University of Nevada, Reno

Myc Protein Expression in Response to Docosahexaenoic Acid

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by

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Myc Protein Expression in Response to Docosahexaenoic Acid Supplementation

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Abstract

Breast cancer is a commonly diagnosed cancer and targets 1 in 8 women in the United States, annually. Certain changes in DNA can induce normal cells to become abnormal, and a contributor to this is a gene called Myc, which has shown to be overexpressed in many cancers, including breast cancer. Myc was one of the first discovered oncogenes and it functions as a transcription factor that regulates nearly 15% of the genome. When Myc is mutated in cancer, called *c*-Myc, it functions abnormally and activates pro-survival and pro-proliferative pathways. Although decades of research have proven that it could serve as a promising chemotherapeutic target because it plays such an important role in cellular division, metabolism, and apoptosis, there has been no therapeutic success. Its dysregulation causes uncontrolled cell growth, which is a hallmark of cancer. Furthermore, overexpression of GLUT glucose transporter proteins can facilitate the development of many cancer types due to higher than normal glucose metabolism, named the *Warburg* effect. Nutritional supplementations, such as omega-3 (n-3) polyunsaturated fatty acids (PUFAs), have been known to lower the chances of developing cancer, especially in breast cancer. Preliminary evidence suggests that docosahexaenoic acid (DHA), an omega-3 fatty acid, has been found to inhibit cancer cell growth and alters the phosphorylation status of Myc. Analysis of Myc phosphorylation sites, which is critical for determining downstream Myc targets will provide a better understanding of the anti-cancer properties of DHA and examine the consequences of these alterations in breast cancer. Contributions to this research that has vast benefits can improve the quality of life for breast cancer victims.

Introduction

While there are many conventional forms of treatment for cancer, including chemotherapy and radiation, often times there are many adverse side effects, such as hair loss, nausea and fatigue. There is no guarantee that these treatments are successful. Not only does chemotherapy directly kill cancer cells, normal tissues are affected as well. The issue today is finding an alternative way to completely eliminate cancerous cells. For many years, it was sought that dietary factors can prevent or even treat cancer. Our lab is focusing on how nutritional supplementation may play a role in preventing the growth of tumors or cancerous cells.

Our lab focuses on combining nutritional supplementation and conventional cancer therapies. We have been investigating polyunsaturated fatty acids (PUFAs), more specifically omega-3 fatty acids, to use in cancer remedies. Omega-3 (n-3) fatty acids have been found to have many health benefits towards the heart, brain and muscles, as well as lower incidences of developing cancer. In the 1970s, epidemiological studies showed that Inuit Eskimos who consumed high amounts of omega-3 fat obtained from fish had less incidences of developing cancer. The lab has determined the docosahexaenoic acid (DHA), an n-3 fatty acid, has been found to inhibit cancer cell growth. Preliminary evidence suggests that DHA alters Myc protein expression and its transcriptional activity in cancer cells.

Myc is a regulator gene that is absolutely essential for survival and plays a large role in proper cell division, which codes for a transcription factor for other genes. Myc was one of the first discovered oncogenes and it functions as a transcription factor that regulates nearly 15% of the genome[1]. Overexpression of Myc can lead to tumor formation[2]. Myc is a potent oncogene and because it serves such an important role in cell division and acts as a transcription factor for

other oncogenes, its dysregulation causes uncontrolled cell growth, which is a hallmark of cancer. This dysregulation will cause Myc to transition from proliferation to differentiation[2,3]. When Myc is mutated it is called c-Myc, the most common Myc isoforms found in cancer, it will persistently function and be expressed abnormally.

Abnormal cancer cell growth can be analyzed by using antibodies specific to c-Myc. Oncogenic activity of c-Myc conserved by threonine 58 (T58) and serine 62 (S62) phosphorylation sites will be used to regulate c-Myc protein stability in response to mitogenic signaling and have been observed in human cancer[4]. This will allow identification of cell proliferation and cell cycle regulation within in cancer cells. Analysis of Myc phosphorylation sites will allow determination of downstream Myc targets that will help further unravel the anti-cancer properties of DHA. A β-actin antibody will also be used to detect β-actin expression levels implicated in the formation of tumor cells[4]. The use of these antibodies will help identify Myc protein expression and how it coincides with cell proliferation and apoptosis.

Myc isoforms act as regulators of cell processes, such as growth, proliferation, and apoptosis. These Myc family residues were originally identified due to their cancer-inducing properties[4]. *c*-Myc is capable of encoding transcriptional factors that can lead to overexpression of this mutated gene associated with tumorigenesis. Additionally, the c-Myc isoform has been found to contain apoptotic properties[6,7]. Analysis of these c-Myc isoforms properties will further help better understand of its roles in apoptosis and tumorigenesis in breast cancer. This project will entail an investigation into how this is happening and examine the functional consequence of alterations of Myc protein and mRNA expression by DHA.

Methods

Isolation of Docosahexanoic Acid (DHA) and Linoleic Acid (LA). Inhibitors were thawed for the cell lysis buffer (Na_3VO_4 was at -20° C and Phosphatase Cocktail was at 4° C). The appropriate amount of cell lysis buffer was made with 1 mL of GTP Lysis Buffer Stock mixed with 20 uL of PMSF, 20 uL of Na3VO4, 10 ul if Aprotonin, 10 uL of NaF, and 10 uL of Protease Cocktail. The media was then aspirated from the cells. Cells were washed twice with ice cold dPBS, which the cells were aspirated after each wash. Accumulated excess PBS was then aspirated. 50 uL of cell lysis buffer was added to the cells, cells were scraped thoroughly, and the cell lysate was collected. Cells were centrifuged at 12,000 x g for 5 minutes. The supernatant was finally transferred to storage and lysates were ready to be assayed for determination of protein concentration.

Quanitification of protein concentration in Docosahexanoic Acid and Linoleic Acid. Quantification of protein concentration in DHA was determined by protein assay. The protein samples and standards were thawed at room temperature. 1 ug, 2.5 ug, 5 ug, 10 ug, and 20 ug of standard in triplicate (1 ug/ul stock was added to 20 ul for 20 ug standard, 10 uL of stock added to 10 uL of dH_2O for the 10 ug standard, and vice versa) were added to a 96-well assay plate. 2 uL of the protein samples and 18 uL of dH_2O were added in triplicate. 25 uL of Protein Assay Reagent A and B were added to all the samples and standards. The standards and samples sat at room temperature for 5-30 minutes. The standards and samples were analyzed via protein assay at optical density 650 nM.

Protein Assay of Docosahexanoic Acid and Linoleic Acid. The protein samples were assayed in standardized amounts of 25 ug and 50 ug. Appropriate amounts of lysis buffer were added to the ethanol standard, DHA and LA (Table 1). Additionally, 7 uL of loading buffer was

added to the standardized 25 ug amount and 8 uL was added to the standardized 50 ug. One set of the 25 ug standardized amounts and one set of the 50 ug were obtained for Western Blot analysis.

Isolation of Docosahexanoic Acid and Linoleic Acid in A-549 and BT-474 cell lines. 50 ug of ethanol, DHA and LA were loaded into gel for Western Blot analysis. The proteins were run at 90V for 2 hours and then rinsed with dH_2O. The gel was then transferred to a Nitrocellulose membrane. 40 mL of the 20x Transfer Buffer Stock was made with 200 mL of methanol and 760 mL of dH_2O. The proteins were then transferred at 35V for 1 hour in NUPAGE transfer buffer. 10 mL of 5% milk in TBS-T (Tris-Buffered Saline and Tween) blocking buffer was used for antibody dilutions and wash volumes. The proteins were blocked for 1 hour at room temperature. The proteins were then washed 3 times with TBS-T for 5 minutes at room temperature. The proteins were treated with secondary antibodies of rabbit in Myc and two phosphorylated c-Myc proteins, as well as treatment of mouse antibody in β-actin. 0.6 g of milk was mixed with 20 mL of TBS-t and 4 mL of mouse secondary antibody. 1.2 g of milk was mixed with 40 mL of TBS-T and 8 uL of rabbit secondary antibody. 4 mL of ECL reagent was used per blot containing our desired proteins. The membranes were finally analyzed in a dark room to expose to film in order to develop the Western Blot results.

Design of Primer sequences for quantitative Real-Time PCR. Primers were designed using Primer3 Input[8], except for published primer set for c-Myc[9] and beta-Actin[10].

Quantification of cDNA in A-549 and BT-474 Cell Lines via quantitative Real-Time PCR. A-494 and BT-474 cells were treated with either 100uM of LA, DHA or an equal volume of ethanol for 4 hours. Total RNA was isolated and purified using RNeasy mini kit (Qiagen) following the manufacturer's protocol. cDNA was then synthesized using cDNA Synthsis VILO

kit (Invitrogen). Quantities of cDNA were measured by quantitative real-time PCR on a CFX96 Real-Time PCR System (Bio-Rad). Reaction used SYBR green FAST master mix (Applied Biosystems). The relative abundance of mRNA was determined by comparative Ct method[7].

Results

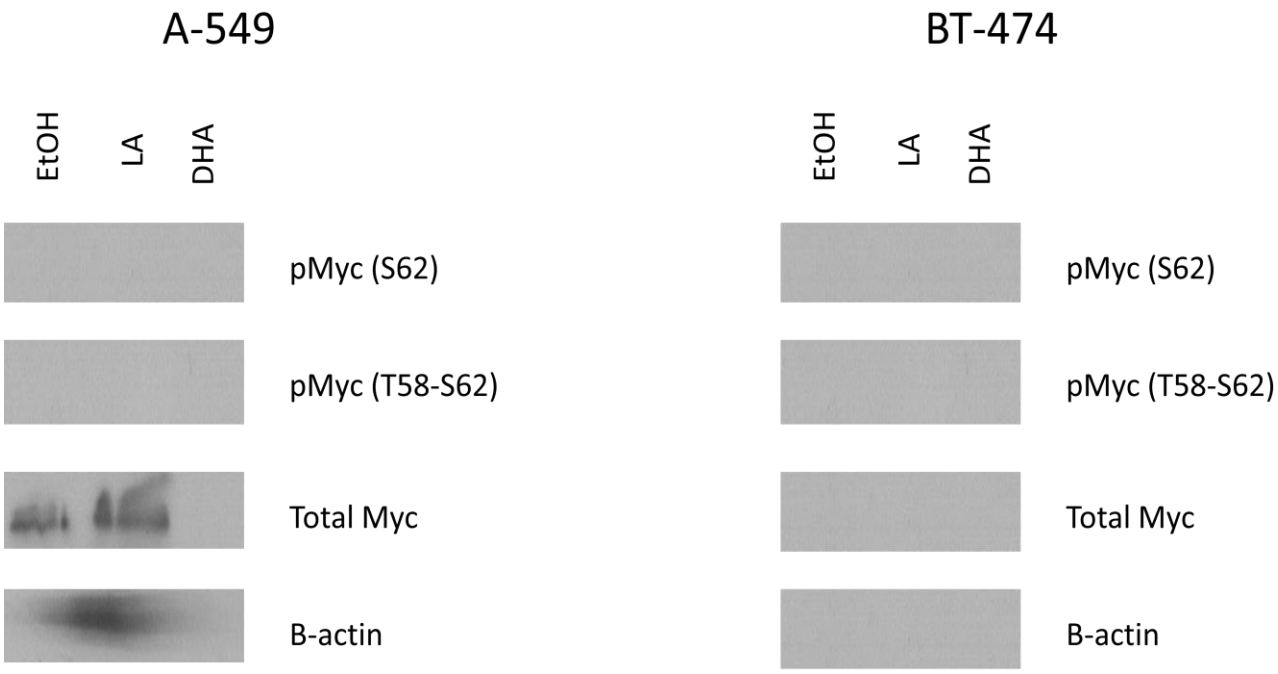

Figure 1: PUFA Effect on Myc Phosphorylation *in vitro*. Two *in vitro* models, A-549 and BT-474 cell lines, were treated with 100uM LA and DHA or an equal volume of ethanol for 48 hours. Cells were lysed and proteins were assayed followed by a separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were electroblotted on a nitrocellulose membranes, followed by immunoblotting of pMyc (S62), pMyc (T58-S62), Myc, and β-actin antibodies. Proteins were determined by Western Blot.

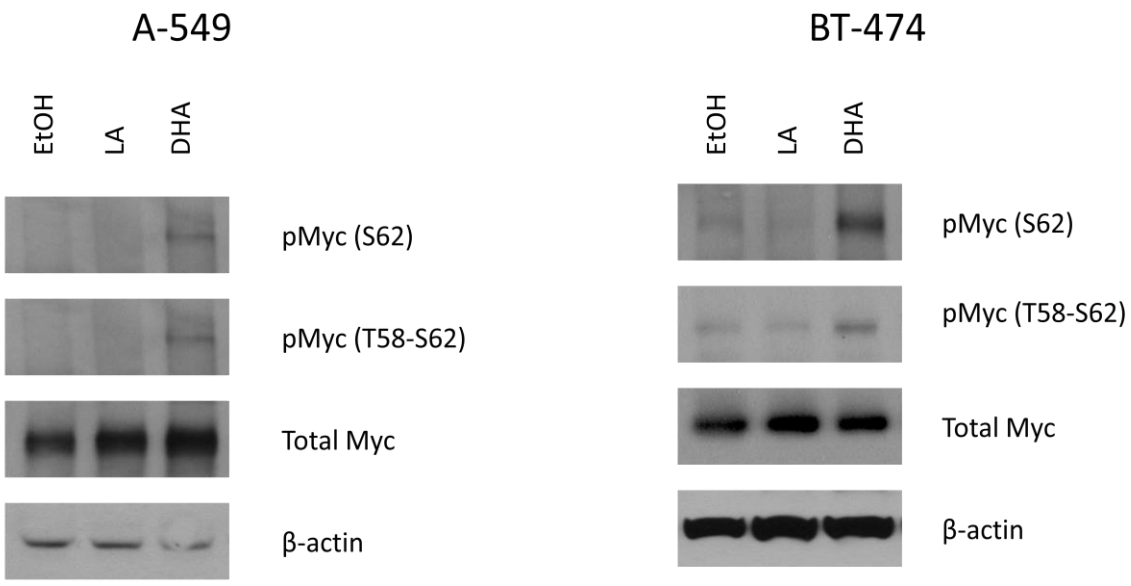

Figure 2: PUFA Effect on Myc Phosphorylation *in vitro*. Two *in vitro* models, A-549 and BT-474 cell lines, were treated with 100uM LA and DHA or an equal volume of ethanol for 48 hours. Cells were lysed and proteins were assayed followed by a separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were electroblotted on a nitrocellulose membranes, followed by immunoblotting of pMyc (S62), pMyc (T58-S62), Myc, and β-actin antibodies. Proteins were determined by Western Blot.

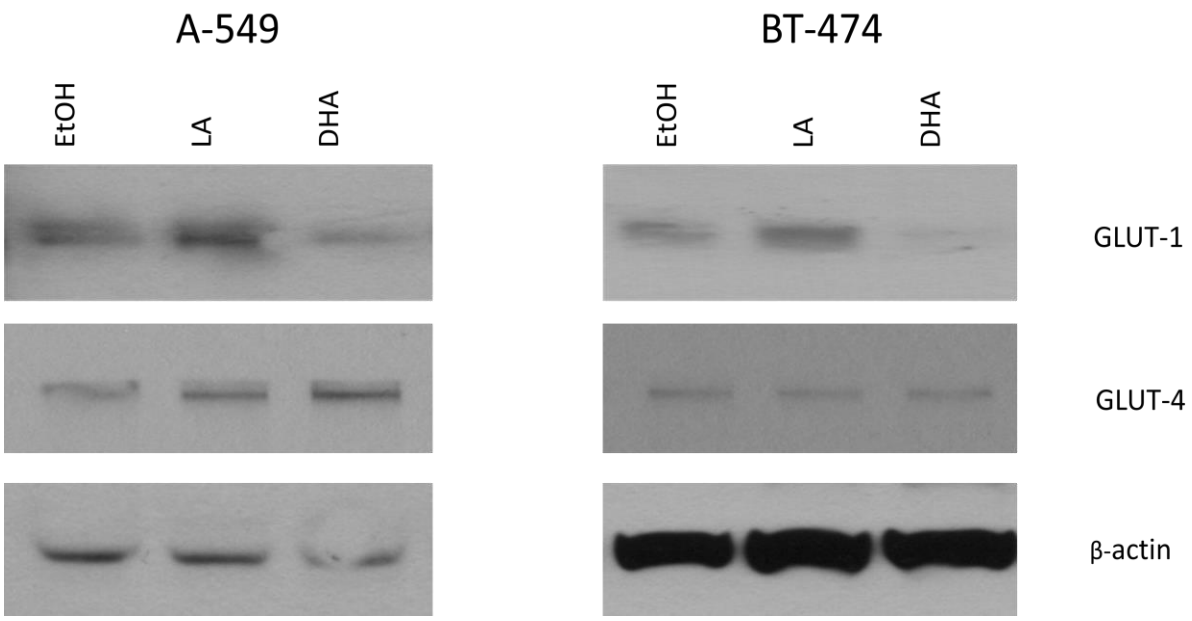

Figure 3: GLUT-Family Protein Expression Upon PUFA Supplementation. A-549 and BT-474 cells were treated with 100uM LA and DHA or an equal volume of ethanol for 48 hours. Cells were lysed and proteins were assayed followed by a separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were electroblotted on a nitrocellulose membranes, followed by immunoblotting of GLUT-1, GLUT-4, and beta-actin antibodies. Proteins were determined by Western Blot.

Table 1: Primer sequences for quantitative real-time PCR. Primers were designed using Primer3 Input[8], except for published primer set for c-Myc[9] and β-Actin[10].

	Gene Primer Sets
	VMYC
Forward	ATCACCATCACACCACCTGA
Reverse	CTCAAATGCACCCGAGAAAT
	c-MYC
Forward	TCAAGAGGTGCCACGTCTCC
Reverse	TCTTGGCAGCAGGATAGTCCTT
	MYCN
Forward	ACACCCTGAGCGATTCAGAT
Reverse	AGGCATCGTTTGAGGATCAG
	MYCL1 variant 1
Forward	CGACCCTGGGAACACTTAA
Reverse	ACCGATTTCCCTGTGTCTTG
	MYCL1 variant 2
Forward	ATGGAGGACAGTTTGGGTGT
Reverse	ACCCTTGCAACAGGAAATTG
	MYCL1 variant 3
Forward	GGACAAGGGATTTGAGGTGA
Reverse	AGGACGAAGGTTTTGGCTTT
	beta-Actin
Forward	CGTCTTCCCCTCCATCG
Reverse	CTCCTTAATGTCACGCAC

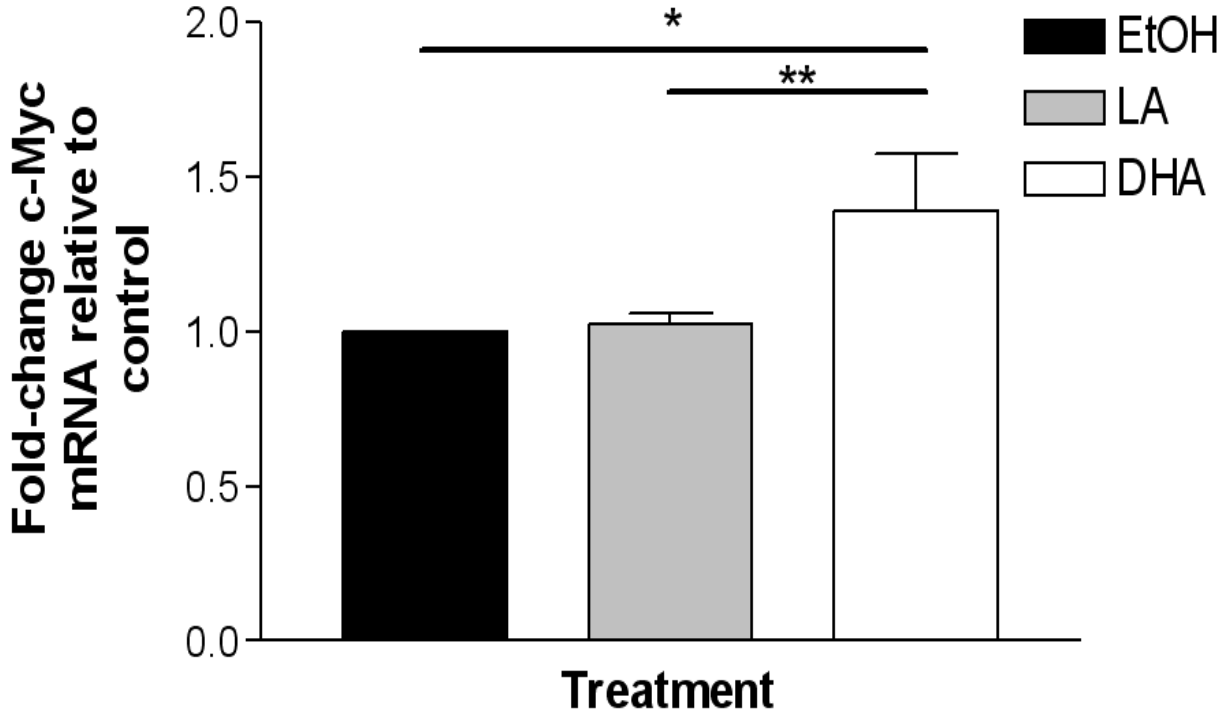

Figure 4: BT-474 cells were treated with either 100uM of LA, DHA or an equal volume of EtOH for 48h. Total RNA was isolated and purified using RNeasy mini kit (Qiagen) following the manufacturer's protocol. cDNA was then synthesized using cDNA Synthsis VILO kit (Invitrogen). Quantities of cDNA were measured by quantitative real-time PCR on a CFX96 Real-Time PCR System (Bio-Rad). The conditions for the reaction have been described by (Mouradian et al., not published). Reaction used SYBR green FAST master mix (Applied Biosystems). The primers that were used are listed in Table 1. The relative abundance of mRNA was determined by comparative Ct method[7]. Data represents n=3 independent experiments ± SD. (*,** indicate P-values < 0.05).

Discussion

Results obtained for both A-549 (human lung cancer adenocarcinoma derived cell line) and BT-474 (human breast cancer cell line) in response to linoleic acid and docosahexanoic acid were undesired. In Figure 1, neither phosphorous c-Myc blots showed any protein expression. This may have been due to short exposure in a tap blot before exposing and developing the film. The results would have turned out more desirable if the phosphorylated c-Myc blots were left overnight in the tap blot prior to developing. Whereas, the β-actin control blot did not show clear results and this may have been due to a lengthy tap blot prior to developing. β-actin is known to have a highly strong signal that it only requires about 30 seconds to 2 minutes in the tap blot before exposure to the film. Finally, the Myc protein blot did not successfully express in neither ethanol, LA and DHA. The bands were difficult to distinguish between ethanol and LA and it shows that there may have been a lack of DHA concentration in the solution. The desired results should result with an upregulation in DHA in both p c-Myc protein residues, while there should be a down regulation in LA. The phosphorous c-Myc protein residues are to assist in investigating the role of T58 and T58/S62 c-Myc phosphorylation sites in cell proliferation. The overall trend of these proteins do not seem to show desired results, thus, we cannot successfully conclude nor determine Myc protein and mRNA expression by DHA. Possible sources of error could have been to due transferring of the protein in the wrong direction away from the blot, not enough transfer time, short exposure times, and not enough wetting or calibration time. Depending upon the type of blot, nitrocellulose versus polyvinylidene difluoride (PVDF), results could be greatly distorted. Nitrocellulose is typically only good for one round of stripping before it begins to break down and can lose some protein. Additionally, phospho-antibodies are hard to work with and do not give a large amount of signal.

However, in Figure 2, we were able to obtain the desired results for the phospho-Myc and total Myc residues in response to LA and DHA. These results obtained are expected for both A-549 and BT-474 cell lines. DHA expresses an upregulation in both cell lines for both phospho-Myc residues, but not for total Myc. These results confirm enhancement of the phosphorylation of Myc and indicates phosphorylation of T58 and S62 are differentially affects c-Myc activity. Since phosphorylation of Myc induces tumorigenesis, these results have shown that DHA prevents the phosphorylation at these sites. Due to this enhancement, we can conclude that DHA is successfully altering Myc and *c*-Myc expression. These alterations exhibit anti-cancer outcomes, such as preventing apoptosis and cell proliferation. Moreover, in Figure 3, DHA expresses a downregulation in both cell lines for the GLUT-1 residue only. There seems to be no significant difference between LA and DHA in the GLUT-4 residue, thus, we can exempt DHA have any affect on GLUT-4 activity. Downregulation of DHA in GLUT-1 illustrates low expression of the GLUT-1 glucose transporter, which results in inhibition of tumor cell growth and glucose transporter protein expression in cancer cells. Overall, recognition of the upregulation in the phospho-Myc residues and downregulation in the GLUT-1 glucose transporter indicates that there may be a correlation between Myc and GLUT in glycolysis and its mechanistic roles in cancer.

Our next approach was designing primers specific to various Myc isoforms. Primer efficiencies were performed to determine how efficient the primers are for other isoforms other than *c*-Myc (Table 1). Primer efficiencies have been performed to determine how efficient the primers are for other isoforms other than *c*-Myc and whether these primers could efficiently select and identify other isoforms. With the proper primer sets unique and specific to Myc, it is possible to determine the mRNA product to a target gene(s), in this case, are the various Myc

isoforms. Having the correct primer pair set is crucial in determining any mRNA product. After selecting a gene of interesting, we use the mRNA sequence (NCBI) of the gene to select the primer pair set, either from used published primers or designing our own primers. Based on the sequence, we select both a forward and reverse primer (which is the reverse compliment of the sequence). Once we received the primer pair set, we resuspended and diluted them to make a working stock. Using known concentrations of cDNA (template), we ran a standard curve of primers (with multiple sets of varied primer concentrations) to determine the optimal primer efficiency to quantitate the mRNA of my experimental groups. However, the qrtPCR results were successfully obtained solely for the BT-474 cell line and not for A-549. Figure 4 shows expected results of increased mRNA expression of *c*-Myc in response to DHA. This suggests that DHA has the ability to inhibit proliferation in human breast cancer cells.

For future directions, having selected the proper primer sets specific to Myc will provide possible findings of Myc isoforms function as well as which other isoforms(s) besides *c*-Myc is responsible in regulating cell growth and apoptosis in breast cancer. We could also determine why Myc is being upregulated and further investigate phosphorylation sites of Myc, which is crucial for determining the downstream targets of Myc. These research findings will provide a better understanding of the anti-cancer properties of DHA and examine the consequences of these alterations in breast cancer. Contributions to this research that has vast benefits by combining nutritional supplementation with conventional cancer therapies can one day improve the quality of life for breast cancer victims.

Literature Cited

1. Zhou, Zi-Qiang, and Peter J. Hurlin. (2001) The Interplay Between Mad and Myc in Proliferation and Differentiation. *TRENDS in Cell Biology.* S10-S14.

2. Henriksson, M. and Luscher, B. (1996) Proteins of the Myc network: essential regulators of cell growth and differentiation. *Adv. Cancer Res.* 68, 109–182

3. Y. Wang, M. Cunningham, X. Zhang, S. Tokarz, B. Laraway, M. Troxell, and R.C. Sears. (2011) Phosphorylation Regulates c-Myc's Oncogenic Activity in the Mammary Gland. *American Association for Cancer Research.*

4. C. Benassayag, L. Montero, N. Colombie´, P. Gallant, D. Cribbs, and D. Morello. (2005) Human c-Myc Isoforms Differentially Regulate Cell Growth and Apoptosis in *Drosophila melanogaster*. *American Society for Microbiology*. 9897-9909.

5. D J Liao and R B Dickson. (2000) *c-Myc* in Breast Cancer. *Endocrine-Related Cancer.* 143–164

6. Barbara Benassi,Maurizio Fanciulli, Francesco Fiorentino, Alessandro Porrello, Giovanna Chiorino, Massimo Loda, Gabriella Zupi, and Annamaria Biroccio. (2006) c-Myc Phosphorylation Is Required for Cellular Response to Oxidative Stress. Molecular Cell 21, 509–519.

7. Livak KJ, Schmittgen TD. (2001) Analysis of relative gene expression data using real time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. Dec;25(4):402-8.

8. Rozen S and Skaletsky HJ (2000) Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S (eds) Bioinformatics Methods and Protocols: Methods in Molecular Biology. Humana Press, Totowa, NJ, pp 365-386.

9. Ozpolat B, Akar U, Steiner M, Zorrilla-Calancha I, Tirado-Gomez M, Colburn N, Danilenko M, Kornblau S, Berestein GL. (2007) Programmed cell death-4 tumor suppressorprotein contributes to retinoic acid-induced terminal granulocytic differentiation of human myeloid leukemia cells. Mol Cancer Res. Jan;5(1):95-108. de Kok JB, Roelofs RW, Giesendorf BA, Pennings JL, Waas ET, Feuth T, Swinkels DW, Span PN. Normalization of gene expression measurements in tumor tissues:comparison of 13 endogenous control genes. Lab Invest. 2005 Jan;85(1):154-9.

10. de Kok JB, Roelofs RW, Giesendorf BA, Pennings JL, Waas ET, Feuth T, Swinkels DW, Span PN. Normalization of gene expression measurements in tumor tissues: comparison of 13 endogenous control genes. Lab Invest. 2005 Jan;85(1):154-9.