIN28 (RT-PCR)		(Wu, Fan et al. 2006)
	KRAS (Luciferase)	Oncogene	(Johnson, Grosshans et al. 2005)
	NRAS (luciferase)	Oncogene	(Johnson, Grosshans et al. 2005)
	LOC561754 (luciferase)	Zebrafish Trim71	(Lin, Hsieh et al. 2007)
	HMGA2 (western blot)	Transcription Factor: non-histone chromosomal high mobility group protein	(Shell, Park et al. 2007)
	CASP3 (western blot)	Caspase	(Tsang and Kwok 2008)
	DICER1 (western blot)	Ribonuclease	(Tokumaru, Suzuki et al. 2008)
	PRDM1 (luciferase)	repressor of beta interferon gene expression	(Nie, Gomez et al. 2008)
	ITGB3 (luciferase)	Integrin	(Muller and Bosserhoff 2009)
miR-1826	N/A		N/A
let-7d	DICER1 (luciferase/western)	Ribonuclease	(Tokumaru, Suzuki et al. 2008)
miR-1979	N/A		N/A
miR-200c	ZEB2 / SIP1 (Luciferase)	zinc finger homeodomain protein	(Park, Gaur et al. 2008)
	ZEB1 / TCF8 (luciferase)	zinc finger transcription factor	(Park, Gaur et al. 2008)
let-7c	Trim71 (Real-time RT-PCR)	mRNA Repression	(Lin, Hsieh et al. 2007)
	MYC(luciferase/western blot/northern)	nuclear phosphoprotein, transcription factor	(Koscianska, Baev et al. 2007)
	HMGA2 (luciferase) (western)	Transcription Factor: non-histone chromosomal high mobility group protein	(Shell, Park et al. 2007; Peng, Laser et al. 2008)
miR-191	N/A		N/A
miR-342-3p	N/A		N/A
miR-26a	SMAD1 (luciferase)	SMAD signal transducer	(Luzi, Marini et al. 2008)
	PLAG1(luciferase) mRNA repression	zinc-finger protein	(Volinia, Calin et al. 2006)
	Ezh2 (luciferase) mRNA repression	PcG family.	(Wong and Tellam 2008)
	SERBP1 (luciferase)	mRNA binding protein	(Beitzinger, Peters et al. 2007)
	TGFBR2 (luciferase)	Serine Threonine protein kinase and TGFB receptor subfamily	(Volinia, Calin et al. 2006)

Table 3-3. Comparison of miRNA expression profiles of MCF-7 cells treated with or without 10 μg/ml Polyphenon 60 green tea extract.

	miRNAs	
miRNA category	Number	Name
Up-regulated following Treatment	7	miR-548m, miR-720, miR-1979, miR-1826, miR-1978, miR-107, let-7a
Down-regulated following Treatment	16	miR-1977, miR-21, miR-92a, miR-25, let-7c, let-7e, miR-342-3p, miR-125a-5p, miR-203, miR-200b, let-7g, miR-454, miR-26b, miR-27b, miR-1469, miR-27a
miRNA Expression in Control only	7	miR-30b*, miR-29a, miR-221, miR-936, miR-1249, miR-200a, miR-424
miRNA Expression in Treatment only	73	miR-585, miR-656, miR-346, miR-876-5p, miR-517c, miR-519d, miR-519e*, miR-519a, miR-124, miR-1284, miR-555, miR-450b-3p, miR-1181, miR-34b*, miR-518f*, miR-492, miR-1471, miR-517*, miR-130a, miR-29c, miR-181a-2*, miR-212, miR-1233, miR-584, miR-1296, miR-760, miR-92a-2*, miR-659, miR-1908, miR-152, miR-1231, miR-602, miR-589*, let-7a-2*, miR-1228*, miR-323-5p, miR-24-2*, miR-551a, miR-1470, miR-513a-5p, miR-214, miR-1237, miR-1300, miR-324-3p, miR-184, miR-1228, miR-564, let-7f-1*, miR-1238, miR-1247, miR-497, miR-629*, miR-17*, miR-517b, miR-106b*, miR-145, let-7c*, miR-769-3p, miR-329, miR-125a-3p, miR-21*, miR-500*, miR-193a-3p, miR-96, miR-27b*, miR-550*, miR-671-5p, miR-424*, miR-143, miR-302f, miR-1259, miR-548f, miR-133b

Table 3-4. Twenty-three miRNAs with aberrant expression levels in MCF-7 cells following exposure to 10 μg/ml green tea Polyphenon 60 treatment for 48 hours ($p<0.01$)

miRNAs	Control (Ck) signal	Treatment (T) signal	log2(T/Ck)	Fold Change
Up-regulated [n=7]				
miR-548m	86.31	328.33	1.92	3.80
miR-720	774.56	2003.79	1.38	2.59
miR-1979	16191.43	25368.35	0.65	1.57
miR-1826	16883.71	23230.82	0.46	1.38
miR-1978	1810.15	2421.58	0.42	1.34
miR-107	1947.77	2421.49	0.31	1.24
let-7a	15302.94	16971.10	0.15	1.11
Down-regulated [n=16]				
miR-27a	542.00	252.93	-1.06	2.14
miR-1469	491.78	270.56	-0.85	1.82
miR-27b	663.73	369.59	-0.81	1.80
miR-26b	1393.07	817.41	-0.77	1.70
miR-454	834.68	512.25	-0.71	1.63
let-7g	1249.24	778.48	-0.68	1.60
miR-200b	1288.41	893.61	-0.55	1.44
miR-203	1161.38	808.24	-0.54	1.44
let-7c	7182.70	5160.29	-0.40	1.39
miR-342-3p	5764.27	4146.29	-0.43	1.39
miR-125a-5p	2813.94	2089.79	-0.53	1.35
let-7e	5258.72	3920.73	-0.42	1.34
miR-25	3003.24	2367.50	-0.34	1.27
miR-92a	3039.63	2538.69	-0.26	1.20
miR-21	57730.34	48852.23	-0.26	1.18
miR-1977	5978.10	5242.92	-0.19	1.14

Table 3-5. Documentation of miRNAs in cancers identified by microarray analysis.

miRNA	**Diseases with opposite expression**	**Diseases with similar expression**
↑ miR-548m		↑ Colorectal Cancer (Piriyapongsa, Marino-Ramirez et al. 2007)
↑ miR-720	↓ Renal Cell Carcinoma (Chow TF 2009)	
↑ miR-1979	n/a	
↑ miR-1826	n/a	
↑ miR-1978	n/a	
↑ miR-107	↓ Head and Neck/Oral Cancer (HNOC) (Wilfred, Wang et al. 2007) ↓ associated with HNF1alpha hepatocellular tumors (Liu, Yeh et al. 2009) ↓ CLL (Pallasch, Patz et al. 2009) ↓ Colorectal Cancer (Xi, Shalgi et al. 2006) ↓ Malignant Melanoma (Schultz et al., 2008) ↓non-alcoholic fatty liver disease (Zheng, Lv et al. 2009) ↓ Non-small cell lung cancer (Takahashi, Forrest et al. 2009) ↓Oral Squamous cell Carcinoma (Kozaki, Imoto et al. 2008)	↑ Pancreatic Tumors (Roldo, Missiaglia et al. 2006) ↑ Alzheimer's Disease (Wang, Rajeev et al. 2008) ↑ Pancreatic Cancer (Volinia, Calin et al. 2006) ↑ Cardiac Hypertrophy (Sayed, Hong et al. 2007) ↑ Esophageal Cancer (Guo, Chen et al. 2008) ↑ Gastric Cancer (Volinia, Calin et al. 2006) ↑ Hepatocellular Carcinoma (Huang, Wang et al. 2009) ↑ Schizophrenia (Beveridge, Gardiner et al. 2009)
↑ let-7a	↓ Lung Cancer (Wang, Weng et al. 2007), ↓ Breast Cancer (Yu, Yao et al. 2007), ↓ Burkitt Lymphoma (Sampson, Rong et al. 2007), ↓ CLL (Marton, Garcia et al. 2008), ↓ Colorectal cancer (Fang, Lin et al. 2007), ↓ Gastric Cancer (Zhang, Wang et al. 2007), ↓ Hepatocellular Carcinoma (Tsang and Kwok 2008), ↓ Laryngeal carcinoma (Long, Sun et al. 2009) , ↓ Malignant melanoma (Schultz,	↑ Cholangiocarcinoma (Meng, Henson et al. 2007), ↑ Hodgkin's Lymphoma (Nie, Gomez et al. 2008),

	Lorenz et al. 2008), ↓ Ovarian Cancer (Shell, Park et al. 2007), ↓ Squamous carcinoma (Tsang and Kwok 2008)	
↓ miR-1977	n/a	
↓ miR-21	↑ Bladder Cancer (Catto, Miah et al. 2009) ↑ Breast Cancer (Si, Zhu et al. 2007) ↑Cardiac Hypertrophy (Sayed, Hong et al. 2007) ↑ Cervical Cancer (Lui, Pourmand et al. 2007) ↑ Cholangiocarcinoma (Meng, Henson et al. 2006) ↑ Cholesteatoma (Friedland, Eernisse et al. 2009) ↑CLL (Fulci, Chiaretti et al. 2007) ↑ Colorectal cancer (Wang, Zou et al. 2009) ↑ Cowden Syndrome (Pezzolesi, Platzer et al. 2008) ↑ Diffuse Large B-cell Lymphoma (Lawrie, Soneji et al. 2007) ↑Duchenne Muscular Dystrophy (Eisenberg, Eran et al. 2007) ↑ Esophageal Cancer (Mathe, Nguyen et al. 2009) ↑ Gastric Cancer (Zhang, Li et al. 2008) ↑ Glioblastoma (Papagiannakopoulos, Shapiro et al. 2008) ↑ Glioblastoma multiforme (Silber, Lim et al. 2008) ↑ Glioma (Corsten, Miranda et al. 2007) ↑ Head and Neck squamous cell carcinoma (Chang, Jiang et al. 2008) ↑ Heart Failure (Thum, Gross et al. 2008) ↑ Hepatocellular Carcinoma (Wang, Majumder et al. 2009) ↑ Laryngeal Cancer (Liu, Wu et al.	↓ Myocardial Infarction (Dong, Cheng et al. 2009)

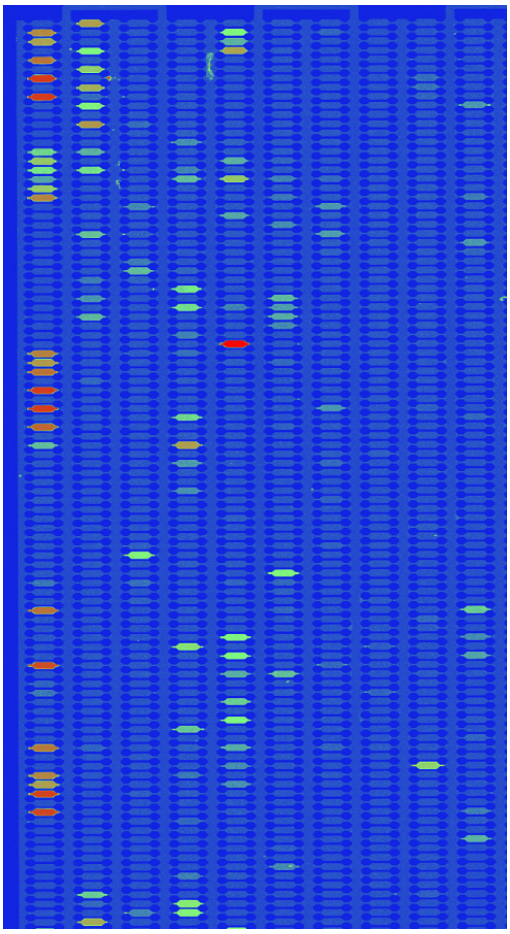
	2009) ↑ Lung Cancer (Seike, Goto et al. 2009) ↑ non-small cell lung cancer (Markou, Tsaroucha et al. 2008) ↑ Oral Carcinoma (Cervigne, Reis et al. 2009) ↑ Pancreatic Cancer (Park, Lee et al. 2009) ↑ Prostate Cancer (Li, Li et al. 2009) ↑ Psoriasis (Sonkoly 2007) ↑ Tongue Squamous cell carcinoma (Li, Huang et al. 2009) ↑ Uterine Leiomyoma (Wang, Zhang et al. 2007) ↑ Vascular Disease (Ji, Cheng et al. 2007)	
↓ miR-92a	↑ hepatocellular carcinoma (Connolly, Tchaikovskaya et al. 2007), ↑ Medulloblastoma (Northcott, Fernandez-L et al. 2009)	
↓ miR-25	↑ Gastric Cancer (Kim, Choi et al. 2009), ↑ multiple myeloma (Pichiorri, Suh et al. 2008)	↓ Colorectal cancer (Xi, Shalgi et al. 2006), ↓ Glioma (Malzkorn, Wolter et al. 2009)
↓ let-7c	↑ Acute promelocytic leukemia (Garzon, Pichiorri et al. 2007) ↑ Cardiac Hypertrophy (Sayed, Hong et al. 2007) ↑ Down Syndrome (Kuhn, Nuovo et al. 2008) ↑ Lung Cancer (Jiang, Lee et al. 2005) ↑ Prostate Cancer (Jiang, Lee et al. 2005) ↑ Uterine Leiomyoma (Wang, Zhang et al. 2007)	↓ Burkitt Lymphoma (Leucci, Cocco et al. 2008) ↓ Lung Cancer (Johnson, Esquela-Kerscher et al. 2007) ↓ Ovarian Cancer (Shell, Park et al. 2007) ↓ Prostate Cancer (Ozen, Creighton et al. 2008) ↓ Uterine Leiomyoma (Peng, Laser et al. 2008)
↓ let-7e		↓ Lung cancer (Lee and Dutta 2007), ↓ Malignant melanoma (Schultz, Lorenz et al. 2008)
↓ miR-342-3p	↑ Prion disease (Saba, Goodman et al. 2008)	
↓ miR-125a-5p		↓ Lung cancer (Wang, Mao et al. 2009)

↓ miR-203	↑Colorectal Cancer (Schetter, Leung et al. 2008) ↑ Neurodegeneration (Saba, Goodman et al. 2008) ↑ pancreatic cancer (Greither, Grochola et al.) ↑ psoriasis (Sonkoly 2007)	↓ ALL (Bueno, Perez de Castro et al. 2008) ↓ CML (Bueno, Perez de Castro et al. 2008) ↓ Esophageal Cancer (Mathe, Nguyen et al. 2009) ↓ Hepatocellular Carcinoma (Furuta, Kozaki et al. 2009)
↓ miR-200b	↑ Cholangiocarcinoma (Meng, Henson et al. 2006), ↑ Ovarian Cancer (Bendoraite, Knouf et al.)	↓ Breast Cancer (Tryndyak, Ross et al. 2009), ↓ Hepatocellular Carcinoma (Tryndyak, Beland et al. 2009), ↓ Lung Cancer (Gibbons, Lin et al. 2009), ↓ Ovarian Cancer (Hu, Macdonald et al. 2009)
↓ let-7g	↑ Colorectal cancer (Xi, Shalgi et al. 2006)	↓ Hepatocellular Carcinoma (Budhu, Jia et al. 2008), ↓ Lung Cancer (Johnson, Esquela-Kerscher et al. 2007), ↓ Malignant Melanoma (Schultz, Lorenz et al. 2008), ↓ Non-Small Cell Lung Cancer (Chin, Ratner et al. 2008), ↓ Ovarian Cancer (Shell, Park et al. 2007)
↓ miR-454	n/a	
↓ miR-26b		↓ Colorectal Cancer (Sayed, Hong et al. 2007) ↓ Hepatocellular Carcinoma (Ji, Shi et al. 2009) ↓Schizophrenia (Perkins, Jeffries et al. 2007)
↓ miR-27b	↑ Cardiac Hypertrophy (Sayed, Hong et al. 2007)	↓ Colorectal Cancer (Xi, Shalgi et al. 2006)
↓ miR-1469	n/a	
↓ miR-27a	↑ Breast Cancer (Guttilla and White 2009) ↑ Cardiac Hypertrophy (Sayed, Hong et al. 2007) ↑ Gastric Cancer (Liu, Tang et al. 2009) ↑ Hepatocellular Carcinoma (Huang, Gumireddy et al. 2008) ↑ Uterine Leiomyoma (Wang, Zhang et al. 2007)	↓ Colorectal Cancer (Xi, Formentini et al. 2006)

Table 3-6. Three most Polyphenon 60-regulated miRNAs and their related cancers and verified targets.

miRNA	Polyphenon 60 Response	Disease Implication	Verified Targets	References
miR-548m	Up-regulated	↑ Colorectal Cancer	None documented	(Piriyapongsa, Marino-Ramirez et al. 2007)
miR-720	Up-regulated	↓ Renal Cell Carcinoma	None documented	(Chow, Youssef et al. 2009)
miR-27a	Down-regulated	↑ Breast Cancer ↑ Cardiac Hypertrophy ↑ Gastric Cancer ↑ Hepatocellular Carcinoma ↑ Uterine Leiomyoma ↓ Colorectal Cancer	ZBTB10 (DNA binding protein) MYT1 (DNA binding protein) SP1, SP3, SP4 (Transcription Factors) PHB (Tumor Suppressor) RUNX (Transcription Factor) FOXO1 (Tumor Suppressor)	(Guttilla and White 2009) (Sayed, Hong et al. 2007) (Liu, Tang et al. 2009) (Huang, Dai et al. 2008) (Wang, Zhang et al. 2007) (Xi, Shalgi et al. 2006) (Mertens-Talcott, Chintharlapalli et al. 2007) (Ben-Ami, Pencovich et al. 2009)

3-1A



3-1B

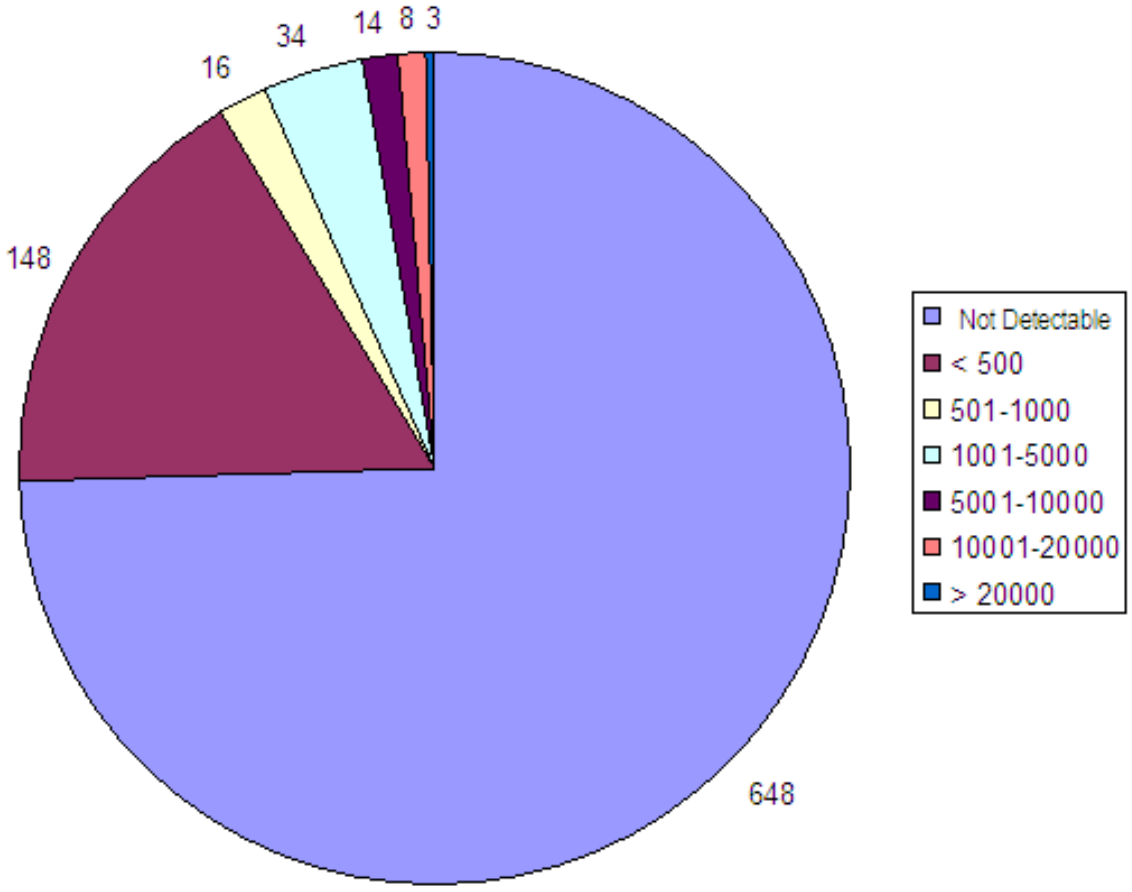


Figure 3-1. miRNA expression profile in human MCF-7 breast cancer cell line. A total of 871 human miRNAs (and functional miRNA*s) present in Sanger miRBase Release 13.0 were analyzed by microRNA microarray. A) The expression profile of 871 miRNAs in MCF-7 cells. The image was displayed in pseudo colors so as to expand visual dynamic range, in which, as signal intensity increases from 1 to 57600 the corresponding color changes from blue to green, to yellow, and to red. B) miRNA distribution based on their signal intensity in microarray analysis. The stronger the signal, the higher the expression of specific miRNAs. The expression level is significantly different among the 871 analyzed microRNAs.

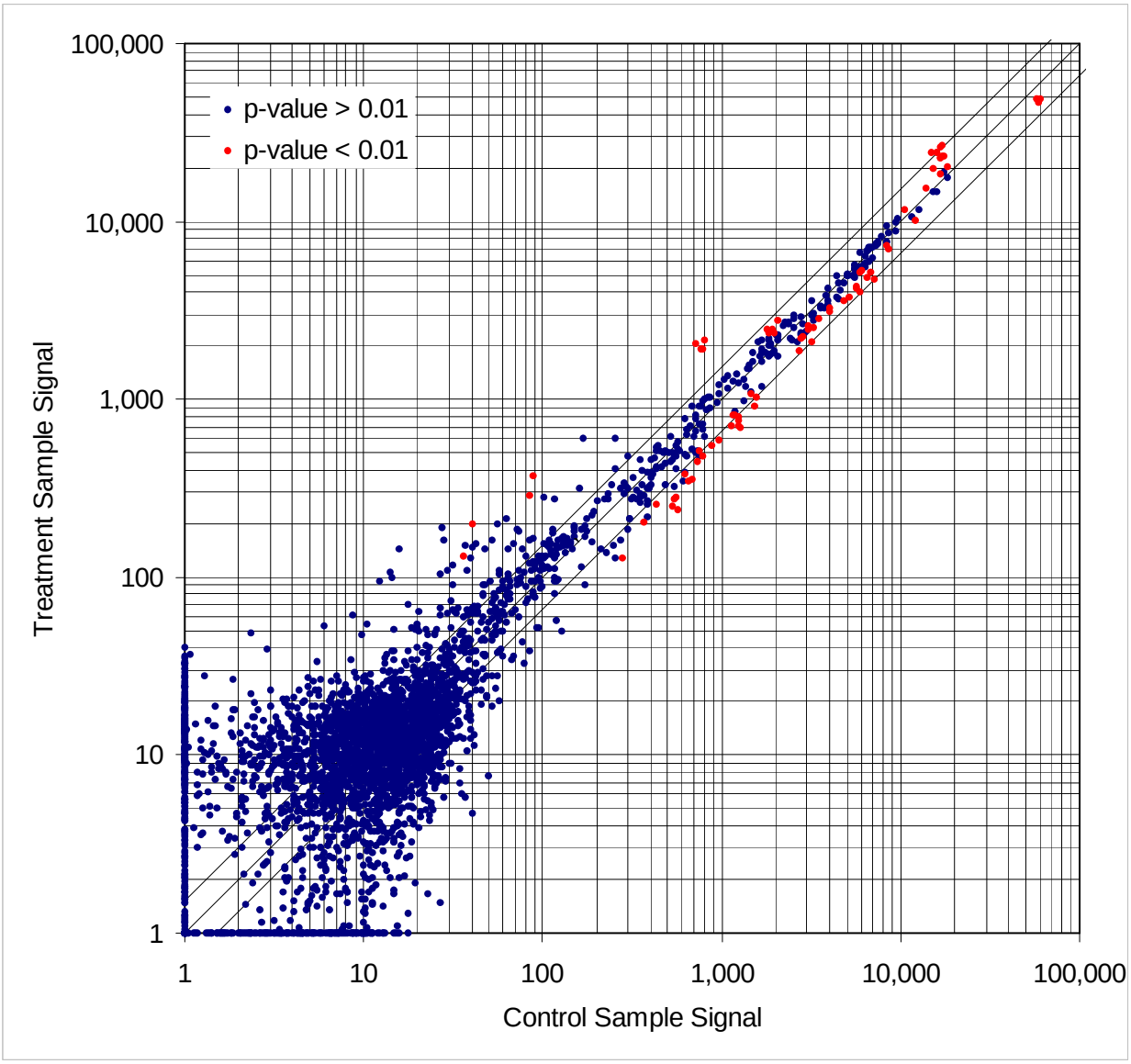


Figure 3-2. miRNA microarray signal distribution between control and Polyphenon 60 treated MCF-7 cells

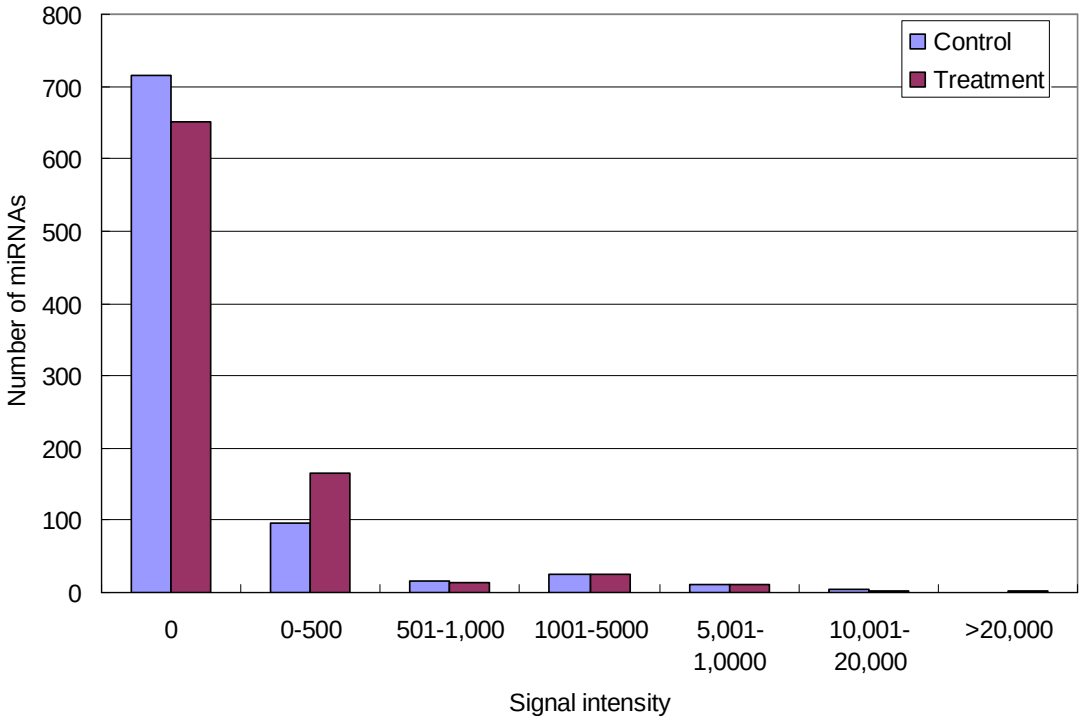


Figure 3-3: miRNA microarray signal intensity distribution for control and 10 μg/ml Polyphenon 60 treated MCF-7 cells

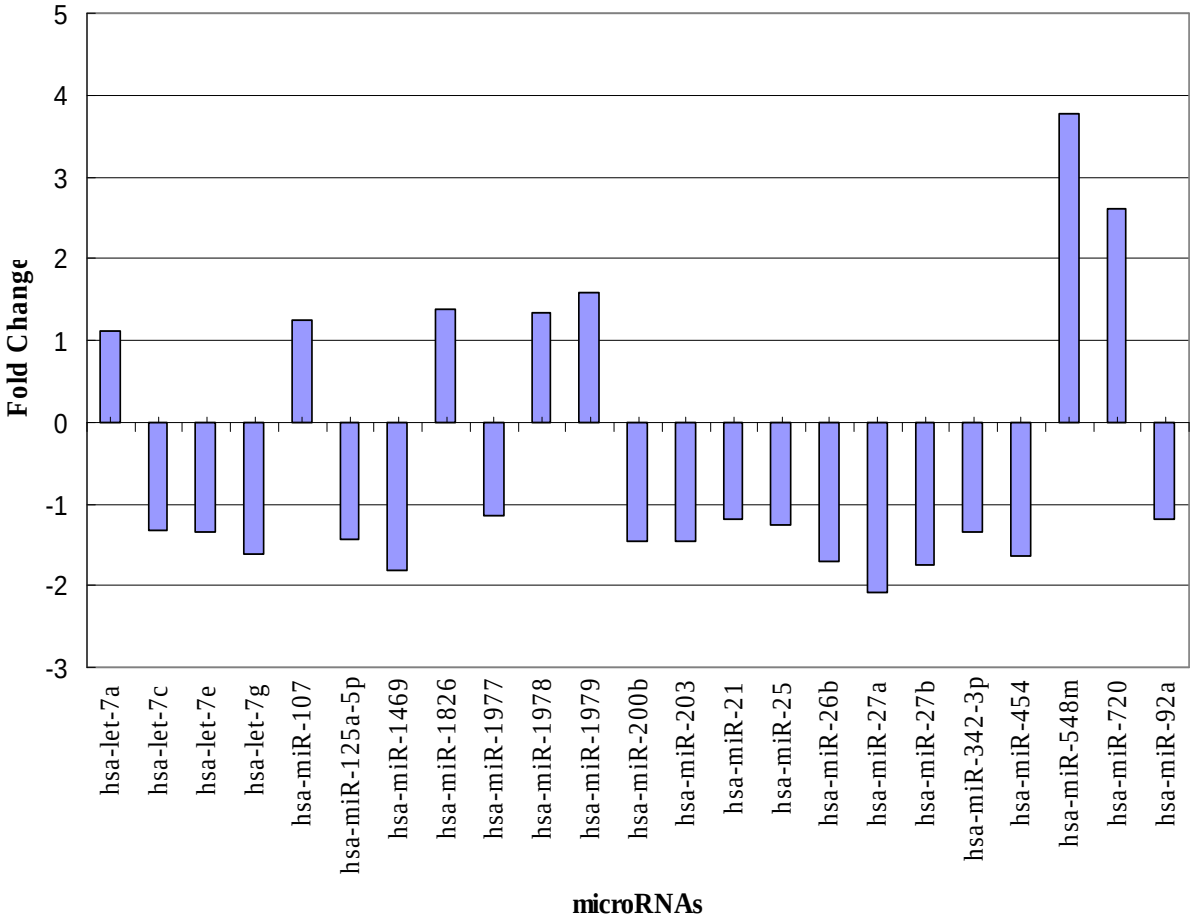


Figure 3-4. miRNAs shown to be differentially expressed between control and Polyphenon 60 treated MCF-7 cells. Y-axis shows fold changes as calculated from signal intensity differences from microarray data.

CHAPTER 4: Effect of Green Tea Polyphenon 60 on the Expression of Selected MicroRNAs in a Dose- and Time-Dependent Manner

Abstract:

The ability of green tea polyphenols to alter miRNA expression levels in MCF-7 breast cancer cells has previously been presented. This study further investigates the relationship between Polyphenon 60 dosage and miRNA responsiveness using let-7g, let-7e, miR-21, miR-26b, miR-27a, miR-92a, miR-548m, and miR-720 previously identified in microarray studies as being differentially expressed following treatment. Additionally, miR-10b, miR-15a, miR-16, miR-200c were investigated based on previous reports of their connection to breast cancer. Several miRNAs showed a change in expression at concentrations as low as 1 μg/ml and did not fluctuate significantly even at the IC80 value of 15 μg/ml. Time-response data was also generated for miR-27a, miR-92a, miR-200c, and miR-720 following 24, 48, and 72 hour treatment with low and moderate concentrations of Polyphenon 60. At low-doses and short treatment times, miR-720 and miR-200c were up-regulated and directly reversed their typical breast cancer expression. miR-27a, an oncomir, was similarly down-regulated. The subtle yet significant changes that low-dose green tea extract can induce in the miRNA expression profile of MCF-7 cells may help substantiate the previous epidemiological claims that green tea effects breast cancer pathogenesis.

Keywords: Green tea; microRNA; quantitative RT-PCR; Polyphenon 60; breast cancer; MCF-7

Introduction

There were over 190,000 new cases of breast cancer diagnosed in the United States during 2009 (Jemal 2009). This accounts for 27% of all incidences of female-specific cancer in America (Jemal 2009). With breast cancer as the second leading cause of death for American women, there exists a wide variety of breast cancer therapies currently offered to patients based on specific diagnostic criteria. The three primary methods of treatment are surgical removal of the tumor, radiation therapy, and chemotherapy. Typically, a combination of these methods is used to ensure near-complete destruction of the tumor. In the case of breast cancer, a lumpectomy can be performed to remove a specific tumor or a mastectomy can be performed to remove the whole breast. Additionally, radiation therapy can be administered to the tumor site, either before or after surgical removal. Radiation is usually in the form of high energy x-ray or gamma rays that cause targeted cell death to both healthy and cancerous cells. A multitude of chemotherapeutics have been developed to specifically interact with various tumor types. Hormone-based chemotherapy has been found to be effective in patients presenting estrogen-receptor positive breast cancer. Tamoxifen is a well-known estrogen receptor antagonist which can interact with the growth-stimulating estrogen receptor, blocking the ligand binding site and preventing the signal cascade causing cell division (Miller, Ghoshal et al. 2008). Other therapies like Taxanes, such as Taxol or Paclitaxel, promote tubulin assembly and induce apoptosis by interfering with mitotic cell division (Tsang and Kwok 2008). Monoclonal antibodies have also been developed to target cytotoxic chemicals to cells differentially expressing specific cell surface receptors or to block the activation of such receptors. Trastuzumab® was developed to target cells over-expressing epidermal growth factor receptor 2 (HER2/*neu*) (Eccles 2001). Other systemic chemotherapies interfere with key mechanisms required for cell division, limiting any rapidly dividing cell growth including hair growth, nail

development and gastric lining replacement, responsible for causing wide-spread side effects (Germano and O'Driscoll 2009). The non-specific targeting of such treatments has lead to the investigation for tumor-specific markers. Up-regulated or over-expressed genes and proteins have been investigated but a new emphasis is being placed on small gene regulatory molecules such as microRNAs (miRNAs). Additionally, negative side-effects from chemical agents have stimulated interest in the development of natural treatments, such as botanical-based therapies. The results presented here seek to combine these two efforts and present critical changes to miRNA expression in breast cancer based on treatment with the natural green tea extract, Polyphenon 60.

miRNAs are small non-coding RNA molecules which down-regulate the expression of target messenger RNAs (Calin and Croce 2006). Since their discovery in 1993, a diverse amount of data has been generated looking at how miRNAs function as gene regulators, with a more recent focus on their role in cancer. Microarray analysis for miRNA expression patterns in various cancer types is slowly generating a list of miRNA profiles that may assist in identifying the tissue of origin for metastases, which aids in the assignment of appropriate therapy (Negrini, Nicoloso et al. 2009). Based on previous reports that miRNAs are differentially expressed in cancer samples, they may also act as internal markers or gene targets for therapeutics (Negrini, Nicoloso et al. 2009). If oncogenic miRNAs can be down-regulated by specific treatments, and tumor-suppressor miRNAs can be induced, it is possible that previously unidentified compounds may have hidden potential in the treatment of cancer.

A variety of cancer-specific miRNAs have been identified, and their presence or absence in tumor samples may lead to improved diagnostic techniques as the technology expands. In the case of breast cancer, a multitude of miRNAs have been investigated but variations due to the

site of origin, level of malignancy, or drug-resistance have generated very specific data implicating miRNAs in various aspects of carcinogenesis (Blenkiron, Goldstein et al. 2007). Primary breast carcinomas have displayed a positive correlation between the level of miR-10b expression in cells and classification of metastatic or invasive behavior (Ma, Teruya-Feldstein et al. 2007). miR-21, miR-9 and miR- 27b have all been identified as being over-expressed in MCF-7 cells (Tsuchiya, Nakajima et al. 2006; Ma, Teruya-Feldstein et al. 2007; Si, Zhu et al. 2007; Frankel, Christoffersen et al. 2008). Microarray work performed by Sempere et al. also indicates a high expression of miR-200, let-7, and miR-92 (Sempere, Christensen et al. 2007). Additional work has examined how drug-resistance affects the miRNA expression of MCF-7 cells. Tamoxifen-resistant MCF-7 cells have been shown to under-express miR-21, miR-342, miR-489, miR-24, miR-27, miR-23, and miR-200 (Miller, Ghoshal et al. 2008). Doxorubicin resistance has been shown to cause similar results, such as a decrease in miR-10, miR-21, and miR-200c (Kovalchuk, Tryndyak et al. 2007). The miR-15a/16-1 cluster has been identified previously as being down-regulated in a variety of cancers and both miRNAs have been shown to act as pro-apoptotic molecules by targeting BCL2 expression (Calin, Cimmino et al. 2008)

For decades, green tea has been epidemiologically linked to a decreased risk of breast cancer. Green tea has been shown to decrease breast cancer cell estrogen receptor expression, increase cell sensitivity to specific chemotherapies and initiate natural cell death (Farabegoli, Barbi et al. 2007). Both estrogen receptor down-regulation and chemotherapy sensitization have obvious merit, as most cancer patients will develop resistance to at least one therapeutic during their course of treatment. The ability to initiate apoptosis in cancer cells with green tea leads to many questions regarding cellular mechanism. If the cytotoxic effect of green tea can be linked to specific miRNAs, it is conceivable that treatments targeting the over-expressed miRNAs can

be developed. It has already been shown *in vitro*, that transfection of cancer cells with specific over-expressing miRNA plasmids can cause specific cellular responses without the widespread systemic effects caused by chemotherapy. The potential impact of these miRNA specific therapies makes miRNA research applicable to a wide variety of medical fields, not limited to cancer research. In our previous study, we identified a set of miRNAs with differential expression after green tea Polyphenon 60 treatment using microarray and qRT-PCR (Chapter 3). In this study, we show that green tea polyphenols are capable of changing the expression of a variety of miRNAs in both dose- and time-dependent manners.

Materials and Methods

Cell lines

The human MCF-7 breast adenocarcinoma cell line was obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). The cells were grown in RPMI 1640 media supplemented with 10% Fetal Bovine Serum, 0.1% gentamycin, and 0.1% bovine insulin (Gibco, Grand Island, NY) and sterilized using a 0.22 µm polyethersulfone filter (Corning Inc., Corning, NY). Cells were grown at 37°C in a 5% CO_2 humidified incubator.

Preparation of Polyphenon 60 treatment

Polyphenon 60 was purchased from Sigma (St. Louis, MO, USA) and dissolved in supplemented RPMI 1640 media prior to each cell treatment. The Polyphenon 60 solution was sterilized using a 0.22 µm polyethersulfone filter. Appropriate dilutions were made from an initial stock concentration of 1000 µg/ml.

Dose- and time- response treatment for miRNA analysis

For the miRNA dose-response analysis, eight 6-well plates were seeded at a cell density of 120,000 cells/ml with 2 ml administered to each dish. After 24 hours, the media was removed and 2 ml of Polyphenon 60 treatments of concentration 0, 1, 3, 5, 6, 7, 10, 15 µg/ml were administered to 6 independent plates. The cells were incubated at 37ºC for 48 hours and were detached using 500 µl of 1X trypsin for 5 minutes. The trypsin was inactivated with 600 µl of RPMI complete and the cells were pelleted at 1000 rpm. RNA extraction was either immediately performed or cells were stored in RNAlater at -20°C. For time-response analysis nine 6-well plates were seeded at 120,000 cells/ml for a total volume of 2 ml. The media was removed after 24 hours and 2 ml of treatment was administered at 0, 3, and 6 µg/ml Polyphenon 60 to 3 independent 6-well plates. After 24 hours, cells from one 6-well plate from each concentration were detached and processed for RNA extraction. The same procedure was done following 48 hours and 72 hours of treatment incubation.

RNA extraction

Total RNA was extracted from the Polyphenon 60 treated and untreated cells using the mirVana™ miRNA Isolation Kit (Ambion) according to manufacturer's instructions. Pelleted cells were either used fresh or RNAlater was removed and 500 µl of Lysis/Binding Solution was added and vortexed with the cells at 1000 rpm. 50 µl of miRNA Homogenate Additive was added to the cells and mixed by vortexing. The mixture was then placed on ice for 10 minutes, 500 µl of Acid-Phenol:Chloroform was added and the cells were vortexed for 60 seconds. The cells were centrifuged for 5 minutes at maximum speed. The aqueous phase was removed and transferred to a new tube and its volume was recorded. 1.25 times the aqueous phase volume of room temperature 100% ethanol was added to the aqueous phase and mixed.

Each sample was transferred to its own filter cartridge collection tube and centrifuged for 15 seconds at 10,000 rpm. The flow-through was discarded and the sample was centrifuged until all of the lysate/ethanol mixture had passed through the filter. miRNA Wash Solution 1 (700 µl) was then added to the filter cartridge and centrifuged for 10 seconds and the flow-through was discarded. 500 µl of Wash Solution 2/3 was then added and centrifuged through the filter as previously done. This was performed twice, with each flow-through discarded. The filter was centrifuged a final time for 1 minute at 10,000 rpm. The filter was then transferred to a new collection tube and treated with 100 µl of 95°C pre-heated nuclease-free water and centrifuged for 10 seconds at maximum speed. The eluate containing the RNA was stored at -20°C. Purity of the sample was measured by analysis of the 260/280 absorbance ratio. Absorbances were taken using a Thermo Scientific NanoDrop 1000.

qRT-PCR analysis of miRNA

Applied Biosystems TaqMan microRNA Assays were performed according to manufacturer's instructions (Chen, 2005). Primers for hsa-let-7g, hsa-let-7e, hsa-miR-10b, hsa-miR-15a, hsa-miR-92a, hsa-miR-16, hsa-miR-21, hsa-miR-26b, hsa-miR-27a, hsa-miR-548m, hsa-miR-720, and reference gene RNU48 were purchased from Applied Biosystems. let-7g, let-7e, miR-21, miR-26b, miR-27a, miR-92a, miR-548m, and miR-720 were previously identified in microarray studies as being differentially expressed following Polyphenon 60 treatment. Additionally, miR-10b, miR-15a, miR-16, miR-200c have previously been linked to breast cancer in other reported sources. The reverse transcription reaction was performed in 15 µL of solution, which contained 500 ng of total RNA (300 ng for time response analysis), 1 mM of dNTPs, 1 µL of MultiScribe Reverse Transcriptase (50 U/µL), 1.5 µL of 10X RT Buffer, 0.188 µL of RNase Inhibitor, and 2 µL of 5X Taqman microRNA RT primer. The RT

reaction was performed using an Eppendorf Mastercycler Personal PCR operated at the following temperature conditions: initial 16°C for 30 minutes followed by 42 °C for 30 minutes, then held for 5 minutes at 85 °C, and stored at -20 °C.

Quantitative real time PCR (qRT-PCR) was done using the Applied Biosystems 7300 Real-Time PCR system using miRNA-specific primers from Applied Biosystems for use with their Taqman MicroRNA Assay kit. Each reaction contained 10 μL of Taqman 2X Universal PCR Master Mix (No AmpErase UNG), 1 μL of Taqman MicroRNA Assays 20X Real Time Primers, and 1 μL of RT PCR product (10-fold dilution from RT PCR reaction). Nuclease-free water was used to adjust the final volume to 20 μL. The qRT-PCR reactions were incubated in a 96-well optical microplate at 95°C for 10 minutes followed by 45 cycles of 95 °C for 15 seconds (to denature DNA) and 60°C for 60 seconds (for primer annealing and extending). Each reaction was done in technical duplicate. In qRT-PCR, small RNA RNU48 was used as the endogenous reference gene for normalizing qRT-PCR results. Relative miRNA expression data was analyzed using the ΔΔCT method. (Zhang, Pan et al. 2007).

Statistical analysis was performed by the one-way Analysis of Variance (ANOVA) test using PASW Statistics 17 software (LSD post-hoc analysis, $p = 0.05$). Statistical significance was assigned to any variation with a p value less than 0.05. The data used for the ANOVA were the ΔΔCT values calculated from qRT-PCR analysis. The control sample replicate ΔCT values were averaged prior to ΔΔCT calculation to minimize deviation based on which control replicate was used to calculate the treatment replicate ΔΔCT value. Three biological replicates were used in the described calculations.

Results

miRNA dose-response to Polyphenon 60 treatment

Twelve separate miRNAs (let-7g, let-7e, miR-27a, miR-720, miR-548m, miR-200c, miR-26b, miR-21, miR-16, miR-92a, miR-15a, and miR-10b) were analyzed for responses in expression levels under various Polyphenon 60 concentrations (0, 1, 3, 5, 6, 7, 10 and 15 μg/ml) (Figures 4-1 - 4-12). Reverse Transcription and quantitative real time PCR were performed to analyze relative miRNA expression levels. Of the twelve analyzed, miR-720 and let-7g showed statistically significant changes in expression across the concentration levels ($p \leq 0.05$). miR-200c showed a significant decrease in fold change between 1 μg/ml and 15 μg/ml treatments. miR-26b had a significant decrease in expression between 1 μg/ml and the 5-7 μg/ml range. miR-16 decreased in expression similarly from 1 μg/ml to 6 μg/ml. miR-720 showed a decrease from 5 μg/ml to 15 μg/ml which was much later than the other three responses. miR-92a showed a decrease in expression from 3 μg/ml to 6 μg/ml. Let-7g showed an up-regulation from the control at 15 μg/ml. The remaining miRNAs tested did not show a significant change in expression over the treatment range and seemed to show expression levels similar to those found in the control sample.

miRNA time-response to Polyphenon 60 treatment

Based on the dose-response study, four miRNAs of interest with high fold changes (miR-27a, miR-92a, miR-200c and miR-720), were used to assess if any time-dependent changes in expression could be identified (Figure 4-13 - 4-20). Cells were treated with 3 μg/ml or 6 μg/ml of Polyphenon 60 for 24, 48 and 72 hours.

At the 3 μg/ml treatment level, the four miRNAs were expressed differently. After 24 hours of treatment, miR-200c was significantly up-regulated by more than a 5.5 fold change

(Figure 4-16). However, the change was decreased to normal with increasing treatment length, which suggests that miR-200c is very sensitive to Polyphenon 60 treatment at an early stage. For miR-720, the expression level was up-regulated after 24 hours of treatment; however, the expression level was down-regulated after 48 and 72 hours. However, this change in expression never significantly varied from the control. (Figure 4-14). In contrast, miR-27a and miR-92a were significantly down-regulated at 24 and 48 hours, respectively. The expression of these two miRNAs returned to normal at the 72 hour time point (Figures 4-15, 4-13). This suggests that different miRNAs respond to green tea polyphenols in different ways.

At the 6 μg/ml treatment level, miR-200c showed a significant increase in expression at the 24 hour time point as was seen with the 3 μg/ml treatment (Figure 4-20). miR-720 showed a higher average fold increase at 24 hours of 6 μg/ml treatment (3.96 fold) than at the 3 μg/ml treatment (1.97 fold) but still had the decrease in expression that was seen at the lower treatment concentration. miR-27a and miR-92a showed no significant differences at the various time points following the 6 μg/ml treatment (Figures 4-17, 4-19).

Discussion

miRNA dose-response to Polyphenon 60 treatment

miR-720 has previously been identified as one of the most up-regulated miRNAs in a microarray of MCF-7 cells treated with 10 μg/ml of Polyphenon 60 for 48 hours (Chapter 3). Those results are further supported by the dose-response data presented here. miR-720 continues to be the most up-regulated miRNA at the 10 μg/ml concentration, but the current data suggests that lower concentrations of approximately 5 μg/ml allow for even greater expression. Previous studies have shown miR-720 to be down-regulated in cases of renal cell carcinoma (Table 4-1)

as well, indicating that its expression may be critical for limiting cancer development (Chow TF 2009). Based on the low bioavailability of plant polyphenols, it is important that the effects of the lower concentration levels be analyzed appropriately. Here it has been shown that low Polyphenon 60 concentrations cause an increase in expression of miR-720, which reduces to a decrease in expression at 15 μg/ml. Further analysis of the potential targets of miR-720 may give greater insight into how this miRNA assists with the anti-oxidant or anti-oncogenic properties of green tea, however, no current research has been done to identify such targets.

Another miRNA whose expression was significantly altered following Polyphenon 60 treatment was miR-200c. At 1 μg/ml, the lowest concentration, miR-200c had a fold-change increase greater than two. This over-expression was significantly decreased at 15 μg/ml, which was the highest concentration tested. miR-200c is a miRNA that was found to be very prominent in MCF-7 breast cancer cells naturally, therefore the ability of Polyphenon-60 to cause an even greater expression and a decreased expression depending on the dosage seems interesting. From previous literature reports, miR-200c is usually down-regulated in breast cancer making this low dose ability to up-regulate the miRNA very important for potential therapy (Gregory PA 2008 May.; Tryndyak, Beland et al. 2009). The ability of miR-200c to negatively regulate a variety of critical genes involved in cancer metastasis and cell division makes its up-regulation of particular interest for the treatment and prevention of cancer.

miR-26b also showed a Polyphenon 60 dose-responsive decrease in expression in MCF-7 cells. At 1 μg/ml, miR-26b was slightly up-regulated but by 5 μg/ml its expression was significantly decreased. Previous literature analysis identified miR-26b as down-regulated in a variety of diseases, including hepatocellular carcinoma (Ji, Shi et al. 2009). The ability of Polyphenon 60 to up-regulate this specific miRNA at the lowest concentration level may

contribute to the subtle differences in disease risks based on consumption of green tea. If very low levels of green tea polyphenols can cause this increase in expression, then it could be proposed that the minimal intake from one cup of green tea may prove to be beneficial.

Another miRNA that showed significant change following polyphenol treatment was miR-16. Similarly to the others mentioned, miR-16 was over-expressed at the 1 μg/ml treatment level and became down-regulated at 6 μg/ml. miR-16 is a part of the miR-15a/16-1 cluster which has been shown to target and down-modulate expression of the anti-apoptotic protein, BCL2 (Calin, Cimmino et al. 2008). Based on the function of this target, miR-16 is considered to be a tumor-suppressor miRNA and has been shown to be down-regulated in a variety of cancers (Lerner, Harada et al. 2009). The ability of green tea polyphenols to up-regulate the expression of miR-16 even at concentrations as low as 1 μg/ml in breast cancer cells may be one of the reasons green tea is proving to be so beneficial in reducing breast cancer development.

miRNA time-response to Polyphenon 60 treatment

miR-200c, which had a dramatic time-dependent decrease in expression ranging from a 5-fold increase to a return to control level expression, has several verified target genes. Such targets include, ZEB1 (deltaEF1) and ZEB2 (SIP1) (Park, Gaur et al. 2008), which are E-cadherin transcriptional repressors implicated in tumor metastasis, TUBB3 neuronal beta tubulin (Cochrane, Spoelstra et al. 2009), which has been implicated in resistance of breast cancer to microtubule binding chemotherapeutics, and BMI1 (Shimono, Zabala et al. 2009), a regulator for stem cell renewal responsible for clonal expansion of cancerous cells (Table 4-2). These previous studies were all done analyzing the effect of miR-200c on breast cancer. Previously presented microarray data identified miR-200c as one of the most over-expressed

miRNAs in the expression profile of MCF-7 cells and gave the initial reasoning behind further investigating how its expression is altered following green tea treatment. The findings presented here show that Polyphenon 60 is able to increase the expression of miR-200c for a short period of time following the start of treatment. Based on previous data indicating that miR-200c is typically down-regulated in breast cancer samples, this ability of green tea to temporarily enhance the expression of this miRNA may be critical in why green tea seems to be anti-oncogenic. Biologically, green tea polyphenols are only in their combined and active forms for a limited amount of time and at very low concentrations if ingested orally through brewed tea. The shortest treatment time and the lowest concentration tested gave the highest amount of miR-200c expression which most closely simulates the effects of drinking green tea.

miR-720 has no previously reported target genes but has been shown to be down-regulated in cases of renal cell carcinoma (Chow TF 2009). The dose-response data indicates that Polyphenon 60 treatment caused an up-regulation at 5 μg/ml. Upon further analysis, it was shown that miR-720 reaches its greatest expression increase at 6 μg/ml after 24 hours of treatment. Again, the shorter treatment time is of interest because of the actual length of green tea polyphenol bioreactivity. Based on these findings and the previous report, green tea may be a treatment option for renal cell carcinoma, as well as breast cancer.

miR-92a also showed a time-dependent change in expression at 3 μg/ml, showing a significant decrease in expression at the 48 hour time point. The temporary down-regulation was more subtle at the 6 μg/ml treatment level, but was still present. Since no experimentation has been done to identify potential targets of miR-92a, it is difficult to hypothesize why this decrease in expression occurs solely after 48 hours. It is possible that the rate of catechin biodegradation allows the expression of a specific active polyphenol, such as EGCG, which alters the expression

of miR-92a only in the absence of the other extract components. Further analysis must be performed to identify if this temporary down-regulation is of any potential therapeutic use.

miR-27a has previously been shown to be up-regulated in breast cancer. Several verified targets have been identified, included cell cycle regulatory molecules, ZBTB10 and Myt-1 (Mertens-Talcott, Chintharlapalli et al. 2007), and the tumor suppressors, FOXO1 (Guttilla and White 2009) and Prohibitin (Liu, Tang et al. 2009). Since miR-27a is able to down-modulate the expression of tumor suppressor genes, it can be classified as an oncogene-like miRNA or oncomir. miR-27a showed a significant decrease in expression at 3 μg/ml following 24 hours of treatment but returned to the levels similar to the control at 72 hours. The initial down-regulation at low doses of Polyphenon 60 may be of initial therapeutic interest, but the increase in expression following continued treatment seems to invoke potential problems.

This investigation was performed to identify the effects of green tea exposure on miRNA expression levels; however, an individual's intake of green tea can vary from one cup a day to continuous exposure throughout ones life. Green tea has been incorporated into a wide variety of products world-wide, ranging from health supplements to candy bars. Analysis of the concentration and time points that seem to cause the most beneficial up- or down-regulation of the key miRNAs critical in limiting breast cancer development indicate that lower doses given for shorter periods of time give the best response. This most closely mimics the effects of drinking green tea and may give further credit to the epidemiological studies linking the decrease in breast cancer incidence found in Eastern countries to the increased intake of green tea. The biological effects of having a constant and high dose intake of green tea may not hold the benefits that moderate use seems to suggest. Therefore, future studies will have to be performed to analyze how the massive incorporation of green tea into the Western diet affects disease rates.

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Table 4-1: Literature analysis of the disease - involvement of key dose response miRNAs

miRNA	**Diseases with miRNA down-regulation**	**Diseases with miRNA up-regulation**
miR-720	↓ Renal Cell Carcinoma (Chow TF 2009)	
miR-200c	↓ Breast Cancer(Gregory PA 2008 May.; Tryndyak, Beland et al. 2009) ↓ Colorectal Cancer(Xi, Shalgi et al. 2006) ↓ Kidney Cancer (Chow TF 2009) ↓ Lung Cancer (Gibbons DL 2009) ↓ Non-alcoholic fatty liver disease (Zheng L 2009) ↓ Oral Squamous Cell Carcinoma (Kozaki, Imoto et al. 2008)	↑Endometrial Cancer (Cochrane, Spoelstra et al. 2009) ↑ Ovarian Cancer (Iorio, Visone et al. 2007) (Cochrane, Spoelstra et al. 2009) ↑Lupus nephritis(Dai Y 2008) ↑ Malignant melanoma (Schultz, Lorenz et al. 2008) ↑ Testicular germ cell tumor(Gillis, Stoop et al. 2007)
miR-16	↓ Chronic lymphocytic leukemia(Raveche, Salerno et al. 2007) ↓ Gastric Cancer (Xia L 2008) ↓ Glioma (Malzkorn B 2009) ↓ Lung Cancer (Crawford M 2009) ↓ Oral Squamous Cell carcinoma (Yu, Wang et al. 2009) ↓ Papillary Thyroid Carcinoma (Gao Y 2009) ↓ Prostate Cancer (Schäfer A 2009)	↑ Hepatocellular carcinoma (Huang, Wang et al. 2009) ↑ Hodgkin's Lymphoma (Van Vlierberghe, Poppe et al. 2008) ↑ Serous ovarian cancer (Nam EJ 2008) ↑Ulcerative colitis (Wu, Zikusoka et al. 2008)
miR-26b	↓ Alzheimer's disease (Hebert, Horre et al. 2009) ↓ Cardiac Hypertrophy(Sayed, Hong et al. 2007) ↓ Hepatocellular Carcinoma (Ji, Shi et al. 2009) ↓Schizophrenia (Perkins, Jeffries et al. 2007) ↓ Oral Squamous Cell Carcinoma (Kozaki, Imoto et al. 2008) ↓ Prostate Cancer (Porkka, Pfeiffer et al. 2007)	↑ Bladder Cancer (Gottardo F 2007) ↑ Epithelial Ovarian Cancer (Zhang L 2008) ↑ Pituitary Adenoma (Bottoni, Zatelli et al. 2007)

Table 4-2: Literature analysis of verified targets for time-response altered miRNA

miRNA	Cancer	Validated targets	Function of target gene	References
miR-200c	Breast Cancer (down-regulated)	ZEB1(deltaEF1) / SIP1(ZEB2)	E-cadherin transcriptional repressors *Implicated in tumor metastasis	(Park, Gaur et al. 2008)
	Breast Cancer (down-regulated)	TUBB3	Class III beta-tubulin- neuron specific *Expression linked to resistance to microtubule binding chemotherapeutics	(Cochrane, Spoelstra et al. 2009)
	Breast Cancer (down-regulated)	BMI1	Stem cell renewal regulator *Responsible for clonal expansion of breast cancer cells	(Shimono, Zabala et al. 2009)
miR-27a	Breast Cancer (up-regulated)	ZBTB10 / Myt-1	*Regulators of G2/M checkpoint	(Mertens-Talcott, Chintharlapalli et al. 2007)
	Breast Cancer (up-regulated)	FOXO1	Tumor suppressor *Transcription factor regulating apoptosis and cell cycle checkpoints	(Guttilla and White 2009)
	Gastric Cancer (up-regulated)	Prohibitin	Transcriptional modulator / potential tumor suppressor *located in the BRCA1 gene region	(Liu, Tang et al. 2009)
miR-92a	No previously reported targets			
miR-720	No previously reported targets			

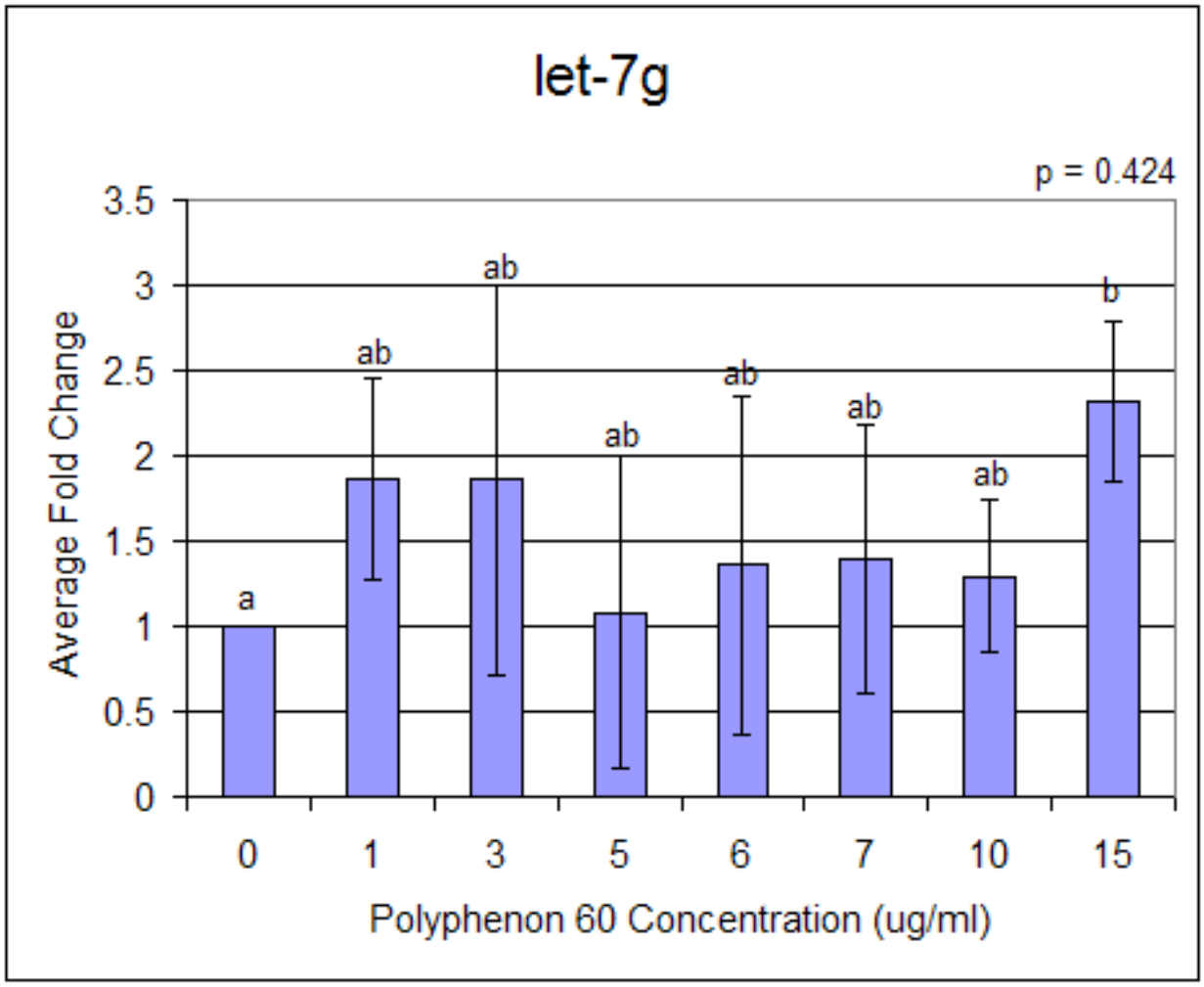

Figure 4-1: qRT-PCR analysis of average fold change expression for let-7g over a range of Polyphenon 60 concentrations from 1 μg/ml to 15 μg/ml. Statistically significant changes in expression between concentrations are designated by single letters ($p \leq 0.05$). p-value shown is the calculated ANOVA value for the complete data set.

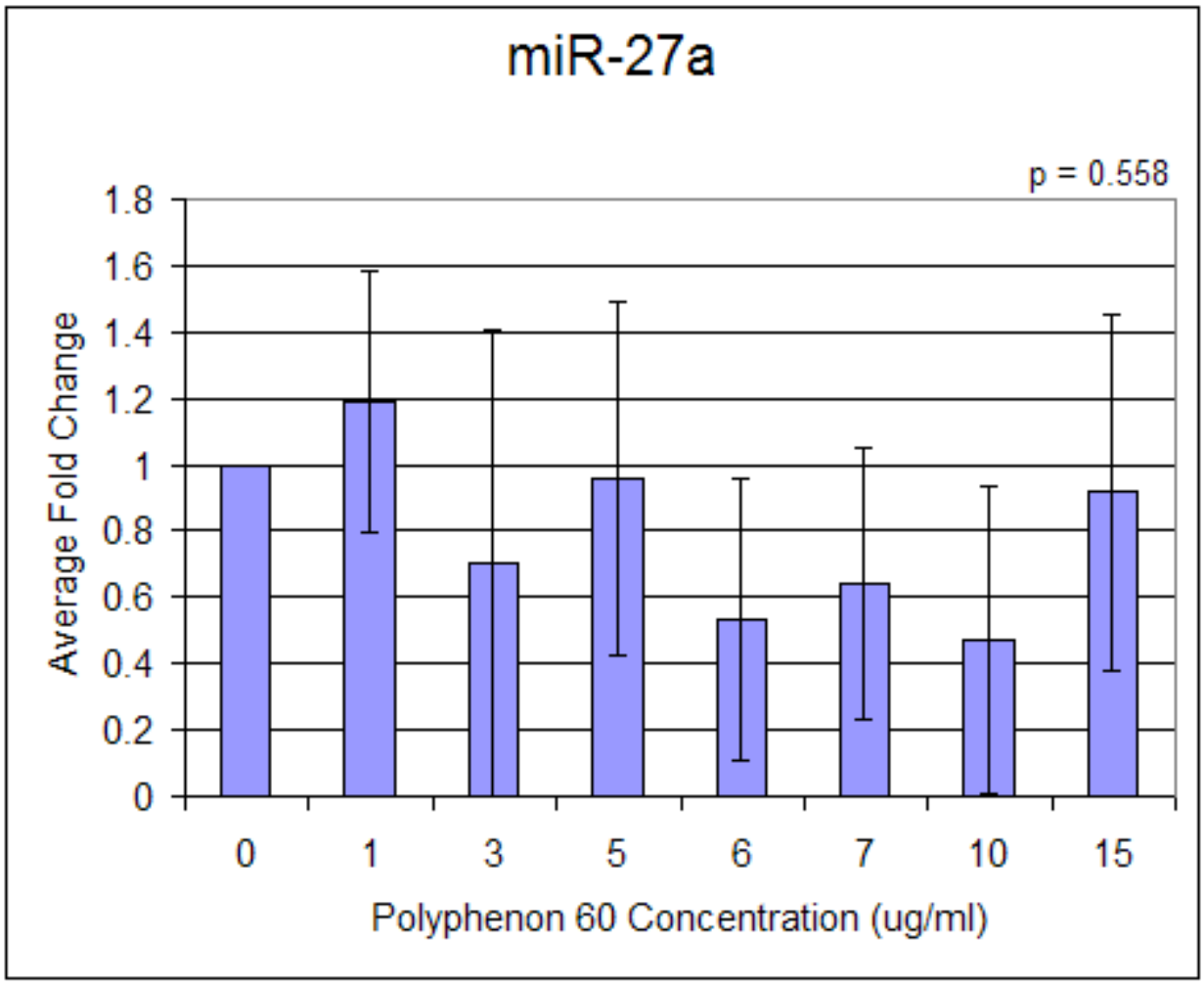

Figure 4-2: qRT-PCR analysis of average fold change expression for miR-27a over a range of Polyphenon 60 concentrations from 1 µg/ml to 15 µg/ml. p-value shown is the calculated ANOVA value for the complete data set.

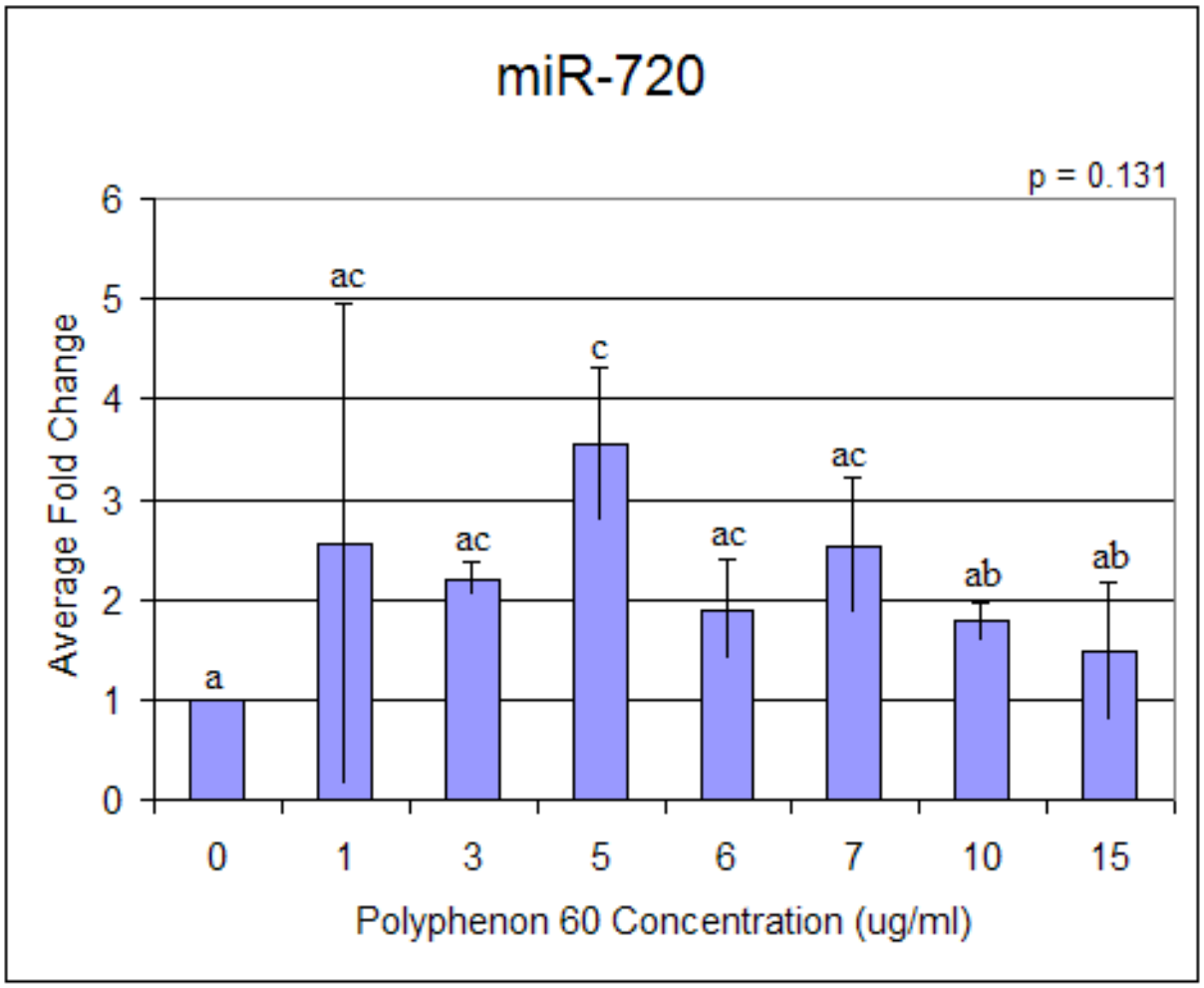

Figure 4-3: qRT-PCR analysis of average fold change expression for miR-720 over a range of Polyphenon 60 concentrations from 1 μg/ml to 15 μg/ml. Statistically significant changes in expression are designated by single letters ($p \leq 0.05$). p-value shown is the calculated ANOVA value for the complete data set.

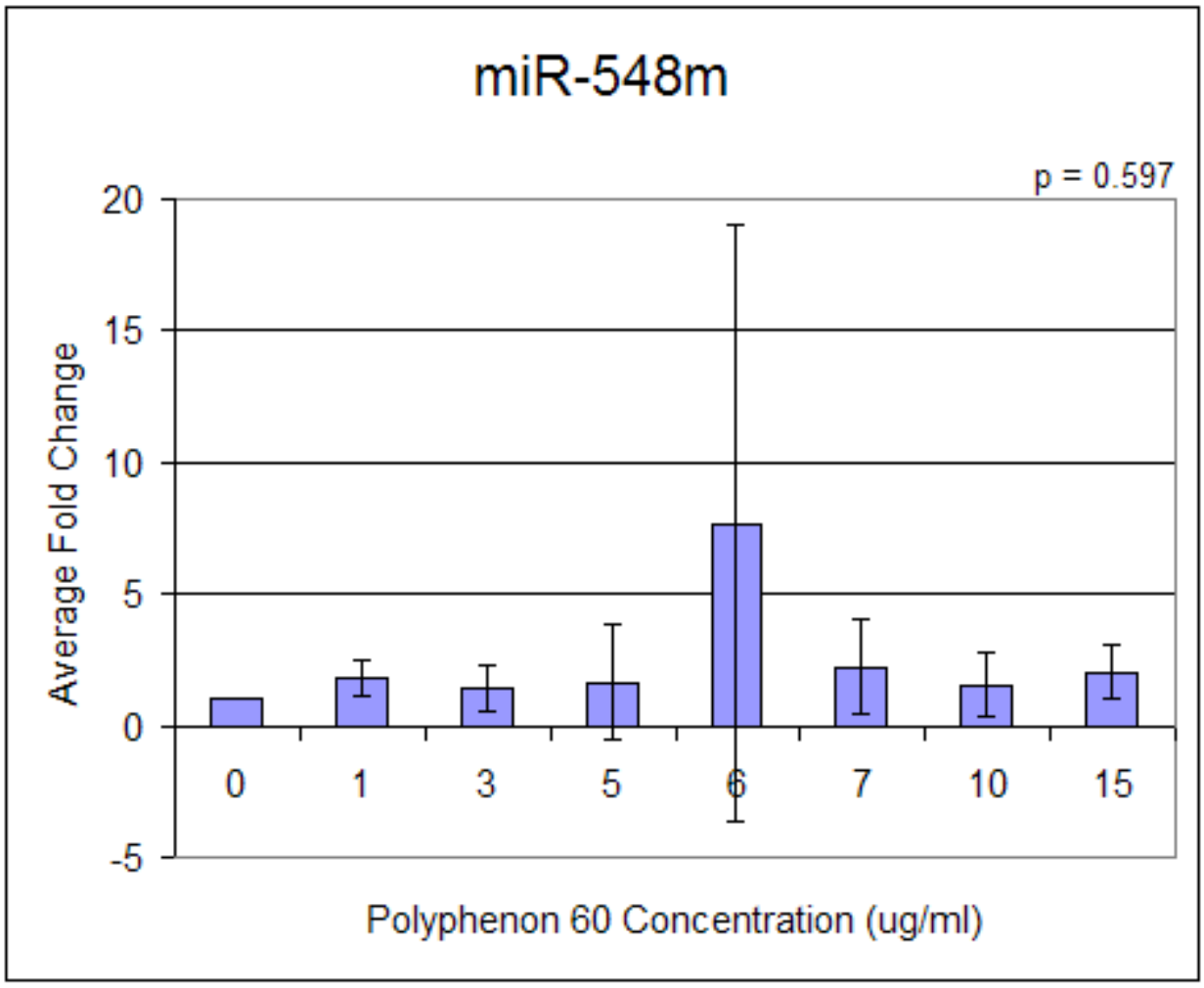

Figure 4-4: qRT-PCR analysis of average fold change expression for miR-548m over a range of Polyphenon 60 concentrations from 1 µg/ml to 15 µg/ml. p-value shown is the calculated ANOVA value for the complete data set.

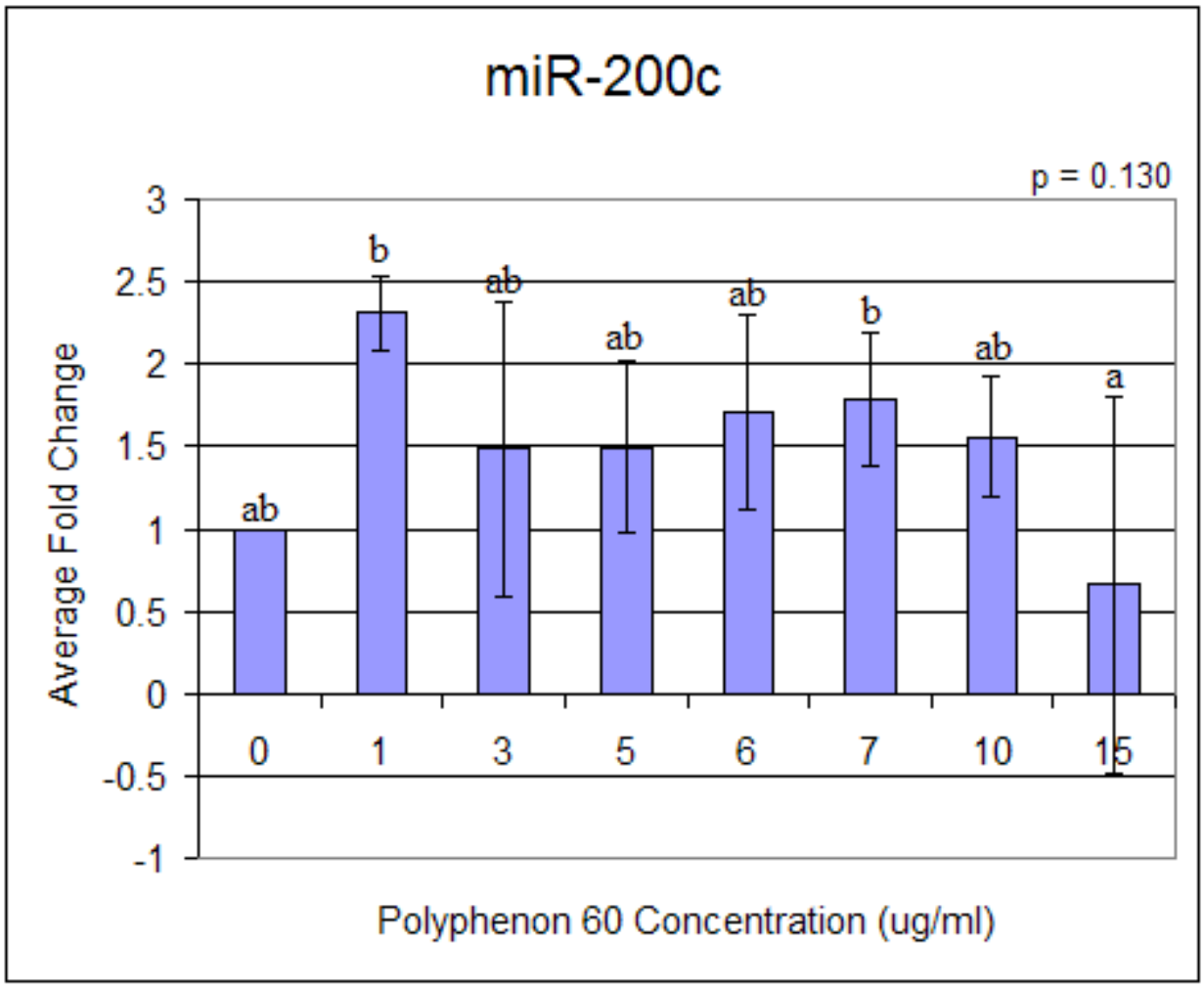

Figure 4-5: qRT-PCR analysis of average fold change expression for miR-200c over a range of Polyphenon 60 concentrations from 1 μg/ml to 15 μg/ml. Statistically significant changes in expression are designated by single letters ($p \leq 0.05$). p-value shown is the calculated ANOVA value for the complete data set.

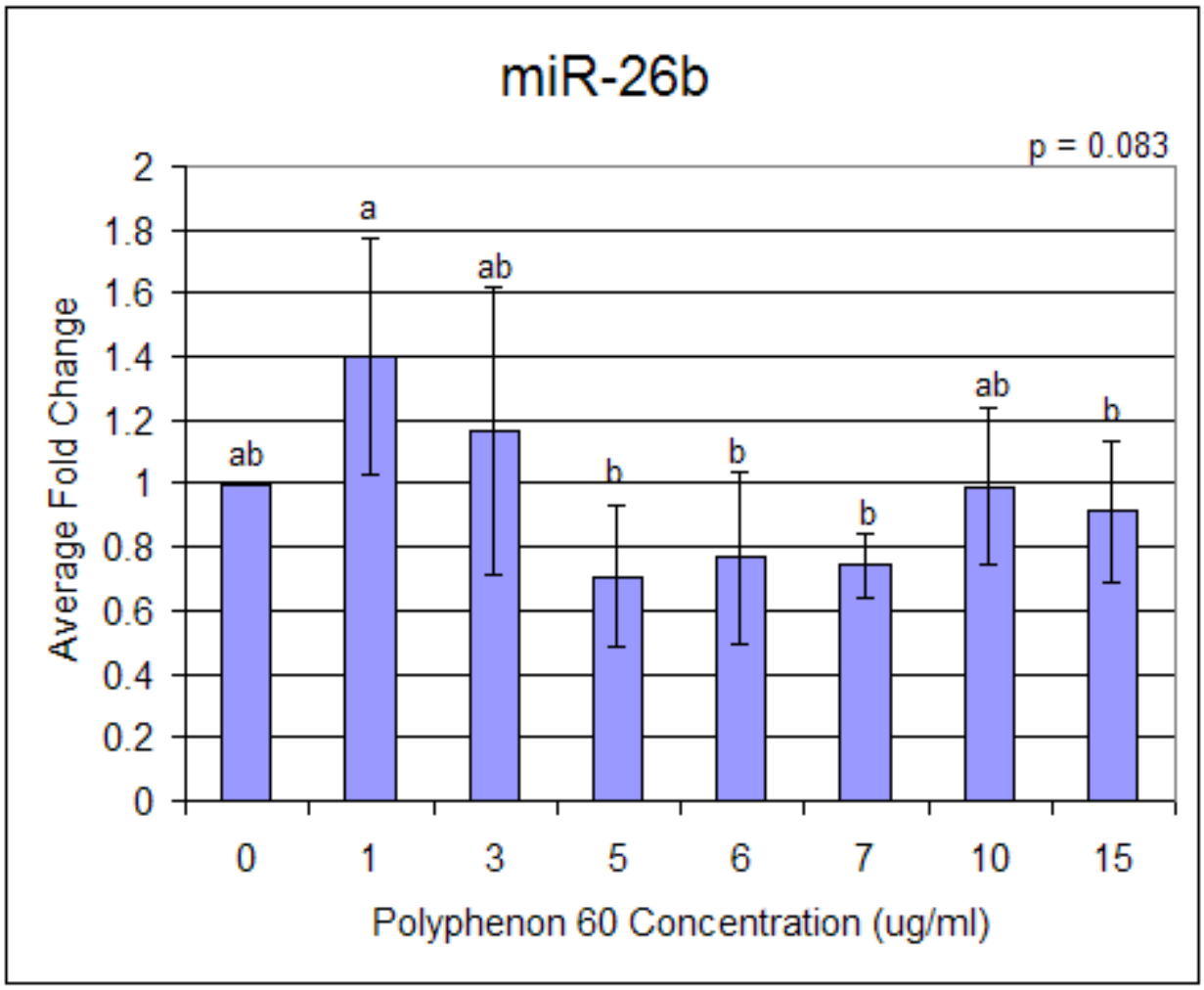

Figure 4-6: qRT-PCR analysis of average fold change expression for miR-26b over a range of Polyphenon 60 concentrations from 1 μg/ml to 15 μg/ml. Statistically significant changes in expression are designated by single letters ($p \leq 0.05$). p-value shown is the calculated ANOVA value for the complete data set.

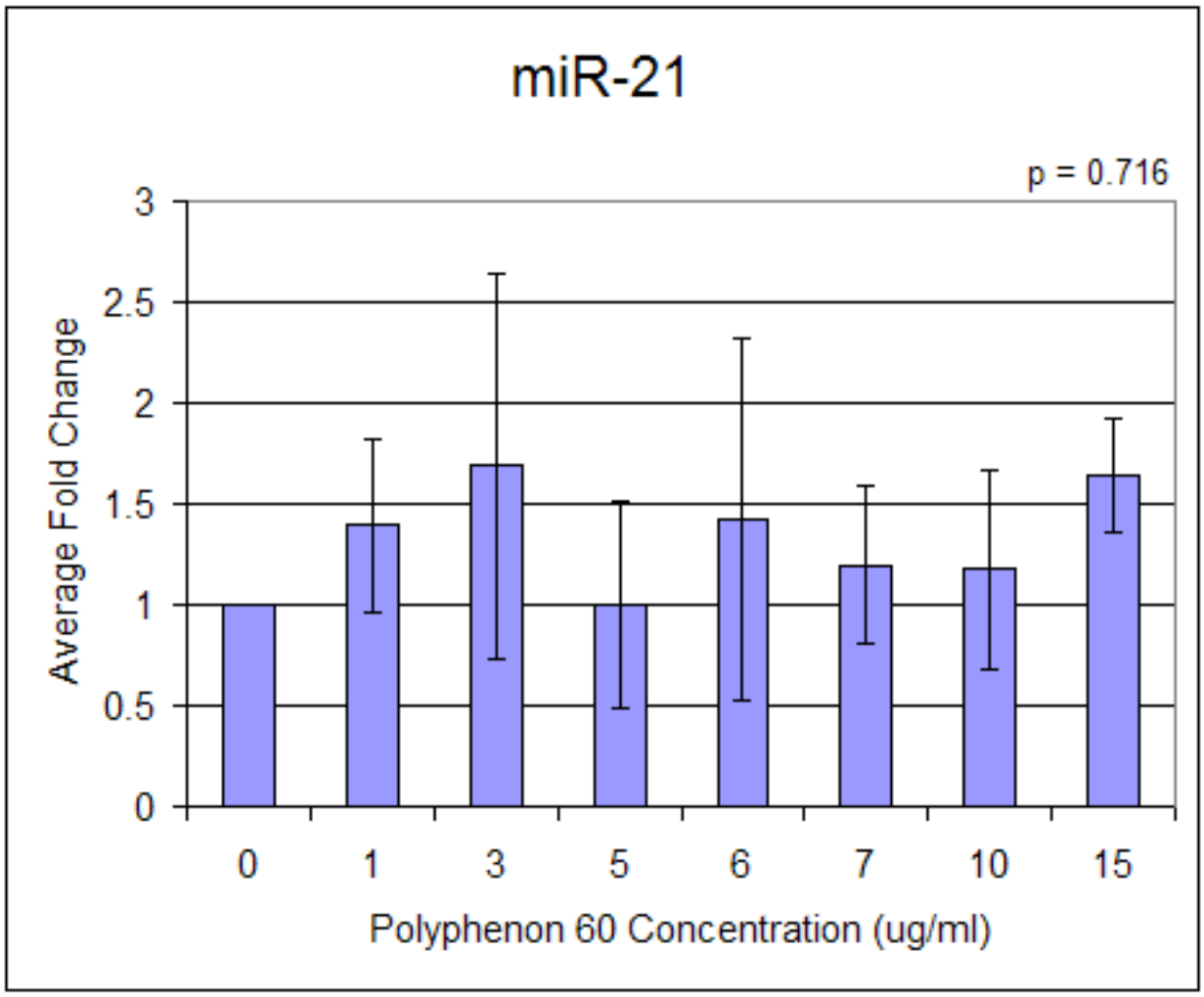

Figure 4-7: qRT-PCR analysis of average fold change expression for miR-21 over a range of Polyphenon 60 concentrations from 1 µg/ml to 15 µg/ml. p-value shown is the calculated ANOVA value for the complete data set.

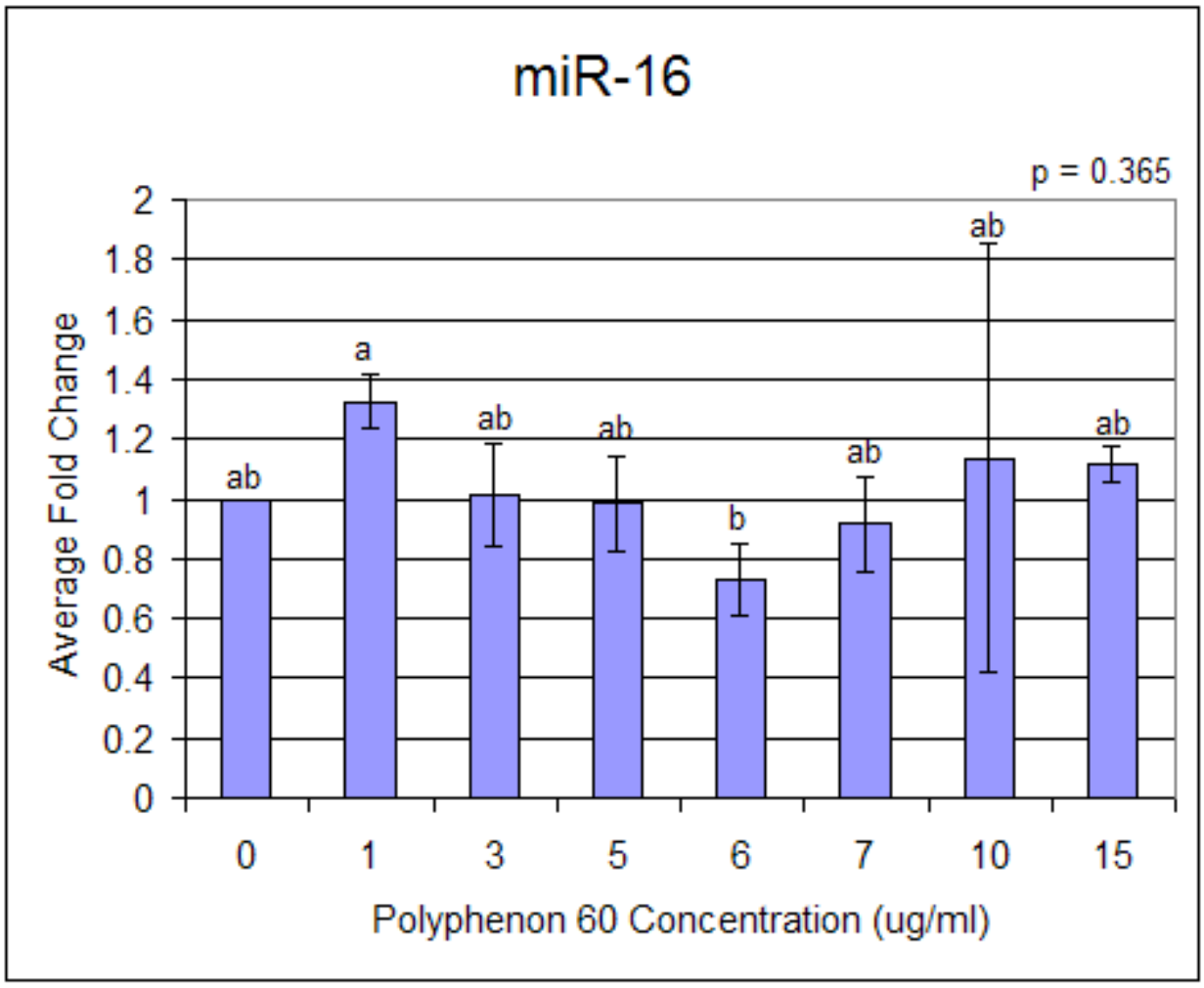

Figure 4-8: qRT-PCR analysis of average fold change expression for miR-16 over a range of Polyphenon 60 concentrations from 1 μg/ml to 15 μg/ml. Statistically significant changes in expression are designated by single letters ($p \leq 0.05$). p-value shown is the calculated ANOVA value for the complete data set.

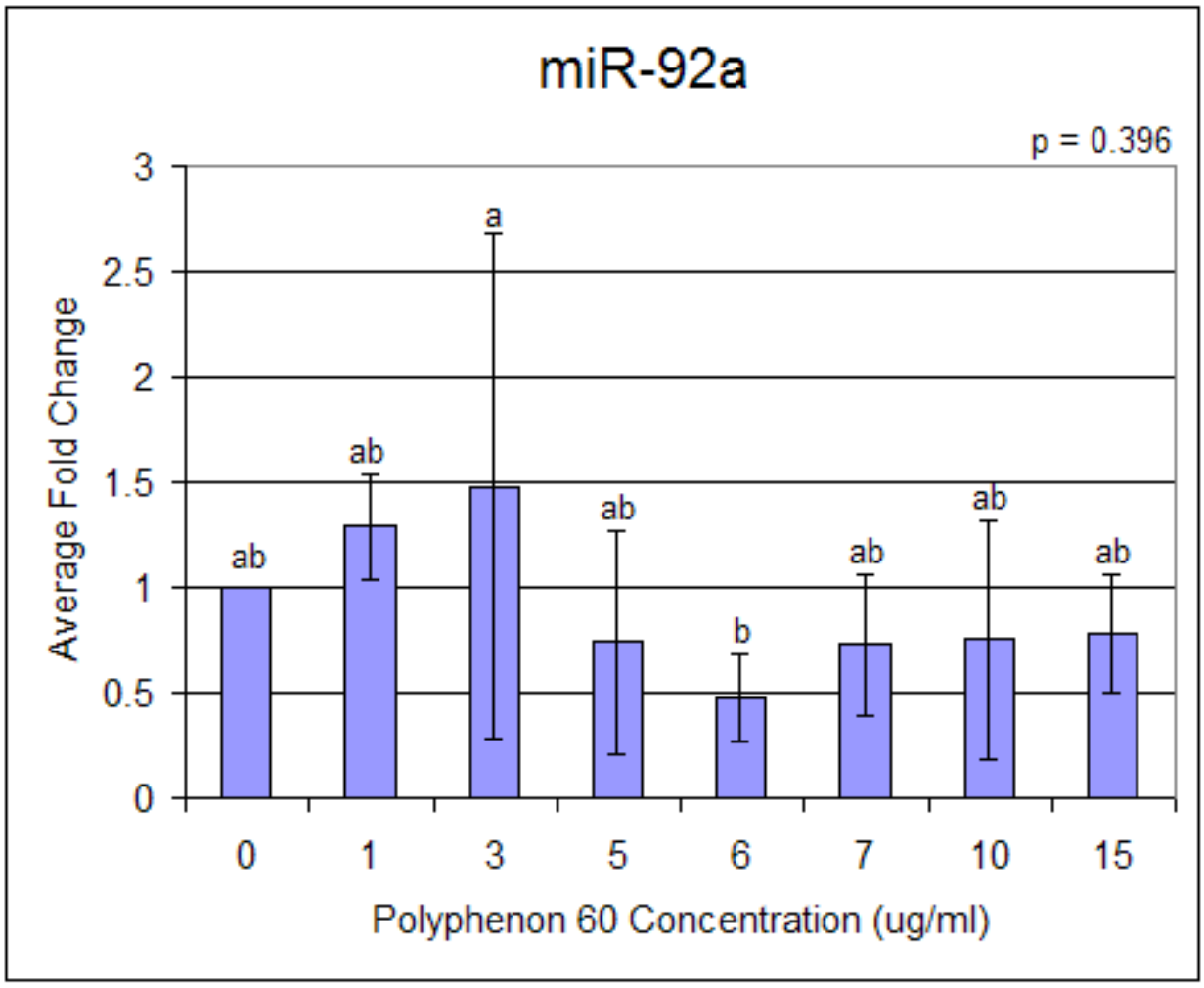

Figure 4-9: qRT-PCR analysis of average fold change expression for miR-92a over a range of Polyphenon 60 concentrations from 1 μg/ml to 15 μg/ml. Statistically significant changes in expression are designated by single letters ($p \leq 0.05$). p-value shown is the calculated ANOVA value for the complete data set.

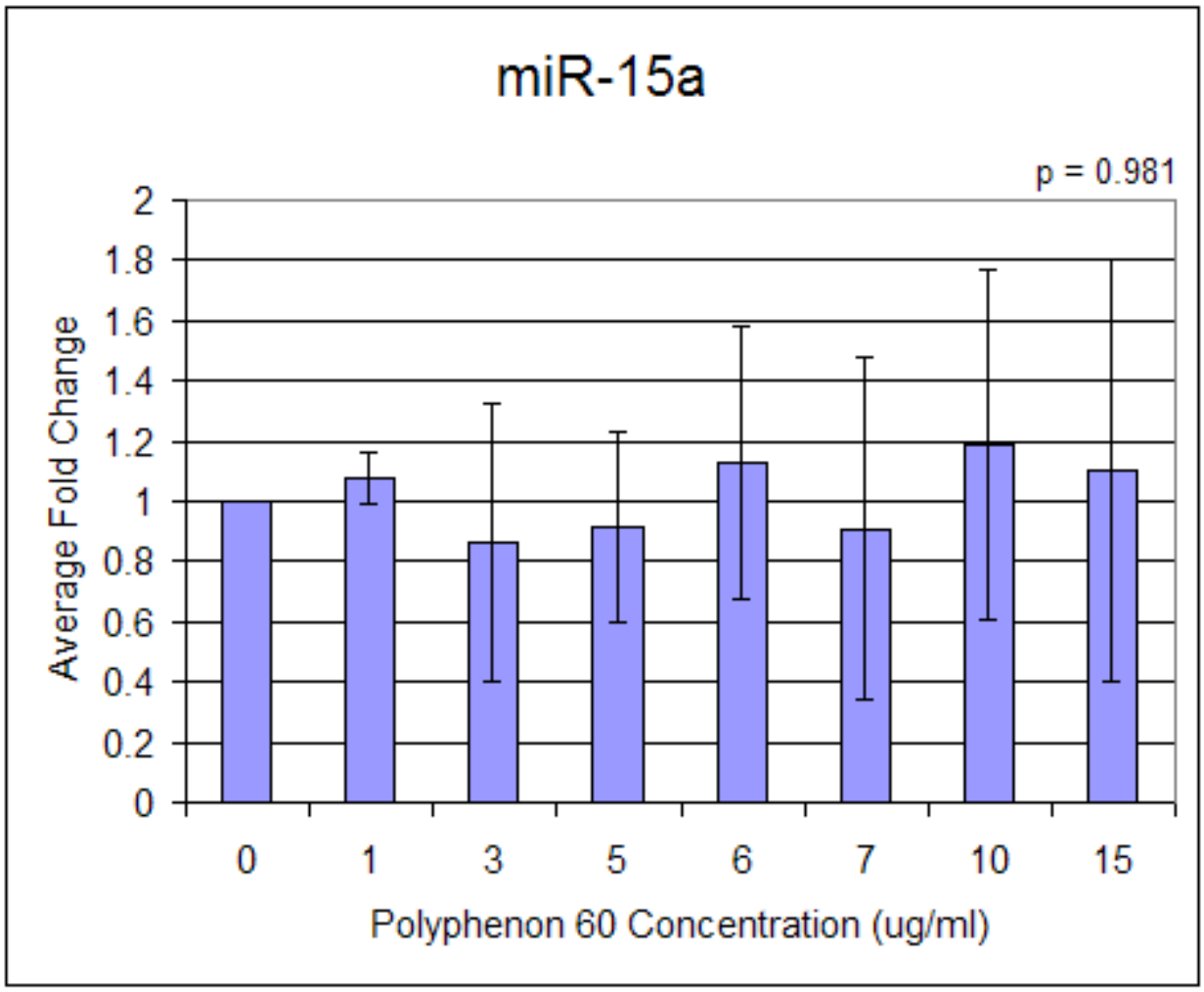

Figure 4-10: qRT-PCR analysis of average fold change expression for miR-15a over a range of Polyphenon 60 concentrations from 1 µg/ml to 15 µg/ml. p-value shown is the calculated ANOVA value for the complete data set.

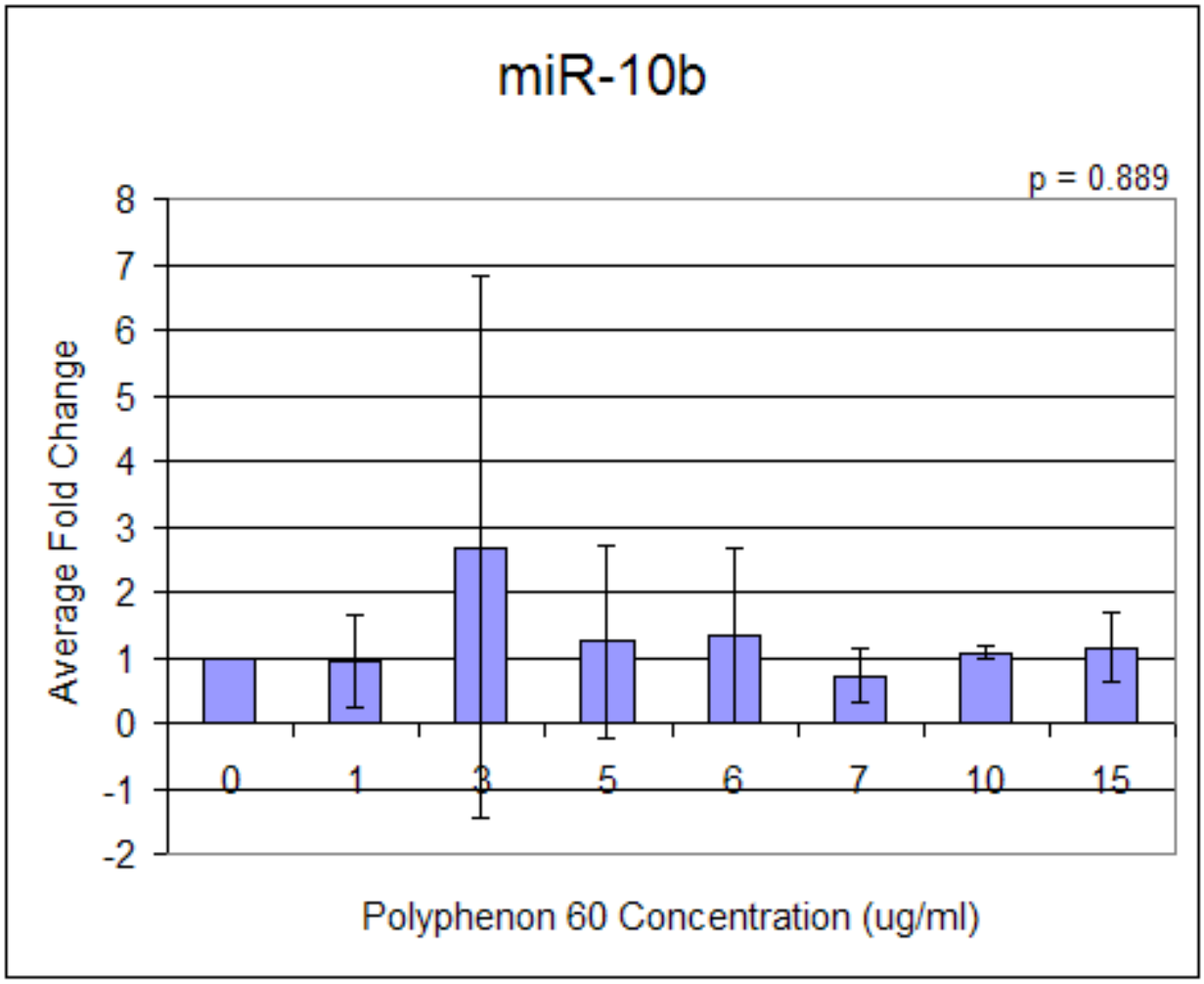

Figure 4-11: qRT-PCR analysis of average fold change expression for miR-10b over a range of Polyphenon 60 concentrations from 1 µg/ml to 15 µg/ml. p-value shown is the calculated ANOVA value for the complete data set.

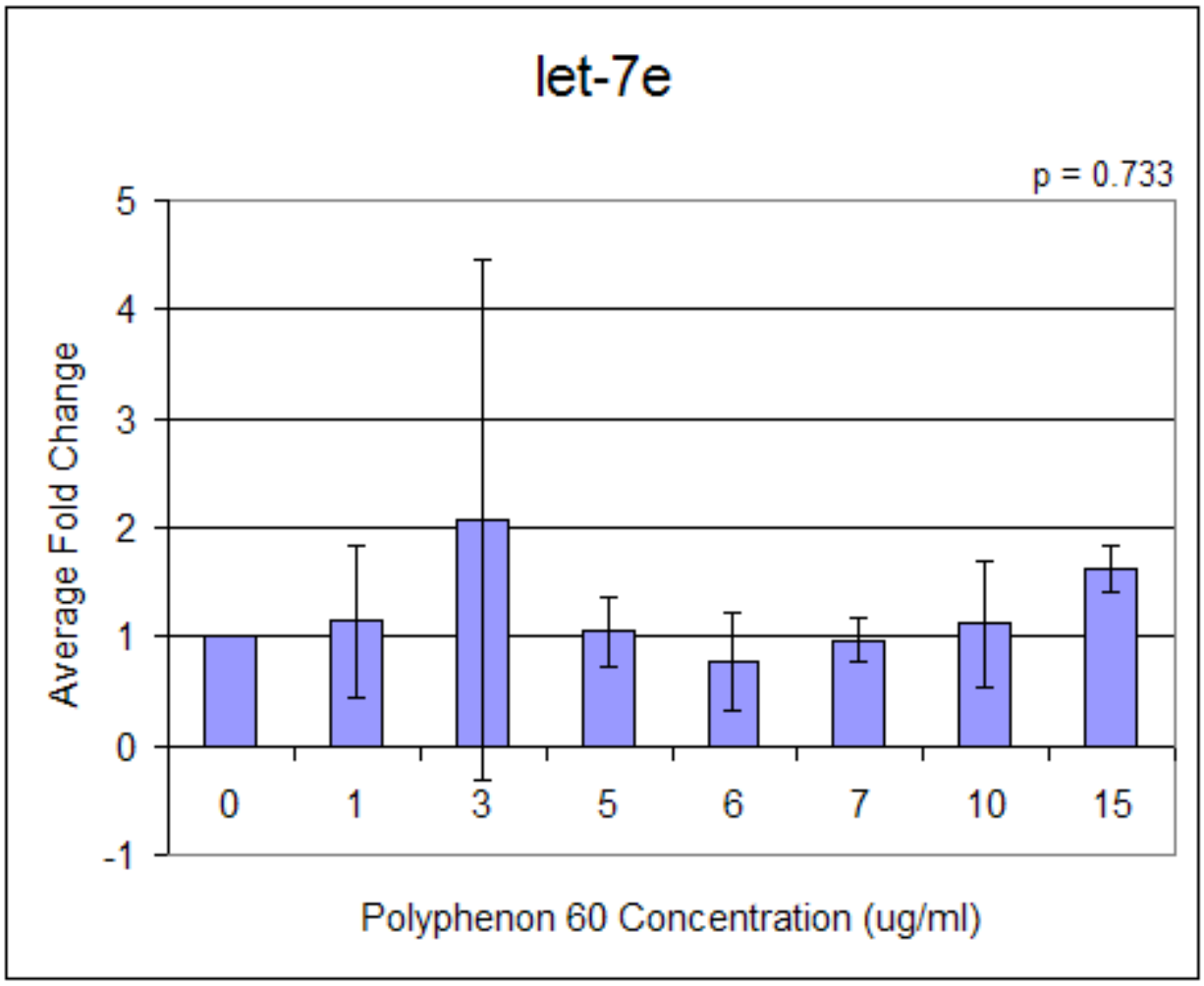

Figure 4-12: qRT-PCR analysis of average fold change expression for let-7e over a range of Polyphenon 60 concentrations from 1 µg/ml to 15 µg/ml. p-value shown is the calculated ANOVA value for the complete data set. p-value shown is the calculated ANOVA value for the complete data set.

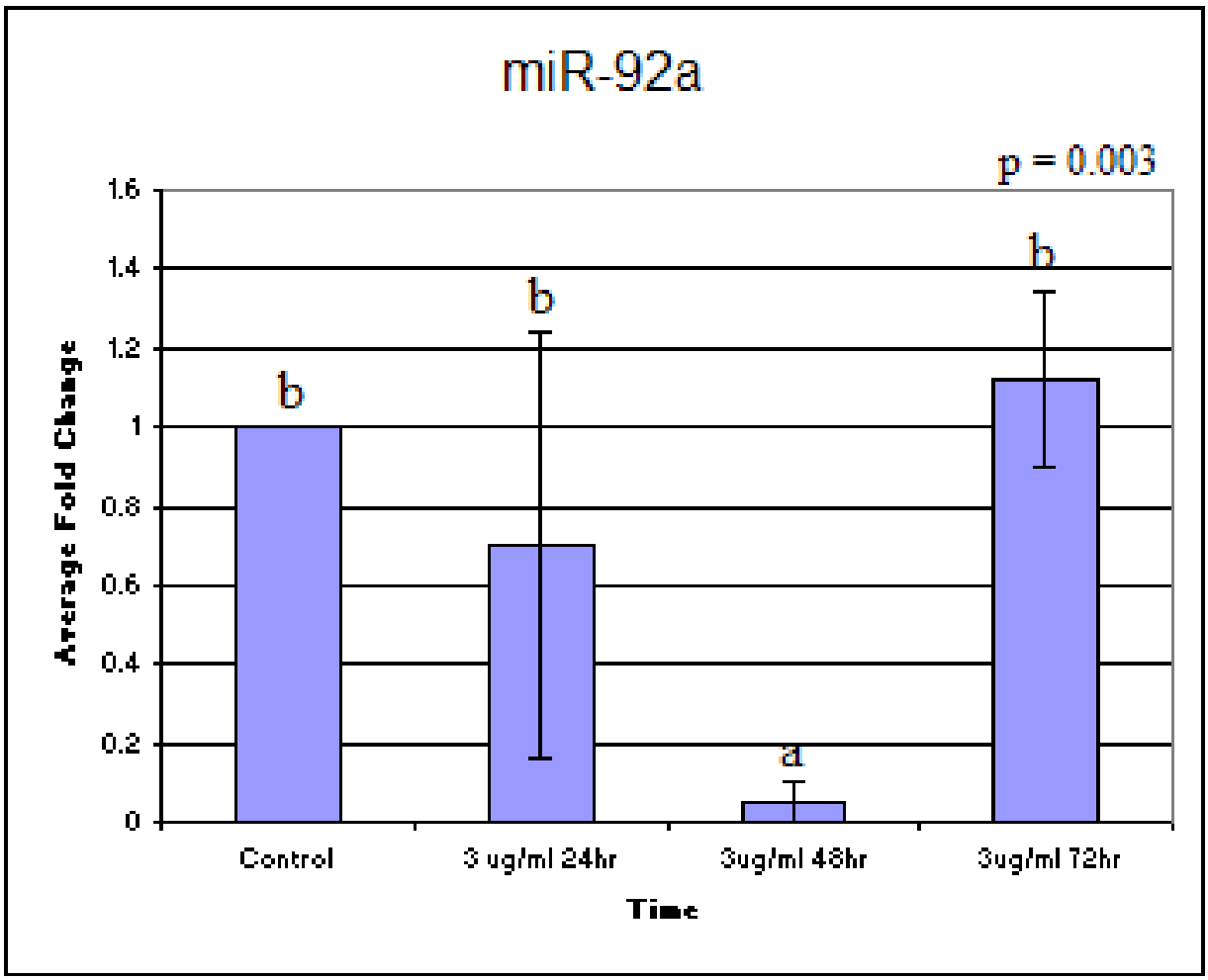

Figure 4-13: Average fold change in expression of miR-92a showing a time response to Polyphenon 60 treatment. Samples were treated for 24, 48 or 72 hours with 3 µg/ml of Polyphenon 60 in cell culture media. Letters denote statistically significant differences in expression for a specific miRNA over time ($p \leq 0.05$). p-value shown is the calculated ANOVA value for the complete data set.

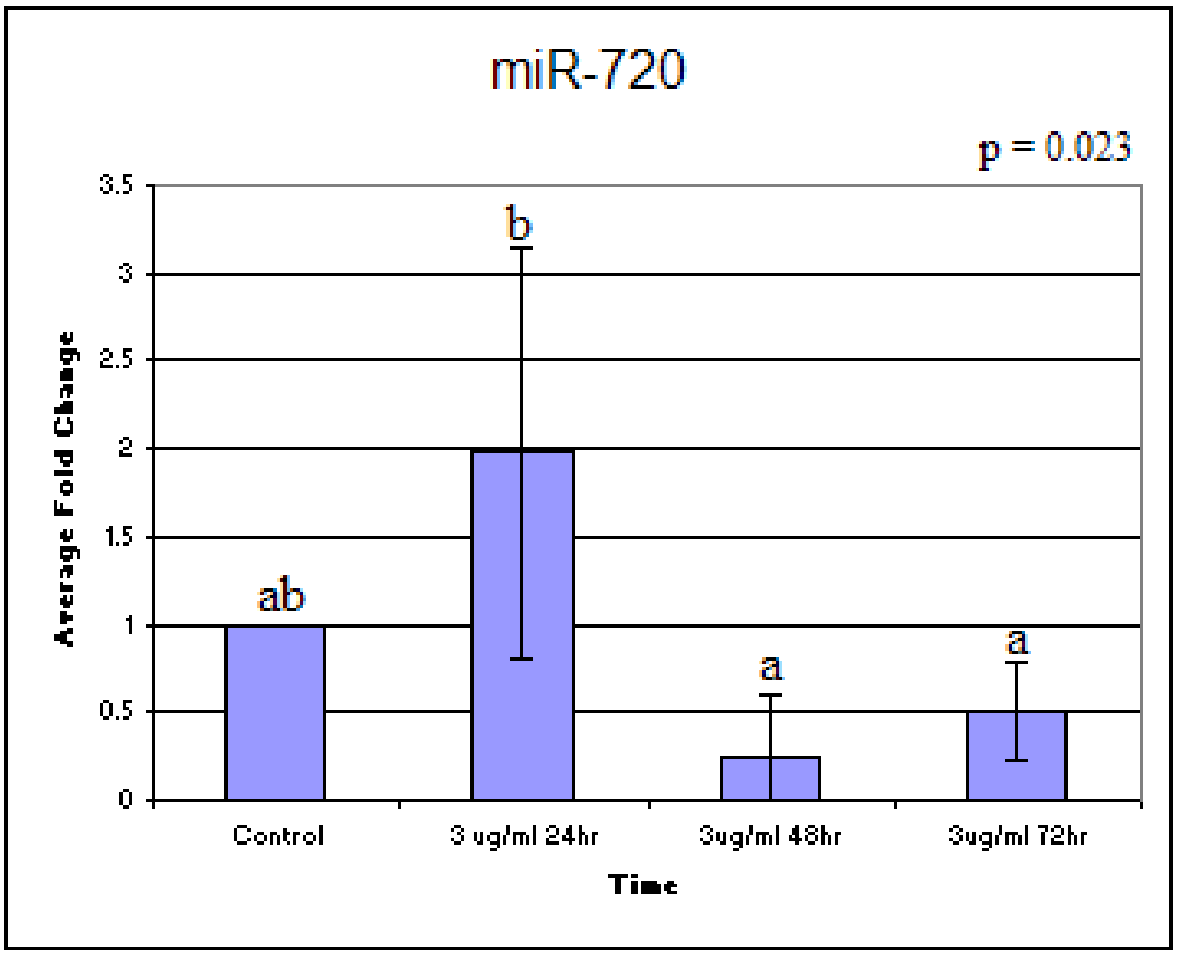

Figure 4-14: Average fold change in expression of miR-720 showing a time response to Polyphenon 60 treatment. Samples were treated for 24, 48 or 72 hours with 3 µg/ml of Polyphenon 60 in cell culture media. Letters denote statistically significant differences in expression for a specific miRNA over time ($p \leq 0.05$). p-value shown is the calculated ANOVA value for the complete data set.

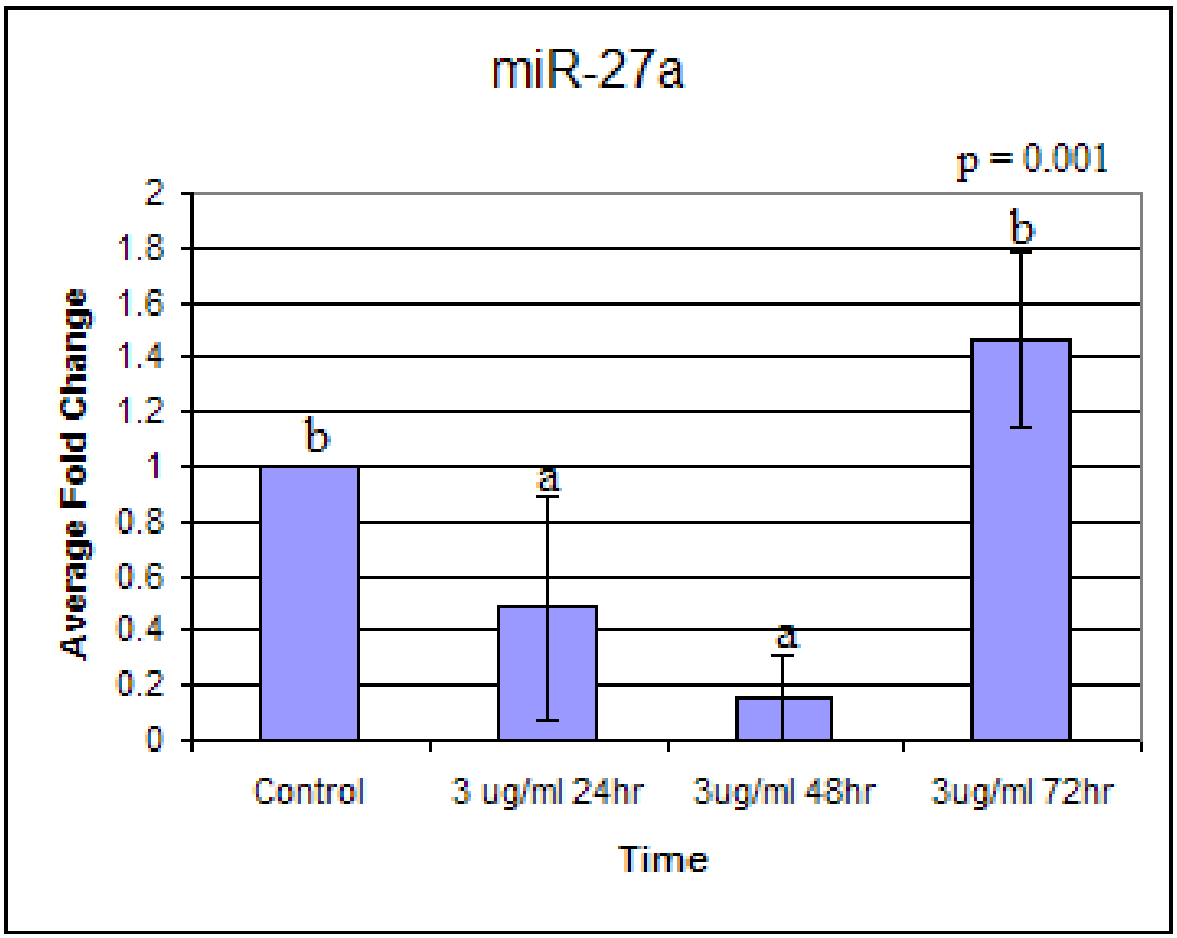

Figure 4-15: Average fold change in expression of miR-27a showing a time response to Polyphenon 60 treatment. Samples were treated for 24, 48 or 72 hours with 3 µg/ml of Polyphenon 60 in cell culture media. Letters denote statistically significant differences in expression for a specific miRNA over time ($p \leq 0.05$). p-value shown is the calculated ANOVA value for the complete data set.

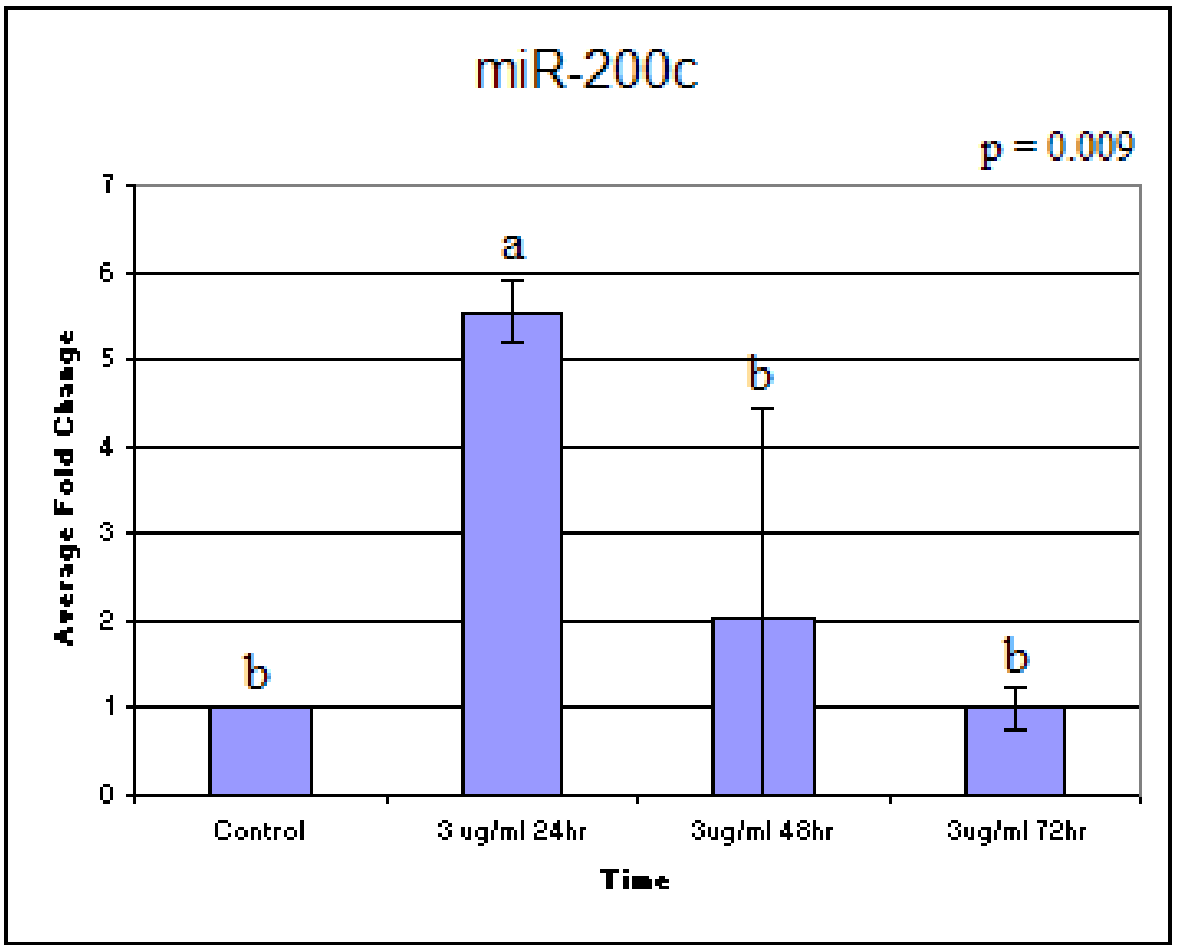

Figure 4-16: Average fold change in expression of miR-200c showing a time response to Polyphenon 60 treatment. Samples were treated for 24, 48 or 72 hours with 3 µg/ml of Polyphenon 60 in cell culture media. Letters denote statistically significant differences in expression for a specific miRNA over time ($p \leq 0.05$). p-value shown is the calculated ANOVA value for the complete data set.

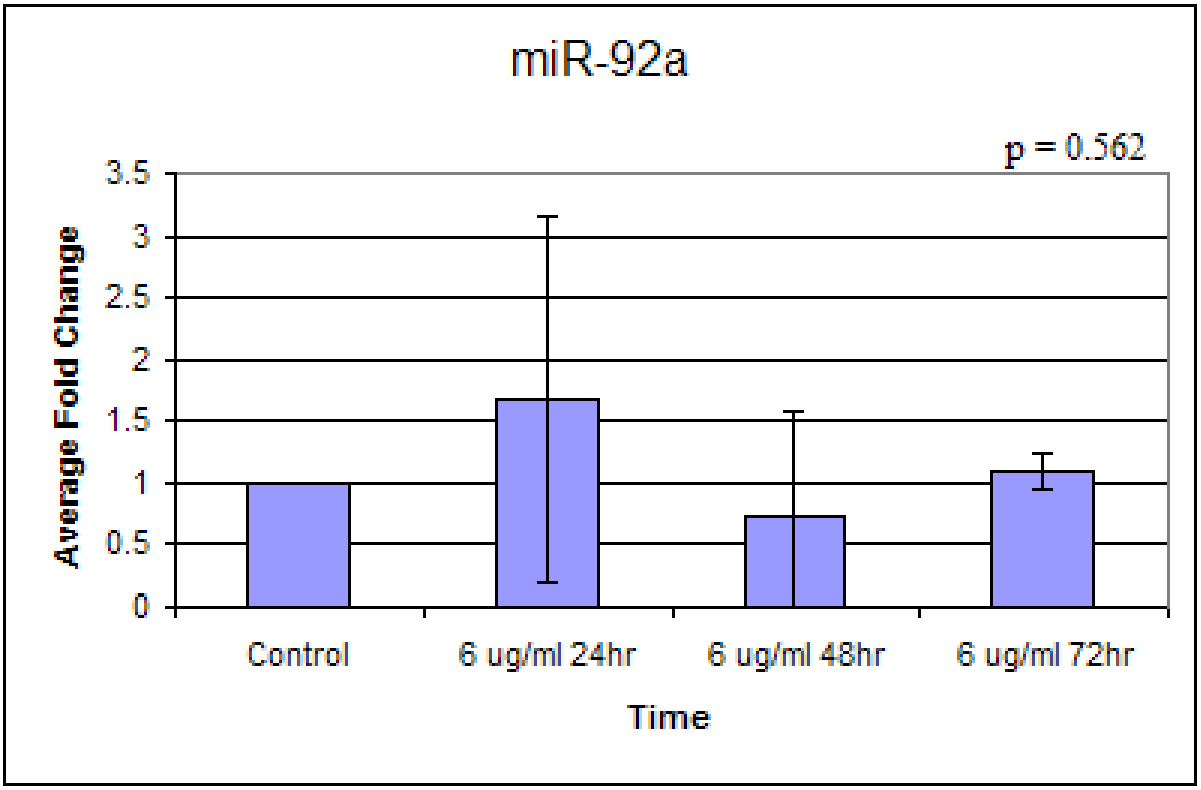

Figure 4-17: Average fold change in expression of miR-92a showing a time response to Polyphenon 60 treatment. Samples were treated for 24, 48 or 72 hours with 6 µg/ml of Polyphenon 60 in cell culture media. p-value shown is the calculated ANOVA value for the complete data set.

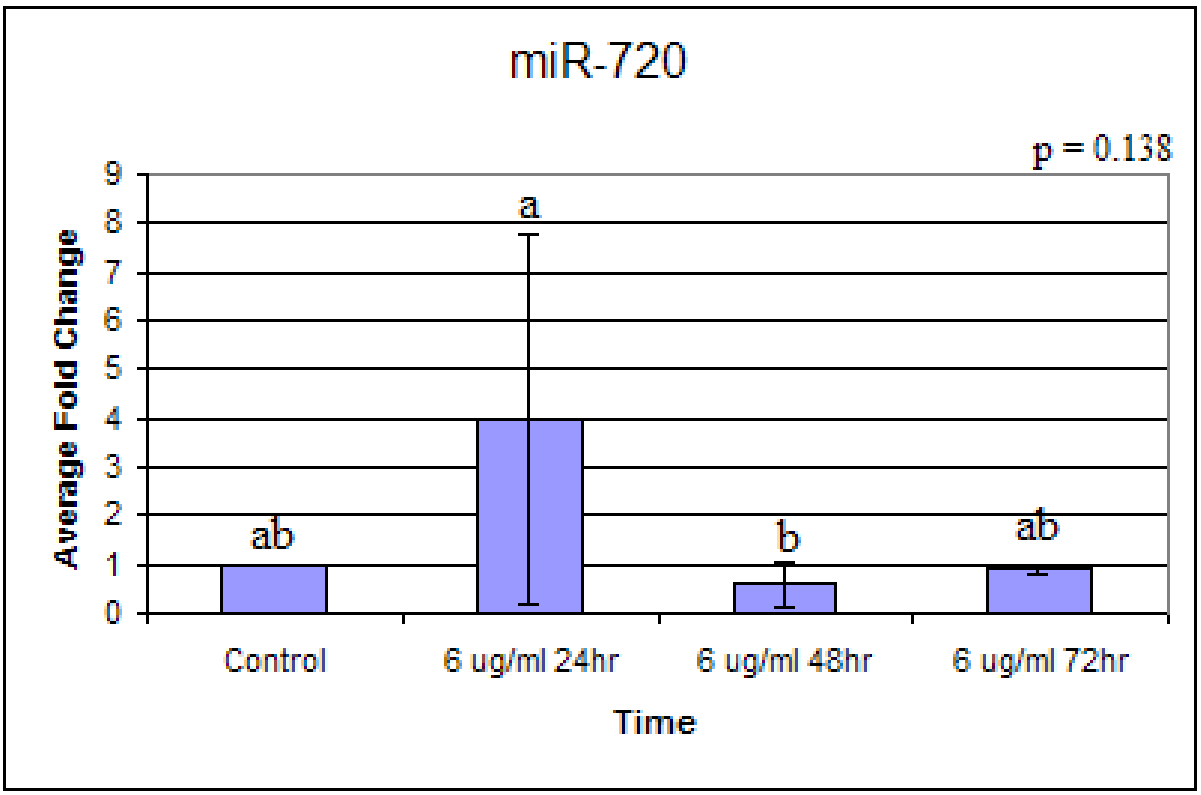

Figure 4-18: Average fold change in expression of miR-720 showing a time response to Polyphenon 60 treatment. Samples were treated for 24, 48 or 72 hours with 6 µg/ml of Polyphenon 60 in cell culture media. Different letters denote statistically significant differences in expression for a specific miRNA over time ($p \leq 0.05$). p-value shown is the calculated ANOVA value for the complete data set.

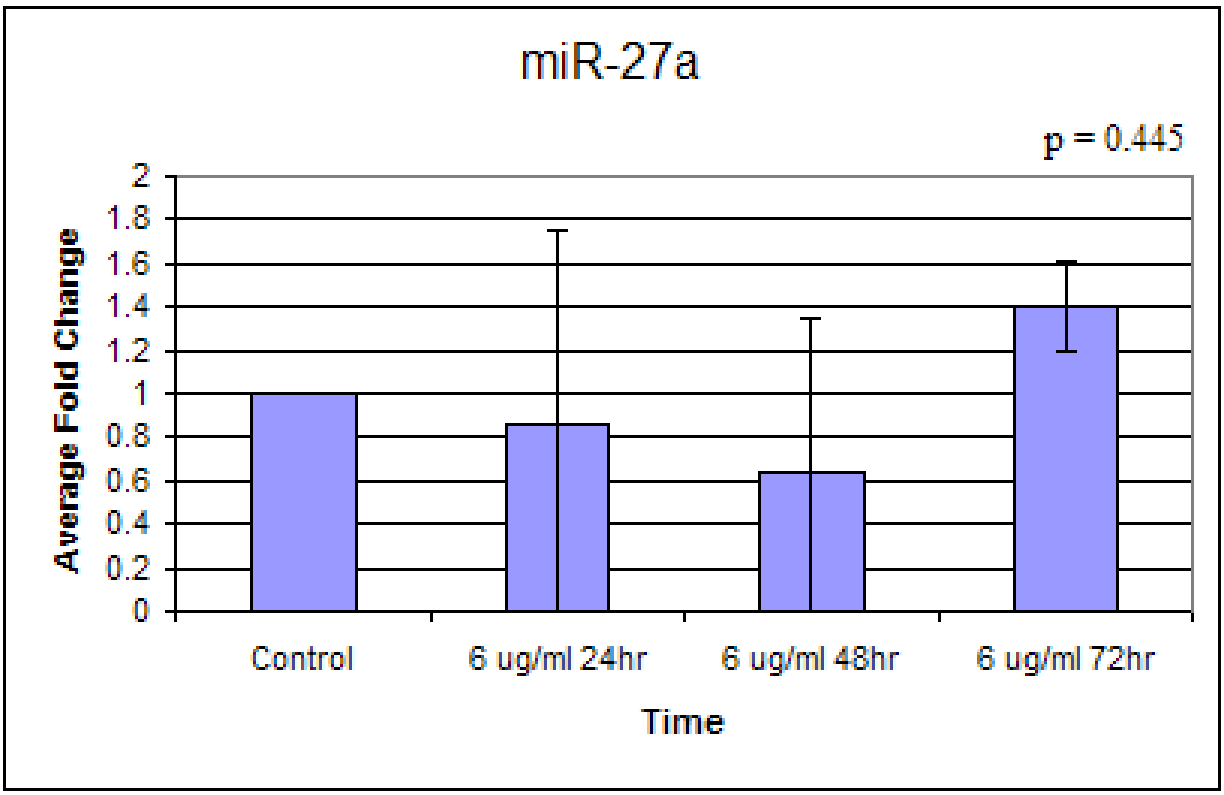

Figure 4-19: Average fold change in expression of miR-27a showing a time response to Polyphenon 60 treatment. Samples were treated for 24, 48 or 72 hours with 6 µg/ml of Polyphenon 60 in cell culture media. p-value shown is the calculated ANOVA value for the complete data set.

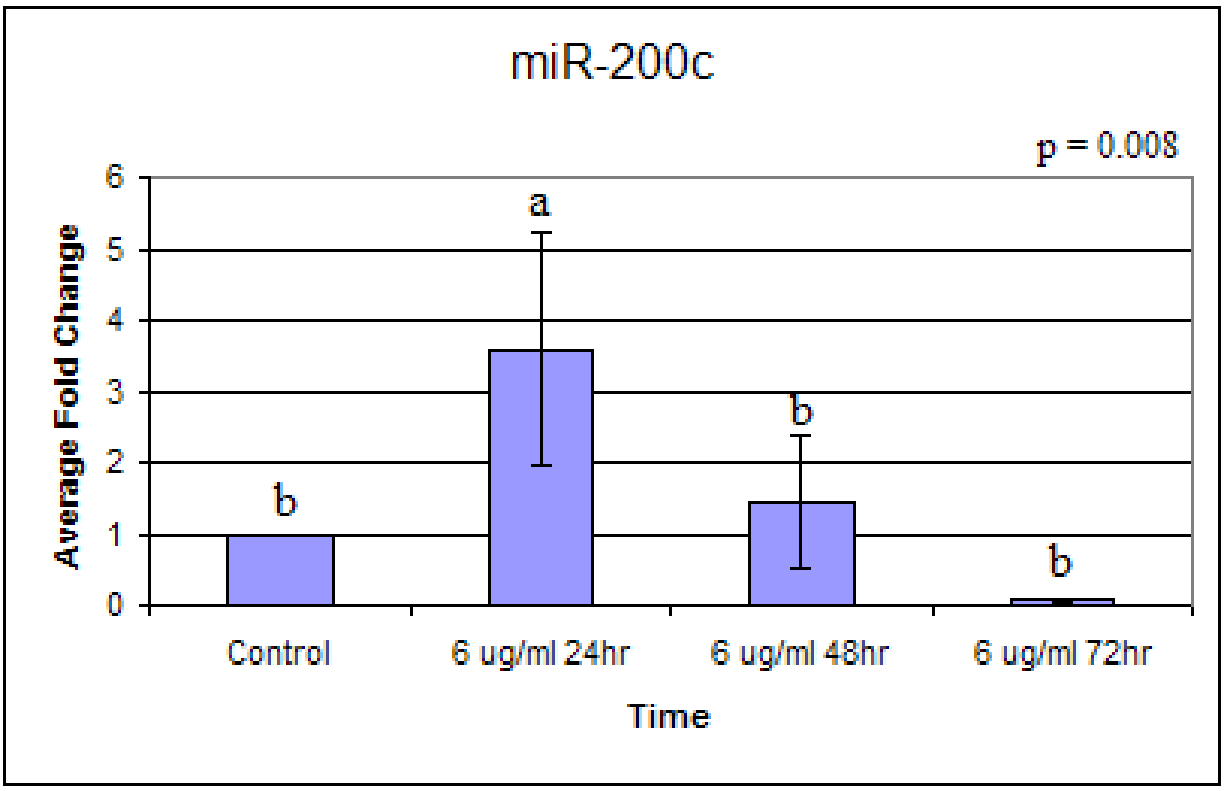

Figure 4-20: Average fold change in expression of miR-200c showing a time response to Polyphenon 60 treatment. Samples were treated for 24, 48 or 72 hours with 6 µg/ml of Polyphenon 60 in cell culture media. Letters denote statistically significant differences in expression for a specific miRNA over time ($p \leq 0.05$). p-value shown is the calculated ANOVA value for the complete data set.