EVALUATION OF GENETIC AND CELLULAR DISPOSITION FOR GEFITINIB TREATMENT IN NON-SMALL CELL LUNG CANCER

ABSTRACT

The epithelial growth factor receptor (EGFR) is a transmembrane receptor which binds to growth factors. The complex stimulates EGFR's tyrosine kinase (TK) to conduct protein phosphorylation leading to cell proliferation. Cancer could result from a malfunction in the EGFR system. Gefitinib is a TK inhibitor capable of reducing tumor size in patients who have the L858R mutation. However, many studies prove that overtime cells become resistant to the inhibitor and other evidence suggest that it can induce epithelial-mesenchymal transition (EMT), consequentially accelerating the disease. In this study, the susceptibility of A549 non-small cell lung cancer (NSCLC) to undergo EMT was evaluated to determine if exposure to Gefitinib would induce transition. Furthermore, a mutational analysis would determine if the subject has an affinity for Gefitinib. It's reported that the subject has markers indicating an inclination of undergoing EMT. It was also found that the subject does not carry the mutation. The introduction of Gefitinib would just accelerate the disease.

INTRODUCTION

Non-small cell lung cancer (NSCLC) is the world's leading cause of death (Giarnieri et al., 2013; Lynch et al., 2004; Mahmood et al., 2017; Narita et al., 2017), it accounts for one third of all deaths from cancer (Sharma et al., 2007). The over expression of epithelial growth factor receptor (EGFR) genes is closely associated with many cancers, including lung cancer (Ranson, 2004). EGFR serves to be the attachment site of extracellular growth factors, the adhesion causes EGFR's tyrosine kinase (TK) subunits to catalyze phosphorylation on target proteins (Giamas & Vorgias, 2007). The protein phosphorylation causes a cascade of events within the cell which promotes cell division. The number of EGFR on the membrane and the cascade system is highly regulated at many levels to control the replication in cells (Krause & Van Etten, 2005). Cancer could result if any level of the complex EGFR regulation system is altered (Giamas & Vorgias, 2007; Krause & Van Etten, 2005). TK inhibitors are potential treatments to control the erratic and unregulated proliferation of cells (Narita et al., 2017).

Gefitinib is a competitive inhibitor drug for the TK ATP binding site in EGFR (Alfieri et al., 2011). Patients with the exon 19 deletions and/or L858R mutations, which affect EGFR protein, report to have a higher sensitivity for the drug, *in vivo* studies have had response rates as high as 70% (Jackman et al., 2009; Sun et al., 2011). Whereas, wild type EGFR patients show a 6.6% sensitivity for the drug (Alfieri et al., 2011). The 70% of the mutant population responded favorably to the drug with a significant reduction of tumor size, however, an overwhelming majority of the subjects became resistant to the drug (Jackman et al., 2009; Ranson, 2004).

A study conducted to examine the relationship between Gefitinid and epithelial-mesenchymal transition (EMT) of NSCLC *in vitro*, demonstrated that long term exposure of mutants EGFR cells to the drug promoted them to undergo EMT earlier, additionally it increased the cell's resistance to the drug (Hu et al., 2017). Therefore, gefitinib exposure is associated with the risk of increasing cell proliferation, inducing EMT and consequently accelerating the disease.

EMT is the process of epithelial cells changing to a mesenchymal phenotype (Giarnieri et al., 2013; Hackett et al., 2009; Mahmood et al., 2017; OTA et al., 2016). It is a natural occurring process during embryonic morphogenesis and epithelial tissue response to stress (Hackett et al., 2009). However it has also been observed as a necessary step for various cancer's metastasis (Giarnieri et al., 2013; OTA et al., 2016). This new mesenchymal stage of the disease increases the cell's ability to move in the body, consequentially its capacity to spread to other organs rises (Giarnieri et al., 2013).

Many phenotypic differences have been observed between mesenchymal and epithelial cells (Mahmood et al., 2017). During the process of EMT, there is a down regulation of the epithelial proteins like E-cadherin (E-cad), and an upregulation of mesenchymal proteins like vimentin (Vim), N-cadherin (N-cad) and alpha smooth muscle actin (SMA). Changes in actin configuration from squamous (epithelial) to filamentous (mesenchymal) can also be observed during the process of EMT (Giarnieri et al., 2013; Mahmood et al., 2017). The process of EMT is regulated by Snail, Slug, and Twist transcription factors (Giarnieri et al., 2013). Snail and Slug belong to the Snail family of zinc finger transcription factors, in epithelial cells they exist within the cytoplasm, however when EMT commences, they move into the nucleus to regulate the transition to a mesenchymal cell (Giarnieri et al., 2013).

Transforming growth factor beta 1 (TGFβ) is a cytokine involved in cellular morphogenesis, embryonic development, wound healing, synthesis of external matrix and apoptosis (Blobe et al., 2000). Like Gefitinib, TGFβ is also known to induce EMT in cells (Kim et al., 2014). In the experiment, TGFβ will be used to see how close A549 NSCL cells are to EMT.

We are testing the ability of these A549 NSCL cells to undergo EMT, since cells that undergo EMT can easily become resistant to chemotherapy drugs and accelerate the disease. We are also determining if the cells are EGFR mutants, since mutants have a greater affinity for Getfitinib.

MATERIALS AND METHODS

Cells, Trypsinization, Counting, Seeding, and exposing cells to TGFβ

The cell line used was A549 NSCLC, cell line passage number P163. The cells were pretreated in media containing no serum. They were washed with PBS and incubated with trypsin at 37°C. The detached cells were transferred to test tubes and spun down to a pellet. The pellet was resuspended in complete media (5%-10% FBS). A hemocytometer and trypan blue dye was used to count the viable cells. Each well, in a 96-well plate, was seeded with 1.2492x10^5 cells/ml. Four petri dishes were seeded with 4.86x10^4 cells/ml. A slide with two etched rings had 1.2492x10^5 cells/ml. 10ng/ml of TGFβ was added to two petri dishes, and one of the etched rings.

Toxicity test

A 1 in 2 series dilution of TGFβ was preformed. TGFβ was added into the 96-well plate from low concentration (column 3) to highest concentration (column 12), column 1 was left blank, and column 2 only has cells. The plate was placed in a 37°C incubator for 24 hours. The media was removed and the cells washed with PBS. The samples underwent an acid phosphatase assay. The plate was visualized at 405nm and 620nm. A percent cell survival versus varying concentration of TGFβ curve was produced from the data collected.

QPCR

RNA from TGFβ treated **(TT)** and non-treated **(NT)** cells were extracted using RNeasy™ Mini Kit, and quantified using a nanodrop. The RNA was made into cDNA using Thermo's Verso's cDNA synthesis kit and random primers. The cDNA underwent QPCR with a primer set composed of sma, E-cad, N-cad and the internal control 18s. The data was normalized using the delta-delta Ct method.

Western Blotting

Protein was extracted from TT and NT cells using RIPA Buffer, a protease inhibitor and phosphatase inhibitors. Two dilutions of protein were made for both TT and NT cells, 1/10 and 1/50. A BCA protein assay was conducted to draw a standard curve using a protein standard, the protein concentration of TT and NT cells was obtained from the curve.

A PAGE-gel electrophoresis was preformed using the TT and NT protein samples. Then the proteins were transferred from the gel to a nitrocellulose membrane. The membrane was exposed to TBS (0.1%) tween containing 5% non-fat dried milk, then it was probed with the primary antibodies recognizing the following proteins: Vim, N-cad, E-cad and the internal control GAPDH. The second antibody, carrying a HRP substrate, was later introduced to the membrane. The nitrocellulose paper was visualized. The data was normalized by GAPDH, the fold difference was calculated, and statistical analysis was performed.

Immunocytochemistry

The cell on the slide with two etched rings were fixed on to the glass with 3.7% methanol free formaldehyde solution in PBS, next it was permeabilized with 0.1% Trion™ X-100 in PBS. The

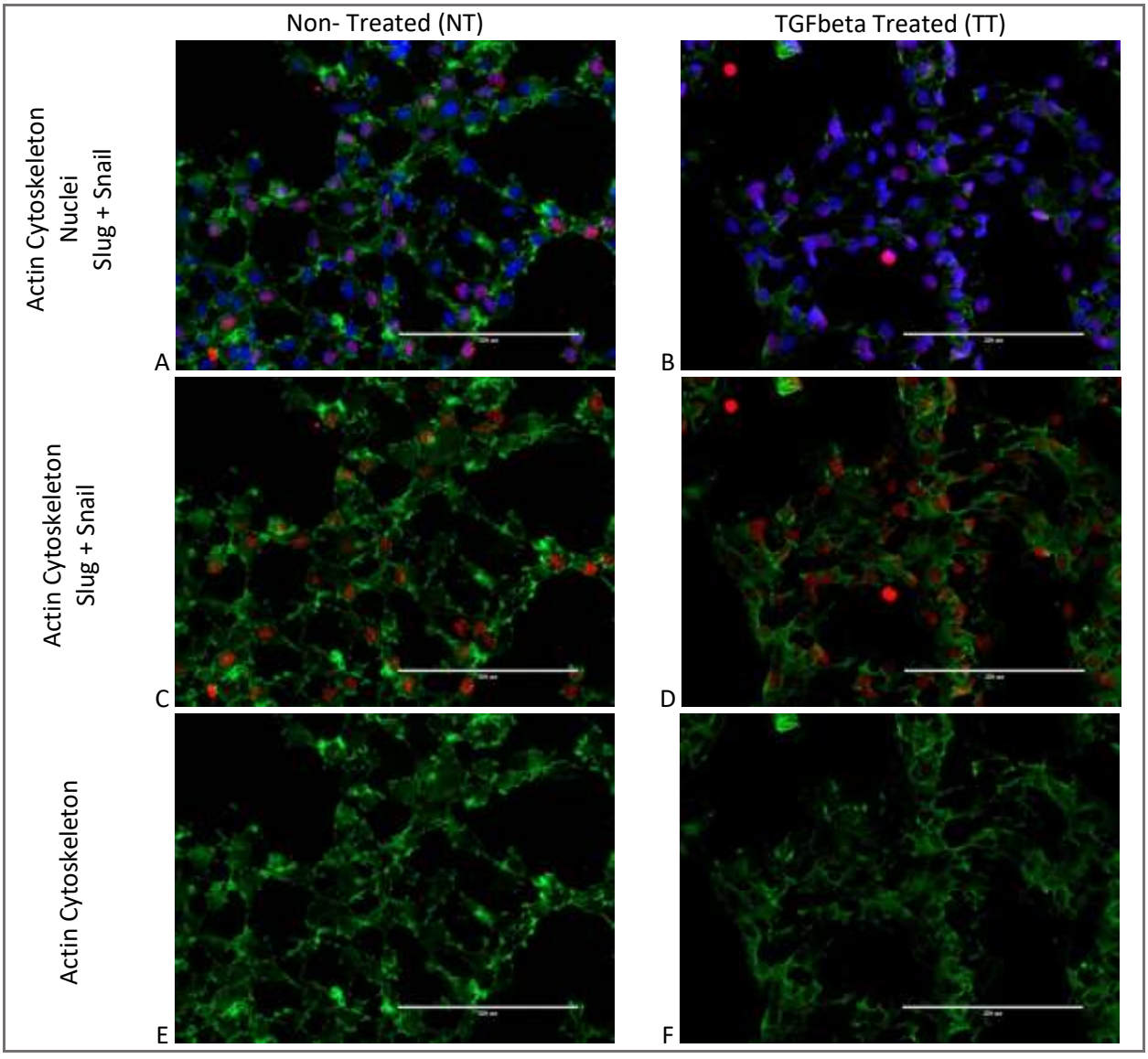
cells were stained with Phalloidin dye, DAPI, and Anti-SNAIL + SLUG antibodies. The slide was viewed under 405nm, 485nm and 594nm wavelengths.

Mutational Analysis

Genomic DNA from A549 NSCLC was extracted, suspended in elution buffer, then quantified using a nanodrop. The genomic DNA underwent PCR using primers surrounding the gene of interest (EGFR). The restriction enzyme Sau96I was used to digest the PCR product. The digested and uncut samples were then loaded and ran on to a 1% agarose gel, then they were visualized.

RESULTS

MORPHOLOGY IMAGES FROM ICC



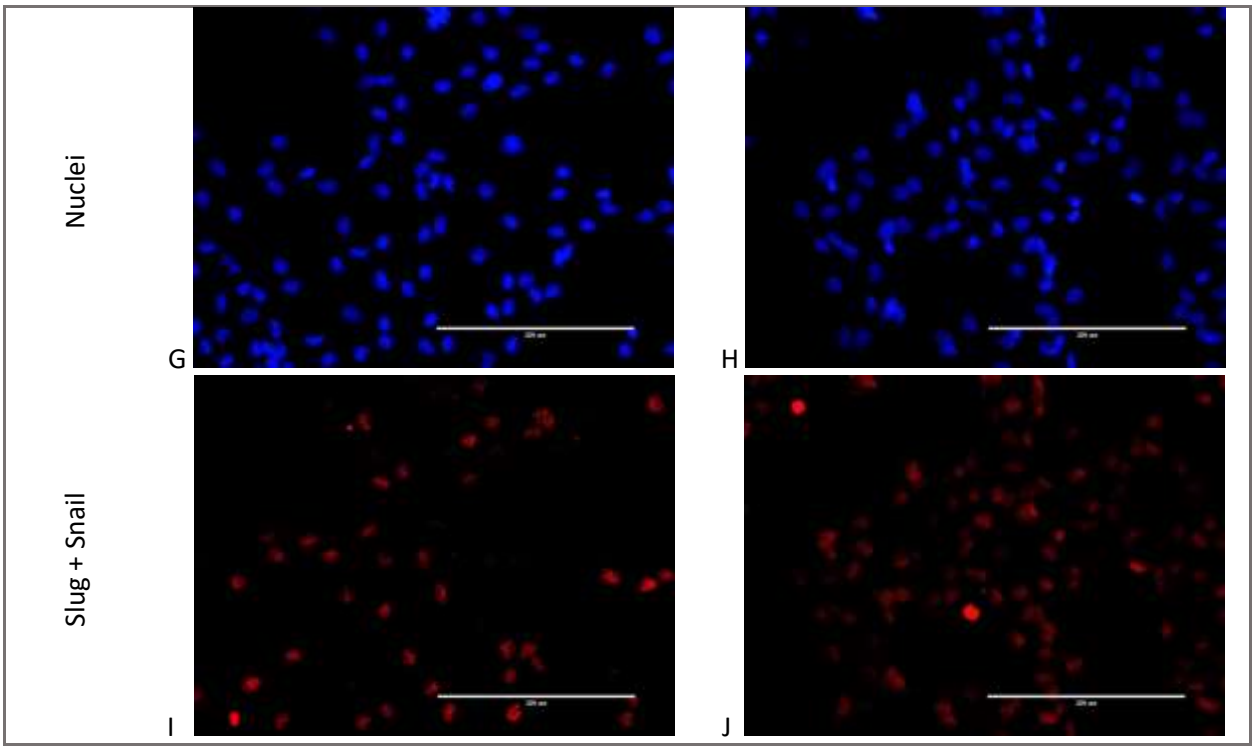


Figure 1- Immunochemistry test showing actin cytoskeleton, Slug , Snail and Nuclei of NT (A,C,E,G,I) and TT cells (B,D,F,H,J). (E&F) Both samples were dyed with 100ul of phalloidin to visualize the actin cytoskeleton under a wavelength of 485nm. (G&H) 100nM DAPI died the nuclei of the cells blue to be visualized at 405nm. (I&J) commercially obtained anti-SNAIL + SLUG marker antibodies stained Snail and Slug transcription factors to see their location within the cell, they were visualized at 594nm. (A&B) Snapshot of the combined actin cytoskeleton, nuclei, slug and snail presence in cells. (C&D) Snapshot of the presence of actin cytoskeleton, slug and snail.

The morphological changes in the cell during EMT can be observed by dying the actin cytoskeleton, nuclei, snail and slug transcription factors. Figure 1 shows the morphological differences occurring in A549 NSCL cells that were treated with TGFβ, to those that were not treated with the drug. In image G and H there is a similar density of nuclei from both samples, however in I and J there is an obvious difference between the amount of snail and slug within the nucleus. J had qualitatively more slug and snail in its nucleus. Snail & slug and the nuclei can be seen overlapping each other in image B. Some snail & slug can be seen overlapping the nuclei in A, however comparatively less than B, the TT cells. Another major dissimilarity between the cells is the structure of their actin cytoskeleton. Image E depicts the cytoskeleton of a NT cell, these cells have a compact structure closer to a square or circle in shape. Unlike E, the cytoskeleton in image F has filaments that elongate the cells. The snail and slug in image C seem to be in the nuclei and not in the cytosol of the cell.

WESTERN BLOT

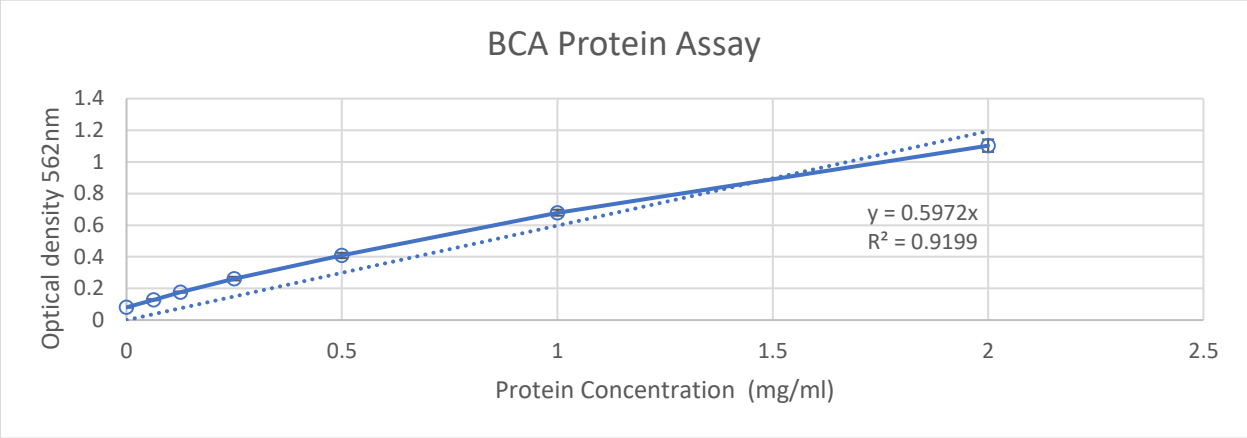


Figure 2- BCA Protein Assay for A592 NSCLC. A standard protein was used to make the standard line. The standard protein under went a 2:1 serial dilution (n=3). The optical density of the samples were compared to the standard and their concentration was found.

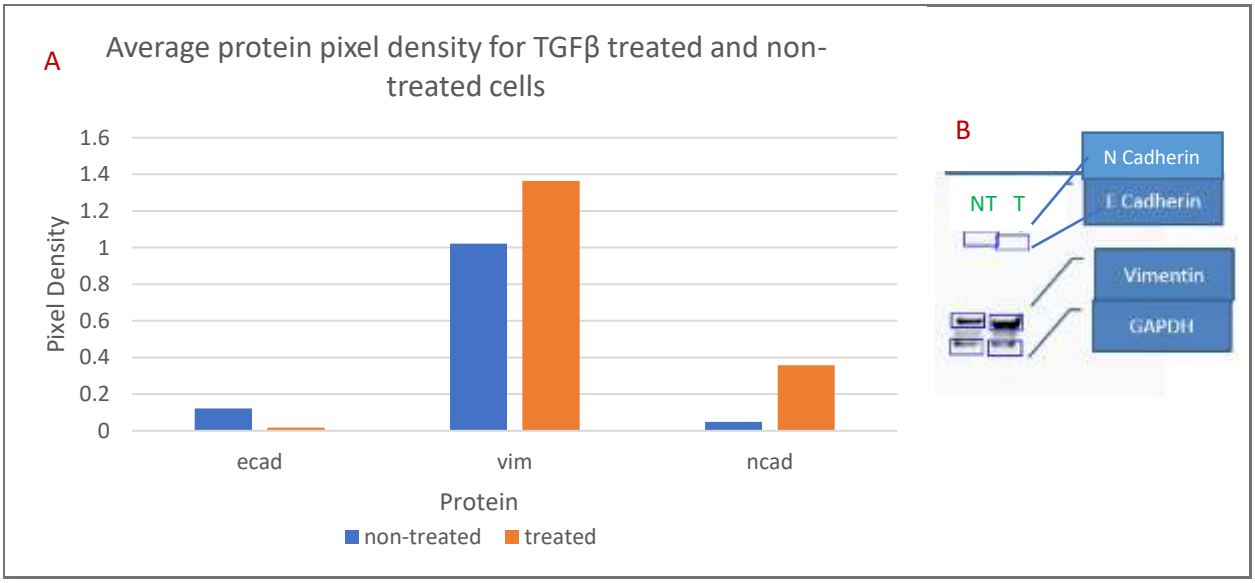


Figure 3- Western blot analysis of E-cad, N-cad, and Vimentin protein expression of TGFbeta treated and non-treated cells. (A) Shows the expression difference between 0.180ug/ul of NT cells, and 0.180ug/ul of TT cells (n=3). Primary antibodies recognizing the following proteins: Vim, N-cad, E-cad and the internal control GAPDH. The second antibody, carrying a HRP substrate was also added. The westernblot was normalized with the protein Glyceraldehyde 3-phosphate dehydrogenase (GAPDH). (B) The gel depicts bands representing E-cad, N-cad, Vim and GAPDH.

Figure 2 -shows how the concentration of the protein was found. A standard curve was created by subtracting the blank from the average OD of each concentration of the control protein. Then a graph was plotted. The concentration of NT and TT samples were compared to the curve. NT 1:10= 0.303ng/ul, NT 1:50= 0.180 ng/ul, TT 1:10=0.510 ng/ul, TT1:50= 0.241 ng/ul.

To detect EMT protein markers, a western blot analysis was performed to quantify the presence of E-cad, N-cad and Vim in TT and NT cells. Figure 3 (A) shows that E-cad had statistically significantly decreased from an average density of 0.122 in NT cell to 0.017 in treated cells, p = 0.0306 (n=3). E-cad decreased by an average of 11.390 folds from NT to treated (sd=9.464). N-cad seems to increase from an average density of 0.049 to 0.359 when exposed to the drug, p=0.1426 (n=3). N-cad experienced an average fold increase of 6.343 (sd= 3.780). Vimentin experienced an average density increase from 1.022 to 1.365, p=0.1670 (n=6). Vimentin's average fold increase is 0.430 (sd=1.785).

TOXICITY TEST

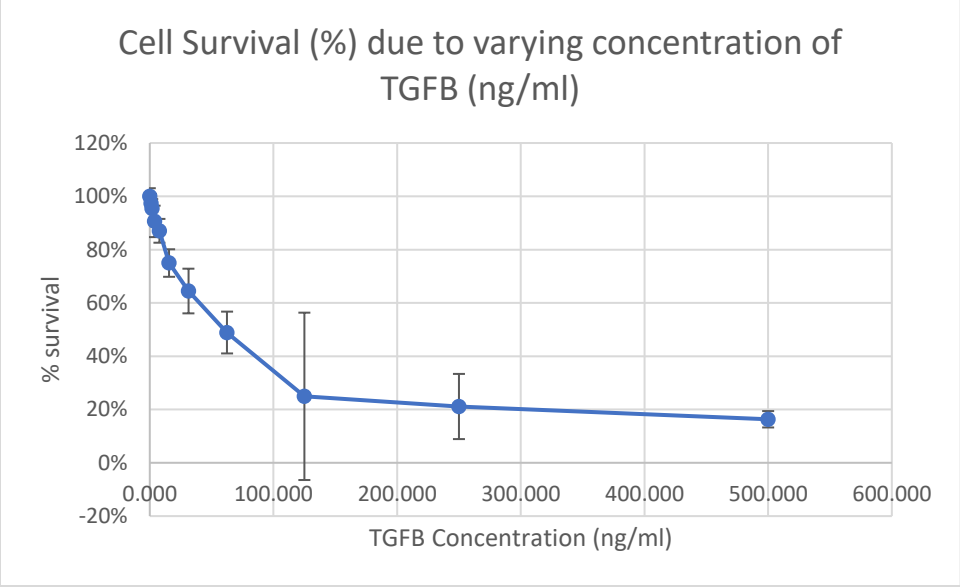


Figure 4- The graph shows the percent survival of NSCLC against various concentrations of TGFβ. NSCLC cells were exposed to Various concentration of TFGβ and placed in a 37°C incubator. Then, the cells underwent an acid phosphatase assay. Finally, they were visualized under a 620nm wavelength. As seen from the graph there is an exponential decrease in %survival as the concentration of the drug increases. The first point in the graph had no TGFβ added to the cell sample.

To determine the appropriate dosage of TGFβ to administer in other experiments within this report, a toxicity test was conducted. Each well held 0.1ml of 1.2492 x10^5 cells/ml. Every TGFβ concentration had 8 samples (n=8). The drug underwent a two-fold dilution. From figure 4 it is apparent that the cell's percent survival is inversely proportional to the drug concentration. There is a large standard deviation (>10%) when the drug had a concentration of 125ng/ul and 250ng/ul. When the concentration of the drug was 0ng/ml there was 100% survival, this is the

control. When there was 500ng/ul of TGFβ there was a 16% survival (sd= 3.07%). There is no steady survival line in the curve

GENE EXPRESSION

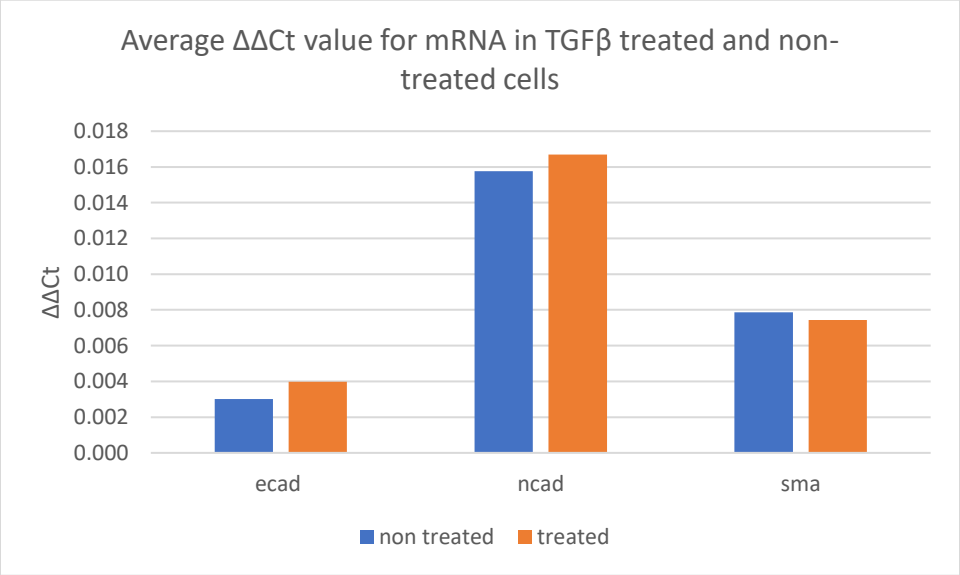


Figure 5- The bar graph shows the average ΔΔCt value for E-cad, N-cad and αSMA mRNA. mRNA was extracted from nontreated and treated cells, quantified, then converted to cDNA and underwent QPCR (n=5). By data analysis, the ΔΔCt was calculated for each EMT mRMA marker.

	ecad	ncad	asma
Average Fold Difference from NT to treated	-0.26827	-1.30474	0.177949
Standard Deviation	4.046881	5.719964	1.812753

Table 1- E-cad, N-cad and SMA fold value. The table shows the average fold value found for each mRNA of interest from NT to TT cells. The fold value was obtained using the ΔΔCt method of normalization.

A gene expression test can tell us how TGFβ influences transcription. From figure 5 we can see that E-cad's NT cells had an average ΔΔCt value of 0.003019 and a TT value of 0.003986 (n=5, p=0.342). E-cad had a fold difference of -0.26827 (table 1). N-cad NT average ΔΔCt value increased from 0.015754 to 0.016686 in TT cells (n=5, p= 0.436) (figure 5). Table1 indicates that N-cad had a fold difference of -1.30474. SMA NT average ΔΔCt value increased from 0.007871 to 0.007441 in TT cells (n=5, p= 0.422) (figure 5). Table one indicates that SMA had a fold difference of 0.177949.

L858R MUTATION ANALYSIS

Figure 6- Gel electrophoresis of EGFR gene, where the L858R mutation could possibly occur. Genomic DNA underwent PCR to extract the gene of interest (GOI) where the L858R mutation occurs. The gene of interest was digested by Sau96I. Both the digested and un-cut sample of the GOI underwent gel electrophoreses. The uncut band is visible at about 220bp, while the digested band is about 180bp.

The GOI is 222bp in length. The wildtype has a single Sau96I restriction cut site at 46bp. The mutated gene has two Sau96I restriction cut sites at 46bp and at 132pb. From figure 5, it's seen that the un-cut fragment is about 220bp in length while the digested band is slightly less, about 180bp. The un-cut band is stronger than the digested band. No other bands were visible in the gel.

DISCUSSION

From the toxicity test, it was evident that TGFβ causes cell death at high concentrations. Figure 4 depicts an immediate decrease in cell survival. The toxicity test should have been repeated narrowing the testing range of TGFβ concentration from 0.0ng/ul to 100 ng/ul to find the concentration before the cell population begins to die. During the experiment a higher concentration of cell was used than anticipated due to human error, a larger density of cells could have cause survivability to worsen. 10ng/ul of TGFβ was utilized in the experiments, as provided by the instructor, it is a concentration of the drug that will not kill the cells.

A standard curve was used to find the concentration of NT and TT protein. This data was then used in the western blot. E-cad showed a statistically significant fold decrease of 11.390 from NT to TT ($p=0.0306$, $n=3$). N-cad showed a statistically insignificant fold increase of 6.343 ($p= 0.1426$, $n=3$). Vim showed a statistically insignificant fold increase of 0.430 ($p= 0.1426$, $n=3$). The western blot demonstrated that when exposed to TGFβ, E-cad was in fact reduced, as described by the literature. The experiment should be replicated because Vim and N-cad's data is not reliable even though they, as expected, increased in density from NT to TT cells.

mRNA EMT markers were analyzed using QPCR. E-cad seems to have increased in density from a NT $\Delta\Delta Ct = 0.003019$ to a TT= 0.003986, yet it has a fold value of -0.26827, meaning it decreased in density ($p=0.342$). The conflicting results could be due to the loss in precision when conducting the fold calculations. Also, in reference to the $p>5$ value, the data failed to

reject the hypothesis that E-cad is higher after EMT. N-cad did increase in density from NT to TT, it has a statistically insignificant fold value of 1.30474 (p=0.436). Similarly, SMA did increase in density from NT to TT, it has a statistically insignificant fold value of 0.177949 (p=0.422). No conclusive findings were obtained from the QPCR since all of the marker's results were statistically insignificant.

The ICC images in figure 1 depict the morphological changes the cell experienced when exposed to TGFβ. The actin cytoskeleton is drastically different between NT and TT. In the NT, actin seems to have formed square/circular arrangements, similar to that of epithelial cells. The TT cells seem to have an elongated spindle like arrangement of actin, congruent of mesenchymal cells. This suggest that EMT did occur in these cells. The nuclei position was determined to find the location of SLUG and SNAIL in reference to the nuclei. From the images (I & J) there seems to be a lower concentration of SLUG/SNAIL within the nuclei of NT cells than in TT. However, the presence of SLUG/SNAIL in the nuclei of the NT sample does not correlate with the characteristics of an epithelial cell. In epithelial cells SLUG and SNAIL would be in the cytosol. This suggest that the NT cells are also going into EMT. During the execution of the experiment the slide flooded constantly, and this could be the reason our control (NT) is taking on mesenchymal markers.

The mutation analysis revealed two visible band of about 222bp for the uncut sample and a band about 180bp long for the digested sample. If the L858R mutation is present then there are expected bands of the following lengths: 90bp, 86bp, and 46bp. If the mutation is not present, there would be 176bp and 46bp bands. According to the results, A549 NSCLC does not have the L585R mutation and therefore does not have the affinity for Gefitinib.

The cell seems to be near EMT, however it is not statistically convincing due to the high p values throughout the experiments. During the western blot E-cad decreased, N-cad increased, and Vim increased. These results are congruent with protein markers of EMT. The QPCR revealed that N-cad and SMA increased, as they do in EMT. However, E-cad results were inconclusive. Finally, the ICC images revealed that actin had an epithelial structure in NT, and a mesenchymal structure in TT. Despite this, SNAIL and SLUG were in the nuclei of both NT and TT cells. TGFβ did cause EMT in A549 NSCLC.

Gefitinib cannot be provided to A549 NSCLC because it does not have the mutation and it is close to EMT. Exposure would result to the acceleration of the disease.

REFERENCES

Alfieri, R. R., Galetti, M., Tramonti, S., Andreoli, R., Mozzoni, P., Cavazzoni, A., … Petronini, P. G. (2011). Metabolism of the EGFR tyrosin kinase inhibitor gefitinib by cytochrome P450 1A1 enzyme in EGFR-wild type non small cell lung cancer cell lines. *Molecular Cancer; London*, *10*, 143. http://dx.doi.org/10.1186/1476-4598-10-143

Blobe, G. C., Schiemann, W. P., & Lodish, H. F. (2000). Role of Transforming Growth Factor β in Human Disease. *New England Journal of Medicine*, *342*(18), 1350–1358. https://doi.org/10.1056/NEJM200005043421807

Giamas, G., & Vorgias, C. E. (2007). Protein kinases as targets for cancer treatment. *Pharmacogenomics; London*, *8*(8), 1005–1016. http://dx.doi.org/10.2217/14622416.8.8.1005

Giarnieri, E., Vitis, C. D., Noto, A., Roscilli, G., Salerno, G., Mariotta, S., … Mancini, R. (2013). EMT markers in lung adenocarcinoma pleural effusion spheroid cells. *Journal of Cellular Physiology*, *228*(8), 1720–1726. https://doi.org/10.1002/jcp.24300

Hackett, T.-L., Warner, S. M., Stefanowicz, D., Shaheen, F., Pechkovsky, D. V., Murray, L. A., … Knight, D. A. (2009). Induction of Epithelial-Mesenchymal Transition in Primary Airway Epithelial Cells from Patients with Asthma by Transforming Growth Factor-[beta]1. *American Journal of Respiratory and Critical Care Medicine; New York*, *180*(2), 122–133.

Hu, Y., Zang, J., Qin, X., Yan, D., Cao, H., Zhou, L., … Feng, J.-F. (2017). Epithelial-to-mesenchymal transition correlates with gefitinib resistance in NSCLC cells and the liver X receptor ligand GW3965 reverses gefitinib resistance through inhibition of vimentin. *OncoTargets and Therapy*, *10*, 2341–2348. https://doi.org/10.2147/OTT.S124757

Jackman, D., Pao, W., Riely, G. J., Engelman, J. A., Kris, M. G., Jänne, P. A., … Miller, V. A. (2009). Clinical Definition of Acquired Resistance to Epidermal Growth Factor Receptor Tyrosine Kinase Inhibitors in Non–Small-Cell Lung Cancer. *Journal of Clinical Oncology*. https://doi.org/10.1200/JCO.2009.24.7049

Kim, Y.-J., Choi, W.-I., Jeon, B.-N., Choi, K.-C., Kim, K., Kim, T.-J., … Ko, H. (2014). Stereospecific effects of ginsenoside 20-Rg3 inhibits TGF-β1-induced epithelial–mesenchymal transition and suppresses lung cancer migration, invasion and anoikis resistance. *Toxicology*, *322*, 23–33. https://doi.org/10.1016/j.tox.2014.04.002

Krause, D. S., & Van Etten, R. A. (2005). Tyrosine Kinases as Targets for Cancer Therapy. *New England Journal of Medicine*, *353*(2), 172–187. https://doi.org/10.1056/NEJMra044389

Lynch, T. J., Bell, D. W., Sordella, R., Gurubhagavatula, S., Okimoto, R. A., Brannigan, B. W., … Haber, D. A. (2004). Activating Mutations in the Epidermal Growth Factor Receptor Underlying Responsiveness of Non–Small-Cell Lung Cancer to Gefitinib. *New England Journal of Medicine*, *350*(21), 2129–2139. https://doi.org/10.1056/NEJMoa040938

Mahmood, M. Q., Ward, C., Muller, H. K., Sohal, S. S., & Walters, E. H. (2017). Epithelial mesenchymal transition (EMT) and non-small cell lung cancer (NSCLC): A mutual association with airway disease. *Medical Oncology; Middlesex*, *34*(3), 1–10. http://dx.doi.org/10.1007/s12032-017-0900-y

Narita, M., Shimura, E., Nagasawa, A., Aiuchi, T., Suda, Y., Hamada, Y., … Narita, M. (2017). Chronic treatment of non-small-cell lung cancer cells with gefitinib leads to an epigenetic loss of epithelial properties associated with reductions in microRNA-155 and -200c. *PLoS One; San Francisco*, *12*(2), e0172115. http://dx.doi.org/10.1371/journal.pone.0172115

OTA, I., MASUI, T., KURIHARA, M., YOOK, J.-I., MIKAMI, S., KIMURA, T., … KITAHARA, T. (2016). Snail-induced EMT promotes cancer stem cell-like properties in head and neck cancer cells. *Oncology Reports; Athens*, *35*(1), 261. http://dx.doi.org/10.3892/or.2015.4348

Ranson, M. (2004). Epidermal growth factor receptor tyrosine kinase inhibitors. *British Journal of Cancer*, *90*(12), 2250. https://doi.org/10.1038/sj.bjc.6601873

Sharma, S. V., Bell, D. W., Settleman, J., & Haber, D. A. (2007). Epidermal growth factor receptor mutations in lung cancer. *Nature Reviews Cancer*, *7*(3), 169–181. https://doi.org/10.1038/nrc2088

Sun, J., Won, Y., Kim, S. T., Kim, J. H., Choi, Y., Lee, J., … Ahn, M. (2011). The different efficacy of gefitinib or erlotinib according to epidermal growth factor receptor exon 19 and exon 21 mutations in Korean non-small cell lung cancer patients. *Journal of Cancer Research & Clinical Oncology; Heidelberg*, *137*(4), 687–694. http://dx.doi.org/10.1007/s00432-010-0928-2