



## Research Article

## Differential Association between HER2/*neu* and Angiogenesis in Breast Cancer

Waleed S. Al Amri<sup>1</sup>, Indra Balachandran<sup>1</sup>, Sudha Thangirala<sup>2</sup>, Arlixer Coleman<sup>1</sup>, Hassan A. N. El-Fawal<sup>1</sup>, Shaker A. Mousa<sup>2</sup>

<sup>1</sup>Albany College of Pharmacy & Health Sciences, USA

<sup>2</sup>The Pharmaceutical Research Institute, Albany College of Pharmacy and Health Sciences, One Discovery Drive, USA

### Abstract

The HER-2/*neu* oncogene encodes a transmembrane tyrosine kinase receptor and a member of the epidermal growth factor receptors (EGFR/ErbB) family. Over-expression of this oncogene is known to contribute to pathogenesis and aggressive progression in breast cancer. On the other hand, angiogenesis is a physiological process of forming new blood vessels from pre-existing ones; however it is essential for tumor growth and transitional from benign stage to malignant form and also fundamental process for metastasis of tumors. In this study the main aim was to investigate whether angiogenesis is the cause for increased aggressive behavior of HER2 overexpression subtype in breast cancer. A variety of approaches were employed to investigate the main aim: quantification of angiogenesis using a mouse cancer cells implanted model; Drabkin's assay for hemoglobin measurement; morphology assessment of cell lines; Bio-Plex analysis of angiogenesis factors angiopoietin-2, follistatin, G-CSF, HGF, IL-8, Leptin, PDGF-BB, PECAM-1 and VEGF; and reverse transcriptase-PCR on VEGFA and HIF-1 alpha genes expression. The data from this study showed that the AU-565 cell line which over-expresses HER2 receptors showed a significant decrease in both tumor weight and hemoglobin measurement when the cells were treated with anti-HER2 implanted in nude mice. Also, the data showed an increased expression of angiogenic factors and genes (mainly VEGF, VEGFA, Angiopoietin, and IL-8) in AU-565 as compared to MCF-7 cells, which have low expression of HER2 receptors. This suggests that breast cancer with HER2/*neu* oncogenes is associated with more angiogenic activities that result in an increased aggressive behavior of this form of cancer.

**Keywords:** Breast Cancer; HER2/*neu*; Angiogenesis; VEGFA/HIF-1 Alpha

**Peer Reviewer:** KomaraiahPalle, PhD, USA Mitchell Cancer Institute, United States; NebojsaArsenijevic, MD, PhD, Departments of Immunology and Experimental Oncology, University of Kragujevac, Serbia

**Academic Editor:** XiaoningPeng, PhD, Department of Internal Medicine, Hunan Normal University, China

**Received:** August 18, 2013; **Accepted:** October 22, 2013; **Published:** November 5, 2013

**Competing Interests:** The authors have declared that no competing interests exist.

**Copyright:** 2013, Al Amri WSet al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

\***Correspondence to:** Waleed S. Al Amri, The Pharmaceutical Research Institute, Albany College of Pharmacy, One Discovery Drive, Rensselaer, NY 12144, USA. Email: [Waleed0401@gmail.com](mailto:Waleed0401@gmail.com)

## Introduction

According to the National Cancer Institute, the number of estimated new cases of breast cancer in the United States in 2011 was 230,480 for females and 2,140 for males. In the same year, the mortality rates attributed to breast cancer in the United States were predicated to be 39,520 for females and 450 for males[1]. Despite ongoing clinical research and the intensive involvement of the medical community, the high prevalence of this disease has declined only slightly in recent years.

Treatment and prognosis are dependent on the accurate classification of receptor status and staging of the breast cancer. The common classification of breast cancer includes: histopathological, grading, staging, receptors status (ex; ER, PR, HER2), and DNA microarray for gene profiling. The newer approach has integrated receptors status and gene profile of the tumor to establish molecular subtypes of breast cancer which are: luminal A, luminal B, triple negative (or basal-like), and human epidermal growth factor receptor 2 (HER2) over-expression. In clinical practice, particular attention has focused on A, B, and triple negative luminal subtypes versus HER2 positive over-expression subtype. The HER2 receptors over-expression is present in 25 to 30 percent of breast cancer[2]. In a study involving 222 invasive breast carcinoma cases, it was reported based on survival rates that the HER2 over-expressing subtype had the worst prognosis due to a high rate of invasion and metastasis [3].

There are many drugs of monoclonal antibodies found to minimize immunogenicity of cancer cells over-expressed with HER2, the

most common is Trastuzumab (commercially known as Herceptin). Trastuzumab however, was found to be associated with many negative side effects when used alone [4].

Angiogenesis on the other hand plays an important role in tumor invasion and metastasis, it occurs because there is either an increase in the pro-angiogenic molecules or decrease in the anti-angiogenic stimuli[5]. Studies have shown that there is a proportional increase in the growth of leukemia and other hematological malignancies related to their degree of angiogenesis[6]. Also, other experimental data suggested that the intensity of angiogenesis in a human tumor could predict the probability of metastasis as an example in cutaneous melanoma[7].

Many angiogenesis genes have been identified in experiments of stimulation of angiogenesis using a variety of different mechanisms. Most commonly, markedly increased expression of VEGFA and HIF-1 Alpha have been studied in breast cancer using the reverse transcriptase-PCR method[8]. Also, it has been found that over-expression of HER2 in human tumor cells are closely associated with angiogenesis as when the VEGF pathway was inhibited, tumor cells growth were suppressed and further experiment has shown that anti-HER2 inhibits tumor cell growth and VEGF expression[9].

In this study the main aim was to study whether angiogenesis is the cause for increased aggressive behavior of HER2 overexpression subtype in breast cancer. Such a finding could possibly lead to a better understanding of breast cancer pathophysiology and result in an improved method of target therapy in the more

aggressive tumors. Also the study could produce new diagnostic and prognostic markers for the clinical staging of breast cancer based on the expression of angiogenesis in the various subtypes.

## Materials and Methods

### Materials

Material used were: human breast cancer cell lines MCF-7, MDA-MB-231, and AU-565 (American Type Culture Collection ATCC, VA, USA); Dulbecco's Modified Eagle Medium DMEM (Invitrogen, Life Sciences, NJ, USA); Roswell Park Memorial Institute-1640 RPMI-1640 (ATCC); Matrigel (BD Biosciences); Anti-HER2 conjugated with fluorescence (BD Biosciences, Becton Dickson); Anti-HER2 inhibitors (Abcam, MA, USA); VILO Master Mix (Invitrogen); Angiogenesis kit (Bio-Rad, CA); Bio-Plex 200 System (Bio-Rad); FACSCaliburFlow cytometry (BD Biosciences); Confocal microscopy TCS SP8 (Leica, NJ, USA); Real time-PCR (Applied Biosystems, Eppendorf, NY, USA).

### Cell Culture

The MCF-7, MDA-MB-231, and AU-565 cell lines were propagated in the cell culture at 37°C, 5% CO<sub>2</sub>. The media used was DMEM with 10% FBS for MCF-7 and MDA-MB-231 and RPMI-1640 with 10% FBS for AU-565.

### Mouse Model

The animal experiment was conducted at the VA Medical Center (Albany, N.Y) animal facility. All experiments were performed in compliance with Public Health Service Policy on HumaneCare and Use of Laboratory

Animals and approved by the VA Medical Center IACUC. At the end of the study the mice were sacrificed humanely. Female nude homozygous mice aged 5-6 weeks with body weights of 20 g were obtained from Taconic Farms (Hudson, NY).

### Standardization of the cell lines

The first step was to sort out the cell lines that are over-expressed with HER2 receptors using flow cytometry technique. We used the SKO3-LU ovarian cell line, known to be over-expressed with the HER2 receptors as a positive control [10]. Cells were taken from cell culture, washed twice with phosphate buffer saline, and then the samples were divided into two sets. Each set of samples contained 1x10<sup>6</sup> cells. The first set of samples was unstained with anti-HER2, while the second set was stained with 50 ul of fluorescence-conjugated monoclonal antibody. Both sets were incubated in the dark for 30 minutes. Then the samples were analyzed and signals were acquired on a FACSCalibur flow cytometry (BD Biosciences). CellQuest software (version 3.2) was used to acquire data. The HER2-positive cells were visualized in a histogram showing fluorescence (APC-positive) events. An acquisition threshold (Laser excitation at 488 nm and 635 nm) was set so that any unwanted events like HER2-negative cells, dead cells and debris are not recorded.

### Morphology Study

The experimental goal of a morphology study was to classify the cell lines according to the Kenny et al. study [11]. MCF-7, MDA-MB-231, and AU-565 cell lines were viewed after 48 hours of the first cell sub-culture using a two dimensional culture

and a stereomicroscope. In addition, the cell lines were stained with cell tracker CMFDA (5-chloromethylfluorescein diacetate) in order for the cells to be visualized under the fluorescent microscope. Morphology descriptions of the cell lines were classified whether round, mass, grape-like, or stellate shapes.

### **Quantification of Angiogenesis in Vivo**

The aim of this experiment was to quantify angiogenesis in a female nude mouse model. One million MCF-7, MDA-MB-231, and AU-565 cells were suspended in 50 ul of media and mixed with 50 ul of matrigel. The total mixture (100 ul) was implanted in the mammary glands (two inoculations) and subcutaneously (two inoculations) in each mouse (total two mice for each cell lines). The control for the HER2 receptors was run on the AU-565 cell line (over-expressed with the HER2 receptors). Each control inoculation consisted of one million of AU-565 cells suspended in 30 ul of media, 20 ul of 0.2 mg/ml anti-HER2 inhibitors, and 50 ul of matrigel, adding to the final concentration of 4 ug of anti-HER2 per one million of cells. After two weeks, the mice were sacrificed and the tumors were harvested, and tumor weights were recorded. Homogenization of the tumors and then a centrifugation step followed. Drabkin's reagent was added and then the samples were incubated for 15 minutes before the hemoglobin concentration was measured using a colorimetric ELISA reader. The absorbance was measured at 540 nm and the hemoglobin concentration was expressed as mg/ml based on comparison with a standard curve.

### **Multiplex Analysis**

The aim of this experiment was to detect the expression level of the following angiogenesis factors using multiplex analysis technique in MCF-7, MDA-MB-231, and AU-565 cell lines: angiopoietin-2, follistatin, G-CSF, HGF, IL-8, leptin, PDGF-BB, and VEGF. Media samples were taken from each cell line after 48 hours from the sub-culturing, and run on a Bio-Plex 200 System using an angiogenesis kit from Bio-Rad. A 96-well plate was used after being pre-wetted with assay buffer. Then, 50 ul of coupled magnetic beads were added in each well, followed by a washing step with PBS performed twice. Lyophilized controls and standards were reconstituted and 8-point serial dilutions of standard were made. Then 50 ul of two blanks (both media RPMI and DMEM), controls, standards, and samples were taken and added in each well accordingly. Each sample was run in triplicate. The samples were incubated and vortexed at room temperature for 30 minutes. Then the samples were washed twice with PBS. Next, 50ul of pre-diluted detection antibodies were added in each well, followed by 30 minutes of incubation at room temperature and washing twice with PBS. Then, streptavidin-PE was added (50 ul) in each well, followed by 10 minutes incubation under agitation. The samples were washed twice and the mixture in each well was suspended in 125 ul of assay buffer. Data acquisition was performed using Bio-Plex Manager Software version 5.0.

### **RT-PCR**

The experimental aim was to determine the expression level of HIF-1 Alpha and VEGFA genes using reverse transcriptase-PCR (RT-PCR). Primer designation for the genes was taken from the National Center for Biotechnology Information NCBI. (Table.1).

**Table 1.** Nucleotides Length, Primers Sequences, and Annealing Temperature for Genes.

Gene	Entrez Gene ID	Length (bp)	Primers	Sequence	Annealing Temp (°C)
HIF-1 Alpha	3091	134	Forward Primer	'5-CGTTTCCTTCGATCAGTTGTC-3'	60.00
			Reverse Primer	'5-TCAGTGGTGGCAGTGGTAGT-3'	
VEGFA	7422	76	Forward Primer	'5-AGGAGGAGGGCAGAATCATCA-3'	60.00
			Reverse Primer	'5-CTCGATTGGATGGCAGTAGCT-3'	

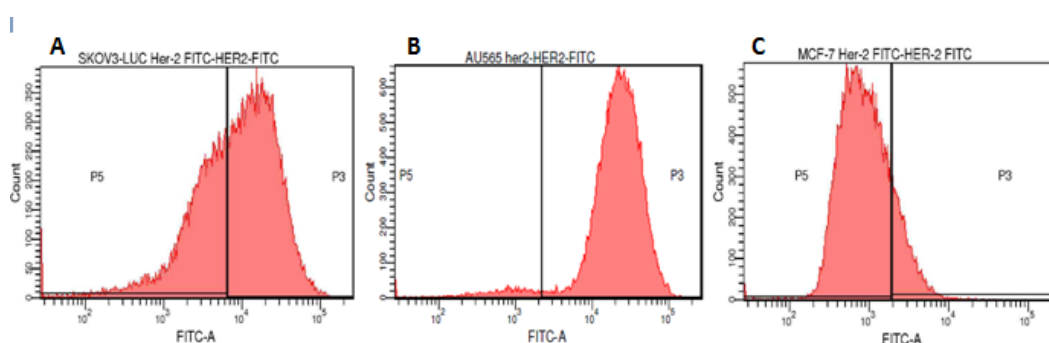
RNAs extraction was performed on MCF-7, MDA-MB-231, and AU-565 cell lines taken from cell culture. Cells were treated with TRIzol to extract the RNAs, after which the RNA was isolated using 100% isopropanol followed by a washing step with 75% ethanol (twice). Next, the RNA was suspended in RNAase-free water and a reverse transcription reaction was performed. Each reaction tube contained: 4 ul of VILO Master Mix, 8 ul of RNAs, and then DEPC-treated water was added to final volume of 20 ul. The mixture was incubated through a series of thermocycler reactions (25°C for 10 mins, 42°C for 60 mins, and 85°C for 10 mins). The resultant cDNA was used for a Real Time-PCR reaction. Each reaction tube contained 1 ul of each forward and reverse primers, 2 ul of cDNAs, 10 ul of SYBR GeneAmp® Fast PCR Master Mix, and then RNA-free water was added for a total volume of 20 ul. After that, the mixtures were processed by real time-PCR using the 9800 Fast Thermal Cycling Profile. The comparative  $C_T$  Method ( $\Delta\Delta C_T$ ) was used to quantify the gene expression. GAPDH was used as the endogenous control. For a valid

$\Delta\Delta C_T$  calculation, the efficiency of the target amplification and the efficiency of the reference amplification were run. In a validation experiment that passed, the absolute value of the slope of  $\Delta C_T$  vs. log input was  $< 0.1$ .

## Results

### Standardization of the cell lines

SKOV3-LUC, positive control, showed 61.5% positivity of HER2 expression. MCF-7 cell line has low expression of the HER2 receptors with 16.6% positivity. On the contrary, AU-565 cells showed an over-expression of HER2, a 91.1% positivity. Hence, AU-565 is considered to be over-expressed with the HER2 receptors. The flow cytometry results were consistent with other studies using immuno-histochemistry because they showed that AU-565 cells over-express the HER2 receptors, while MCF-7 cells are negative for expression[12].



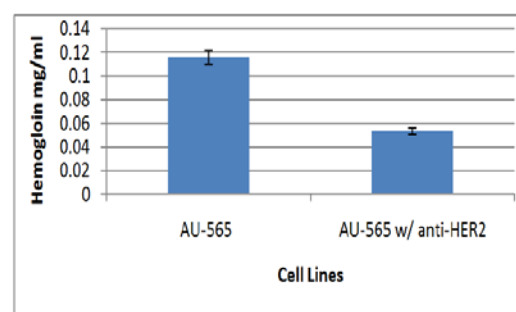
**Figure 1** (A) SKOV3-LUC as a positive control with anti-HER2 staining. Note: gate P3 (positive gate) showed positivity 61.5%. (B) AU-565 cells with anti-HER2 staining showed 95.1% toward gate P3 (high expression), while it showed 4.9% negativity. (C) MCF7 cells with anti-HER2 staining showed 16.6% toward gate P3 (minimal expression), while it showed 84.4% toward gate P5 (negative gate).

**Table 2** Tumor weights of the breast cancer cell lines after harvest from mice after two weeks.

Tumor's Weight in mg			
	AU-565	AU-565 w/ anti-HER2	MCF-7
	35.3	11	14.5
	30.9	11.9	20.9
	31	12.1	26.9
			19.7
			16.7
			8.5
<b>Average</b>	<b>32.4</b>	<b>11.7</b>	<b>19.7</b>

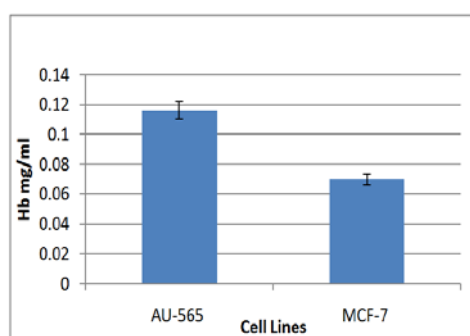
### Angiogenesis Quantification in Vivo

The results of Drabkin’s assay for hemoglobin measurement after the tumors were harvested from mice (two weeks incubation) showed significant variance between AU-565 cell line, which expresses HER2, and the AU-565 cell lines treated with anti-HER2 (Figure 2). The average tumor weight resulting from the AU-565 cell line after treatment with anti-HER2 showed a notable decrease (Table 2) compared with AU-565. The tumors resulting from the AU-565 cell line had more tumor growth and hemoglobin concentration than MCF-7 by 24.3% and 24.7%, respectively. (Table 2 and Figure 3).



**Figure 2** Hemoglobin concentration of tumors of AU-565 cell line and AU-565 treated with HER2 inhibitors after implanted in mice. AU-565 with HER2 inhibitors showed a 37.3% decrease compared to the AU-565 cell line.

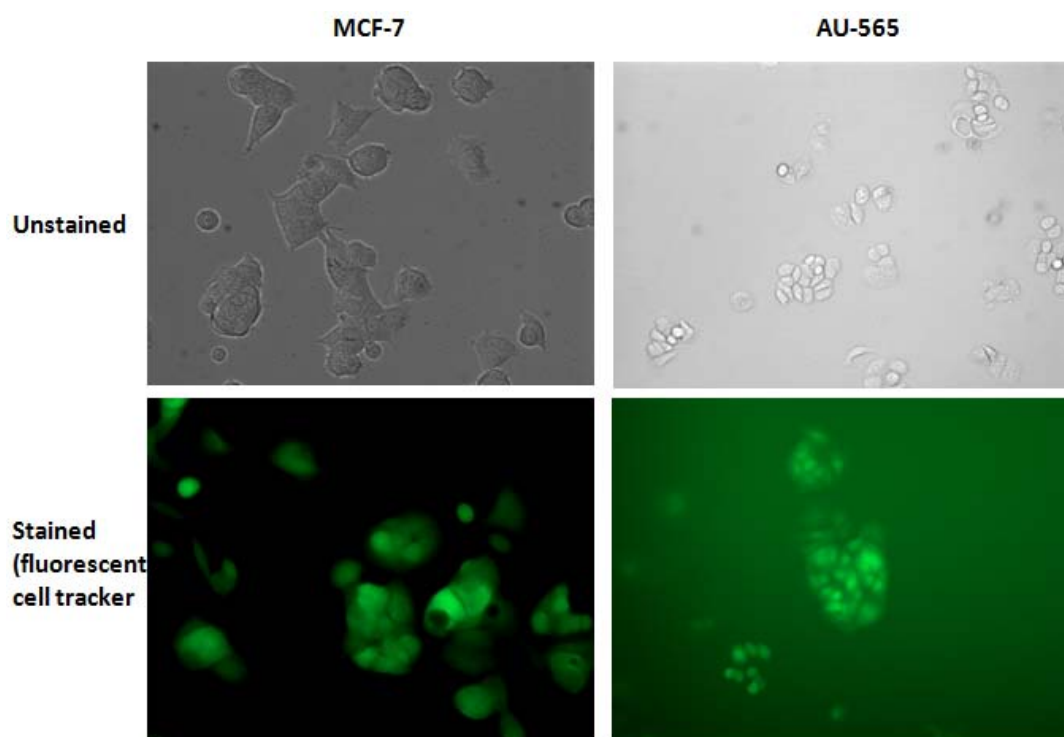




**Figure 3** Hemoglobin concentration of tumors AU-565 and MCF-7 cell lines were implanted in nude mice. AU-565 cells showed a more hemoglobin's concentration than MCF-7 cells by 24.7%.

## Morphology Study

The MCF-7 cell line showed the Mass class morphology as the cells formed colonies with round colony outlines. They are distinguished from the round morphological grouping by their disorganized nuclei and filled colony centers. While, the AU-565 cell line exhibited a Grape-like class morphology, since these cells formed colonies with poor cell-cell contacts and were distinguished by their grape-like appearance, (Figure 4).



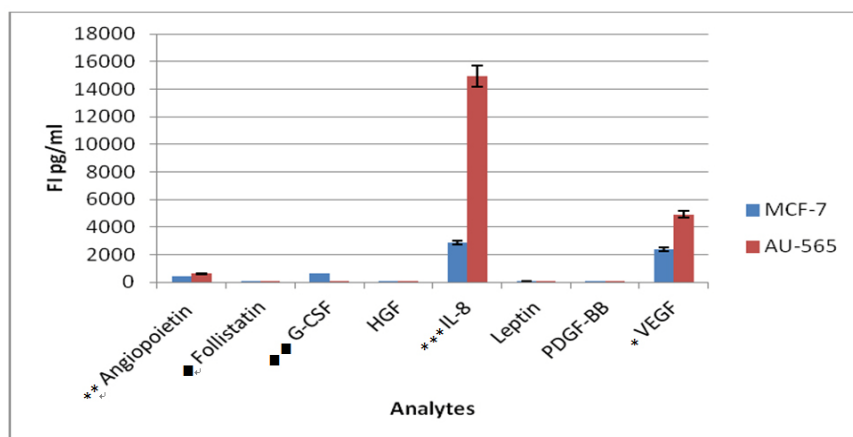
**Figure4** Morphologies of MCF-7 and AU-565 cell lines pictured after 48 hours of first cell sub-culture using two dimensional cell culture and stereomicroscopy. Cells were stained with cell tracker CMFDA (5-choromethylfluorescein diacetate).

## Bio-Plex Analysis

The results of the Bio-Plex angiogenesis assay showed that AU-565 cells which over-express HER2 had the predominance expression for

most of the angiogenic factors studied. However, it showed only a significant expression of Angiopoietin, IL-8, and VEGF. While, MCF-7 cells showed less expression for most of the angiogenic factors studied

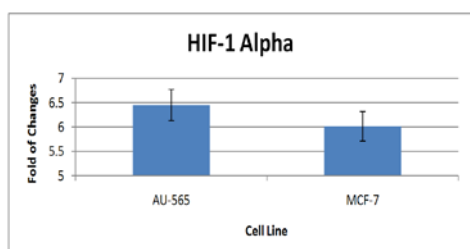
(Figures 5).



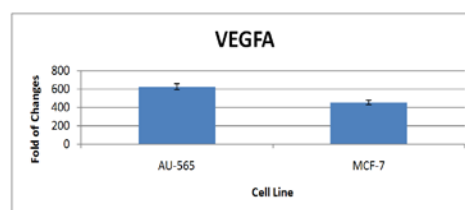
**Figure 5** 48 hours measurement of angiogenesis factors. The AU-565 cell line exhibited a significant higher expression of Angiopoietin, IL-8, and VEGF than MCF-7 cell line by 17.1%, 67%, and 34%. \* P-Value less than 0.05 (AU-565 more than MCF-7); \*\* P-Value less than 0.02 (AU-565 more than MCF-7); \*\*\* P-Value less than 0.01 (AU-565 more than MCF-7); ■ P-Value less than 0.05 (MCF-7 more than AU-565); ■■ P-Value less than 0.01 (MCF-7 more than AU-565)

## RT-PCR

Overall, the results of reverse transcriptase-PCR indicated that the MCF-7 and AU-565 cell lines exhibited more expression of VEGFA than HIF-1 alpha genes. AU-565 cell line, which over-expresses the HER2 receptors, showed higher expression of both VEGFA and HIF-1 Alpha, than MCF-7 cell line (Figures 6 and 7). The validation experiment for VEGFA and HIF-1 Alpha genes showed the slope values were 0.063 and 0.087, respectively (less than 0.1), which indicates our  $\Delta\Delta C^T$  calculation for VEGFA and HIF-1 Alpha are valid.



**Figure 6** RT-PCR expression of VEGFA gene. AU-565 cell line showed comparable expression of VEGFA to MCF-7 cell line.



**Figure 7** RT-PCR expression of HIF-1 Alpha. The AU-565 cell line showed comparable expression of HIF-1 Alpha to MCF-7.

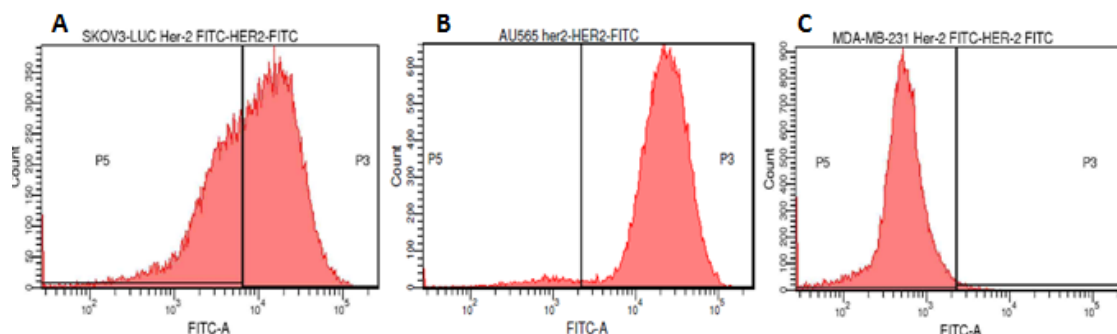
## MDA-MB-231 Cell line

For further data generating we looked at MDA-MB-231 cell line which exhibits an aggressive proliferation rate. The flow cytometry results showed that MDA-MB-231 is low expresses HER2 receptors around 1.1% in comparison to 61.5% SKOV3-LUC (control) and 84.4% AU-565 (figure 8). The vivo study showed that the tumors resulting from the MDA-MB-231 cells after implanted in mice had higher in both tumor weight and hemoglobin concentration by 13.8% and 25.2%, respectively than AU-565 cell line



(Table 3 and Figure 9). The morphology of MDA-MB-231 cell line showed Stellate (elongated) projections that often bridge multiple cell colonies (Figure 10). In addition, Bio-Plex analysis showed MDA-MB-231 cells had a higher expression of follistatin, G-CSF,

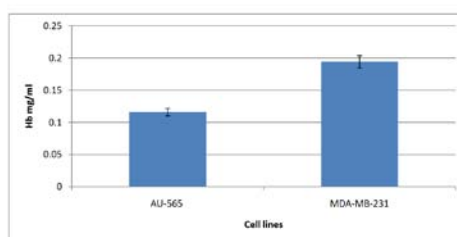
HGF, leptin, and PDGF-BB angiogenesis factors than AU-565 cells (Figure 11). In contrast, the RT-PCR experiment showed that MDA-MB-231 cells had less expression of VEGFA and HIF-1 Alpha genes compared with AU-565 cell lines (Figure 12 & 13).



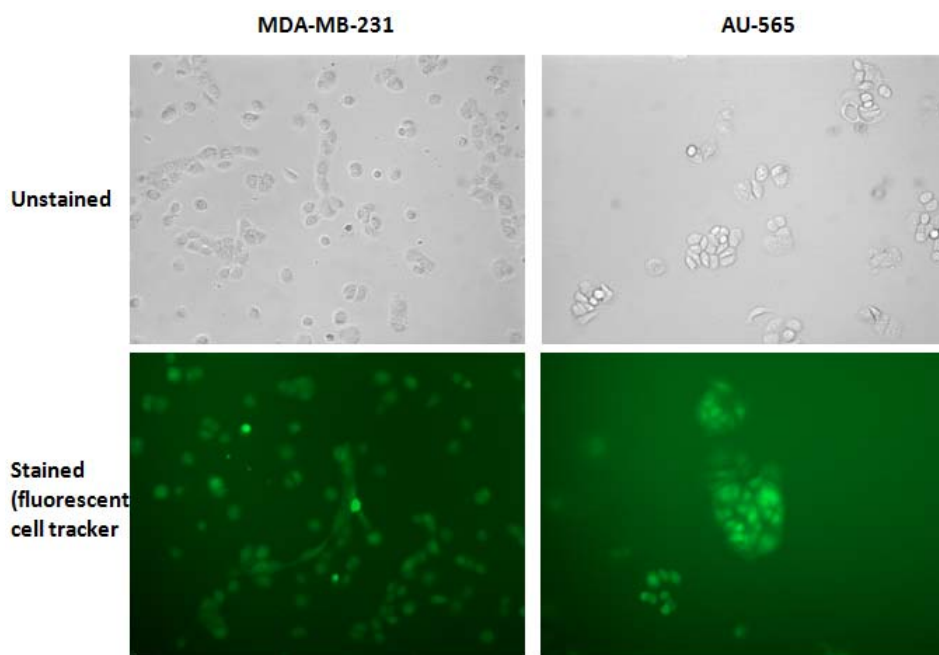
**Figure 8** (A) SKOV3-LUC as a positive control with anti-HER2 staining. Note: gate P3 (positive gate) showed positivity 61.5%. (B) AU-565 cells with anti-HER2 staining showed 95.1% toward gate P3 (high expression) (C) MDA-MB-231 cells with anti-HER2 staining showed 1.1% toward gate P3 (positive), while it showed 98.8% negativity.

**Table 3** Tumor weights of the breast cancer AU-565 and MDA-MB-231 cell lines after harvest from mice after two weeks. Note, highlighted weights indicate the tumors were implanted in the nude mice mammary glands.

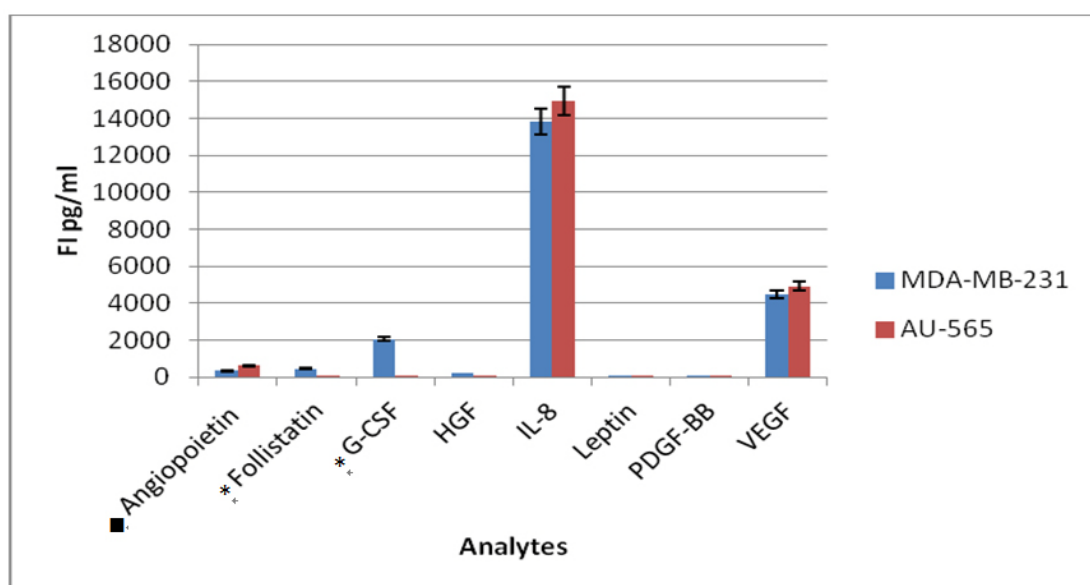
Tumor's Weight (mg)		
	AU-565	MDA-MB-231
	35.3	45.6
	30.9	38.9
	31	43.1
		44.5
		49.6
		35
Average	32.4	42.8



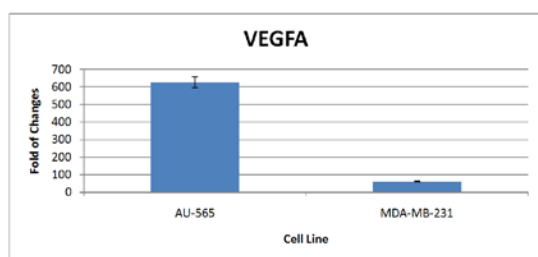
**Figure 9** Hemoglobin concentrations of tumors after AU-565 and MDA-MB-231 cell lines were implanted in nude mice. MDA-MB-231 showed more hemoglobin concentration by 13.8% than AU-565 cell line.



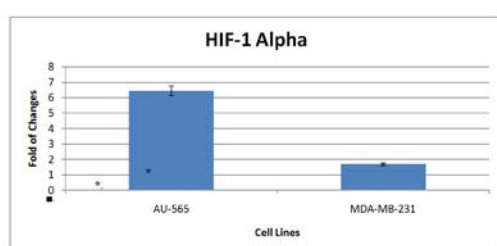
**Figure 10** Morphologies of MDA-MB-231 and AU-565 cell lines pictured after 48 hours of first cell sub-culture using two dimensional cell culture and stereomicroscopy. Cells were stained with cell tracker CMFDA (5-chromethylfluorescein diacetate).



**Figure 11** 48 hours measurement of angiogenesis factors. MDA-MB-231 cells showed a higher expression of follistatin, G-CSF, HGF, leptin, and PDGF-BB than AU-565 by 84.0%, 97.0%, 31.4%, 23.5%, and 18.8%, respectively. While, AU-565 cells showed more expression of Angiopoietin, IL-8, and VEGF compared to MDA-MB-231 cells by 28.5%, 3.8% & 4.6%. \*P-Value is less than 0.05 (MDA-MB-231 more than AU-565); ■P-Value is less than 0.05 (AU-565 more than MDA-MB-231)



**Figure 12** RT-PCR expression of VEGFA gene. The AU-565 cell line had a higher expression of VEGFA approximately 82.5% higher than MDA-MB-231 cell line.



**Figure 13** RT-PCR expression of HIF-1 Alpha. The AU-565 cell line showed more expression of HIF-1 Alpha than MDA-MB-23 cell line around 58.5%.

## Discussion

### Angiogenesis Quantification in Vivo

In order to have a complete appraisal of angiogenesis in breast cancer cell lines, we investigated angiogenesis in vivo and vitro. In vivo study nude mice were used to quantify angiogenesis by implanting the tumor cells subcutaneously and in mammary glands, after two weeks, tumor weights were recorded in refection of tumors aggressiveness and the amount of hemoglobin is a proportional to vascularity formation of the tumors. AU-565 cell line which expresses HER2 showed significant increase of tumor weight and hemoglobin measurement when compared with AU-565 cells treated with anti-HER2 inhibitors and MCF-7 cell line which low expresses HER2. This finding is consistent

with other cell lines which also over-express the HER2 receptors (Sk-Br-3 and BT-474 cell lines), the proliferation rate was inhibited when the cells incubated with anti-HER2/*neu*(c-erbB-2 mouse IgG1 MAbs, Triton) at 0.025, 0.25, and 2.5  $\mu$ g/ml concentrations for 24, 48, and 72 hours [13]. This finding indicates that HER2 oncogenes contribute in proliferation rate of tumor cells by stimulating of angiogenesis formation.

### Cell Lines Morphology Study

Kenny's study reported that the breast cancer cell lines in general exhibited a distinct morphology class and some of these morphologies were associated with tumor cell invasiveness and originating from metastases [11]. In our study, the MCF-7 cell line exhibited a Mass class morphology which is frequently associated with a lesser capability to invade and metastasize. MCF-7 cells exhibiting this morphology are consistent with the low expression of the HER2 receptors and less proliferating rate by these cells.

While, AU-565 cell line showed a Grape-like class morphology, which forms colonies with poor cell-cell contacts and are distinguished by their grape-like appearance. Kenny et al. noted in their study that cell lines that exhibited this type of morphology were isolated from tumor metastases [11]. The AU-565 cell line was confirmed in our lab to over-expresses the HER receptors. In general, these cells formed less closely associated colonies with reduced cell-cell adhesion as compared to cell lines of the other morphologies. This may reflect, in part, the acquired ability of these cells to break away from their neighbors in the primary tumor over the course of their evolution as they acquired the ability to metastasize.

## Bio-Plex Analysis

In this study we used the Bio-Plex Pro Human Angiogenesis Multiplex Assay to compare the expression of angiopoietin-2, follistatin, G-CSF, HGF, IL-8, leptin, PDGF-BB, and VEGF in breast cancer cells lines that over-express HER2 (AU-565) and one that does not (MCF-7). Our results showed that the expression level of these markers in the MCF-7 and AU-565 cell lines are markedly elevated as compared with normal physiological donors' plasma[14]. AU-565 cells showed a significant expression of Angiopoietin-2, IL-8, and VEGF. Past studies have indicated that the levels of IL-8 and VEGF are significantly increased in colon cancer patients[15]. Similarly, Angiopoietin-2 had the highest expression by real time-PCR in Sk-Br-3 breast cancer cells which is a similar cell line to AU-565 cells[16].

## RT-PCR

VEGF-A and HIF-1 alpha genes are known for their significant stimulation of angiogenesis. VEGF-A induces endothelial cell proliferation, promotes cell migration, inhibits apoptosis and induces permeability of blood vessels[17]. HIF-1 alpha is a critical regulatory protein of cellular response to hypoxia and is closely related to the triggering of the angiogenic process[18]. Expression analysis of VEGF-A and HIF-1 alpha genes using quantitative reverse transcriptase-PCR suggested a significant variance between cell lines that over-expresses HER2 (AU-565) and the one that minimally expresses HER2 (MCF-7). The results of this study showed that AU-565 cells have a significant over expression of VEGF-A as compared with MCF-7 cell line. This finding was consistent with a study done on tGLI1 and VEGF-A as tGLI1 expression is proportional to expression of VEGF-A; they found that in

BT-474 cell line (also over-expresses with HER2) has high expression of tGLI1 than MCF-7 cells which promotes secretion of VEGFA [19].

The HIF-1 Alpha gene was relatively less expressed in the MCF-7 and AU-565 cell lines. In other studies, the expression of HIF-1 $\alpha$  protein and HIF-1 DNA-binding activity was found to be significantly higher in stable transfectants of mouse NIH 3T3 cells expressing HER2 than MCF-7 under non-hypoxic conditions [20]. Our results correlated with previously published studies where AU-565 cells showed more expression of HIF-1 $\alpha$  than MCF-7 cells. However, this assay needs a large scale of normal cell lines to determine the normal expression of VEGF-A & HIF-1 $\alpha$ .

## Is HER2 only factor contributes in aggressive behavior of angiogenesis in tumors?

In this study we looked at MDA-MB-231 which shows aggressive proliferating rate. MDA-MB-231 cell line is a triple negative (ER, PR, HER2) and also expresses the both epidermal growth factor (EGF), and transforming growth factor alpha (TGF alpha)[21]. Also, these cells are positive for the TP53 mutation[22]and this would explain the reason this cell line has more proliferation rate than AU-565 cell line which expresses HER2 receptors. The results of angiogenesis quantification in vivo, showed that MDA-MB-231 had slightly higher (statistically non-significant) in both tumor weight and hemoglobin's measurement. Also, the morphology of MDA-MB-231 exhibited Stellate class which associates with a high degree of invasion capability. This finding consistent with molecular profile of this cell line for example Lin28 [23] and P53 mutation [24].MDA-MB-231 cell line also showed a predominant expression of follistatin, G-CSF,

HGF, leptin, and PDGF-BB by Bio-Plex analysis which are consistent with their implication in the progression and metastasis of prostate cancer because it showed significant expression of HGF and PDGF-BB[25]. In contrast, MDA-MB-231 cells showed less expression of VEGFA and HIF-1 Alpha genes than AU-565 cells in the RT-PCR experiment, and this suggest that HER2 interact more closely with VEGFA and HIF-1 Alpha for stimulation of angiogenesis. Overall, the results indicated that MDA-MB-231 cell line is as aggressive as AU-565 cell line (which over-expresses HER2 receptors) and there are others factors other than HER2 contribute to the aggressiveness of angiogenesis in the tumors like P53 mutation, over-expression of EGF and TGF alpha.

## Conclusion

Our data showed that the AU-565 cell line which over-expresses with the HER2 receptors showed a significant decrease when the cells were treated with anti-HER2 inhibitors in both tumor weight and hemoglobin measurement after they were implanted in nude mice in vivo. Also, it showed variable expression of angiogenesis factors and genes (mainly; VEGF, VEGFA, Angiopoietin, and IL-8) as compared with MCF-7 cell line which does not over-express HER2. This suggests that breast cancer with HER2/*neu* oncogenes is associated with more angiogenic activities which results in more aggressive behavior of this form of cancer. However, this data needs to be verified on a larger scale with more breast cancer cell lines in vivo and in vitro. Also, evaluation of angiogenesis by using histopathological methods and immuno-histochemical markers on clinical samples will provide additional solid data to these findings.

## Abbreviations

DMEM, Dulbecco's Modified Eagle Medium; ELISA, Enzyme Linked Immunosorbent Assay; FBS, Fetal Bovine Serum; G-CSF, Granulocyte Colony-Stimulating Factor; HER2, Human Epidermal Growth Factor Receptor 2; HGF, Hepatocyte Growth Factor; HIF-1 Alpha, Hypoxia Induced Factor-1 Alpha; IL-8, Angiopoietin-2, Follistatin 8; PDGF-BB, Platelet-Derived Growth Factor-BB; PECAM-1, Platelet Endothelial Cell Adhesion Molecule-1; RPMI-1640, Roswell Park Memorial Institute-1640; VEGF, Vascular Endothelial Growth Factor; VEGFA, Vascular Endothelial Growth Factor-A

## Acknowledgement

The authors would like to thank the following persons for their valuable contributions: Kelly Keating, PhD; Binshan Shi, PhD; Murat Yalcin, PhD; Laura Stellato; Barbara Zaffo, MS

## References

1. American Cancer Society. Breast Cancer Facts & Figures 2011-2012. Atlanta: American Cancer Society, Inc.
2. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer: correlation of relapse and survival with amplification of the HER2-2/*neu* oncogene. *Science*. 1987, 235:177-259
3. Demir H, Turna H, Can G, Ilvan S. Clinicopathologic and prognostic evaluation of invasive breast carcinoma molecular subtypes and GATA3 expression. *J BUON*. 2010, 154:774-856
4. Carter P, Presta L, Gorman C, Ridgway J, Henner D, Wailee T, Wong W, Rowland A, Kotts C, Carver M. Humanization of an anti-HER2 antibody for human cancer



- therapy. *ProcNatlAcadSci U S A*. 1992, 89:4285-4296
5. Mousa SA. Anticoagulants in thrombosis and cancer: the missing link. *Expert Rev Anticancer Ther*. 2002, 22:227-260
  6. Vacca A and Ribatti D. Bone marrow angiogenesis in multiple myeloma. *Leukemia*. 2005, 20:193-199
  7. Srivastava A, Laidler P, Hughes LE, Woodcock J, Shedden EJ. Neovascularization in human cutaneous melanoma: a quantitative morphological and Doppler ultrasound study. *Eur J Cancer ClinOncol*. 1986, 22:1205-1211
  8. Bryan PS, Milan R, George WS, Jason DR, Lang L, Anna MS, Suzanne L, Anne TN, Bradley AH, Michael S, Todd S, David AF. Association of polymorphisms of angiogenesis genes with breast Cancer. *Breast Cancer Res Treat*. 2008, 111:157-163
  9. Kumar R. and Yarmand-Bagheri R. The role of HER2 in angiogenesis. *SeminOncol*. 2001, 16:27-32
  10. Bacus SS, Kiguchi K, Chin D, King CR, Huberman E. Differentiation of cultured human breast cancer cells (AU-565 and MCF-7) associated with loss of cell surface HER-2/neu antigen. *MolCarcinog*. 1990, 6:350-412
  11. Kenny PA, Lee GY, Connie AM, Richard MN, Jeremy R. Semeiks, Paul TS, Katrin L, Eva HL, Mary W P, Joe WG, and Mina JB,. The morphologies of breast cancer cell lines in three-dimensional assays correlate with their profiles of gene expression. *MolOncol*. 2007, 11:84-96
  12. Department of Surgery, County Hospital, Kalmar, Sweden. Survival rate is improving in HER2 positive breast cancer patients. Published on March 26, 2010. <http://www.news-medical.net/news/2010/0326/Survival-rate-is-improving-in-HER2-positive-breast-cancer-patients.aspx>.
  13. Brodowicz T, wiltschke C, Budinsky A, Krainer M, Steger G, Zielinski C. Soluble HER-2/neu neutralizes biological effects of anti-HER2/neu antibody on breast cancer cells in vitro. *Int J Cancer*. 1997, 73:875-879
  14. V. Gupta L. Ma, J. Fedynshyn and W. Tan. Bio-Rad Laboratories, Inc. Hercules, CA94547
  15. Li M, Zhang Y, Feurino L, Wang H, Fisher W, Brunicardi C, Chen C, Yao Q. Interleukin-8 increases VEGF and neuropilin expression and stimulates ERK activation in human pancreatic cancer. *Cancer Sci*. 2008, 4: 733-737
  16. Sfiligoi C, Luca A, Cascone I, Sorbello V, Fuso L, Ponzzone R, Biglia N, Audero E, Arisio R, Bussolino F, Sismondi P, De Bortoli M. Angiopoietin-2 expression in breast cancer correlates with lymph node invasion and short survival. *Int J Cancer*. 2003, 4:466-512
  17. Sun K, Wernstedt Asterholm I, Kusminski CM, Bueno AC, Wang ZV, Pollard JW, Brekken RA, Scherer PE. Dichotomous effects of VEGF-A on adipose tissue dysfunction. *ProcNatlAcadSci U S A*. 2012, 15:5874-5882
  18. Harada H, Inoue M, Itasaka S, Hirota K, Morinibu A, Shinomiya K, Zeng L, Ou G, Zhu Y, Yoshimura M, McKenna WG, Muschel RJ, Hiraoka M. Cancer cells that survive radiation therapy acquire HIF-1 activity and translocate towards tumour blood vessels. *Nat Commun*. 2012, 17:783-791
  19. Kurebayashi J, Otsuki T, Kunisue H, Mikami Y, Tanaka K, Yamamoto S, Sonoo H. Expression of Vascular Endothelial Growth Factor (VEGF) Family Members in Breast Cancer. *Jpn J Cancer Res*. 1999, 90:977-981
  20. Laughner E, Taghavi E, Chiles K, Mahon P, Semenza G. HER2 (neu) Signaling Increases the Rate of Hypoxia-Inducible Factor 1 $\alpha$  (HIF-1 $\alpha$ ) Synthesis: Novel Mechanism for HIF-1-Mediated Vascular Endothelial Growth Factor Expression. *Mol Cell Biol*. 2001, 12: 3995-4004
  21. Kao J, Salari K, Bocanegra M, Choi Y, Girard L, Gandhi J, Kwei K, Boussard T, Wang P,



- Gazdar A, Minna J, Pollack J. Molecular Profiling of Breast Cancer Cell Lines Defines Relevant Tumor Models and Provides a Resource for Cancer Gene Discovery. *PLoS one*. 2009, 7:6146-6181
22. Brinkley BR, Beall PT, Wible LJ, Mace ML, Turner DS, Cailleau RM. Variations in cell form and cytoskeleton in human breast carcinoma cells in vitro. *Cancer Res*. 1980, 9:3118-3147
23. Meyer T, Marshall JF, Hart IR. Expression of alpha v integrins and vitronectin receptor identity in breast cancer cells. *Br J Cancer*. 1998, 4:530-536
24. Byzova TV, Kim W, Midura RJ, Plow EF. Activation of integrin alpha (V) beta (3) regulates cell adhesion and migration to bone sialoprotein. *ExpCell Res*. 2000, 2:299-308
25. Danni Li, Hanching Chiu, Vinita Gupta, Daniel W. Chan. Validation of a multiplex immunoassay for serum angiogenic factors as biomarkers for aggressive prostate cancer. *ClinChimActa*. 2012, 20:1506-1511