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Title: Profiling of Human Normal and Cancer Cell Lines Using Phenotype Microarray Analysis

Introduction

The occurrence of cancers in the human body is marked by dysregulation of the various signaling pathways within the cancerous cells. Proteins within one network communicate and provide a tight regulation for cell growth and mismanagement of the signaling pathways results in an aberrant regulation. When dysregulation occurs, a loss of the signaling pathways' ability to regulate itself entails, leading to cancerous growth and cell survival. The pathway studied is the mTOR (mammalian Target of Rapamycin) pathway (Fig. 1). This pathway regulates a variety of processes such as cell growth proliferation, motility, survival, protein synthesis, and transcription (*1*). The mTOR pathway was analyzed using Phenotype MicroArray technology (BIOLOG, Inc.). This method provides a platform to comprehensively analyze cellular phenotypes in response to genetic changes or chemical treatments (*2*). It is a powerful tool in the field of functional genomics as it provides information about phenotypic variations observed as a result of gene over-expression or gene silencing. The overall aim of our research was to determine the differences exhibited by cell lines derived from women cancers (breast, uterine, and ovarian) in response to exposure to anti-cancer agents using Phenotype MicroArray panels, cell proliferation, and Immunologic based assays; and to determine whether these anti-cancer agents target the mTOR signaling pathway in these cells. There have been no previous studies reported exploring mTOR signaling in these cell lines using the novel Biolog Phenotype MicroArray platform.

Experimental methods

Cell Culture: The cell lines HEK293 (human embryonic kidney), OV90 (ovarian adenocarcinoma), TOV112D (ovarian adenocarcinoma), KLE (uterine adenocarcinoma), MES-SA (uterine sarcoma), SKBR (breast adenosarcoma) were obtained from ATCC (American Type Culture Collection). HEK 293 was cultured in cultured in Dulbecco's Modified Eagle's Medium (DMEM, high glucose) supplemented to a final concentration of 2 mM glutamine, 50 units/ml penicillin, 50 units/ml streptomycin, and 10% heat-inactivated fetal bovine serum (FBS, Invitrogen). OV90 and TOV112D were cultured in 1:1 mixture of MCDB 105 medium containing a final concentration of 1.5 g/L sodium bicarbonate and Medium 199 containing a final concentration of 2.2 g/L sodium bicarbonate supplemented to a final concentration of 2 mM glutamine, 50 units/ml penicillin, 50 units/ml streptomycin, and 15% heat-inactivated fetal bovine serum (FBS, Invitrogen). SKBR and MES-SA were cultured in McCoy's modified medium supplemented to a final concentration of 2 mM glutamine, 50 units/ml penicillin, 50 units/ml streptomycin, and 10% heat-inactivated fetal bovine serum (FBS, Invitrogen). KLE was cultured in DMEM F12 medium supplemented to a final concentration of 2 mM glutamine, 50 units/ml penicillin, 50 units/ml streptomycin, and 10% heat-inactivated fetal bovine serum (FBS, Invitrogen). Cells were incubated at 37°C in 5% CO_2 and maintained on 60mm plates.

Dose Response Treatment: Cells from 60mm maintenance plates were transferred to 48 well plates upon reaching 90% confluency. Cells were then treated with various anticancer reagents (celastrol, gossypol, azathioprine, berberine chloride, miltefosine, and etoposide) in triplicates at concentrations- 0, 0.05, 0.1, 0.2, 0.5, 1, 2 and 5 µM. 24 hours post treatment cell viability was measured by the MTT Cell Proliferation Assay.

Cell proliferation assays: MTT powder was dissolved in PBS solution to a final concentration of 5mg/mL. This solution was then combined with cell growth media and 270 µl of solution was added to each well. Absorbance at 570 nm was monitored using a plate reader (Biotek Instruments, VT).

ELISA detection of phospho-p70 S6K (Thr389): To assess inhibition of mTOR pathway cells were treated with anticancer reagents for 24h and lysates were prepared. Equal quantities of protein were analyzed using a phospho-p70 S6K (Thr389) Instant One Elisa assay (eBioscience) according to the kit's protocol.

Results and Conclusions

Phenotype Microarray assays' are cell-based assays used to establish profiles of mammalian cells in response to a variety of treatments. The platform uses 96 well plates pre-loaded with carbon-energy and nitrogen substrates, ions, hormones/cytokines and anti-cancer agents. We utilized these arrays to profile a variety of women's cancer cell lines to screen for agents that would inhibit the growth of these cells. For this purpose, the panels PM-M11, 12, 13 and 14 anti-cancer panels were utilized, each panel containing 23 different reagents. 92 different anti-cancer agents were thus tested using four different panels on five human cancer cell lines (ovarian- TOV 112D and OV 90; breast- SKBR; and uterine-MES-SA and KLE) and one normal control cell line (HEK293). Cells were seeded on the panels and growth was analyzed 24-36h post seeding after being treated with increasing concentrations of these agents. A reduction in growth was considered significant if the cell density was found to decrease by at least 50% compared to the untreated controls. As shown in Table 1, of the 92 reagents tested, a majority of them were selective in inhibiting growth in a cell-line specific manner, i.e. some reagents only impacted normal cells, some only cancer cells while some effected all cell lines. Our subsequent experimentation involved monitoring the impact of six

reagents (Celastrol, Etoposide, Berberine Chloride, Miltefosine, Gossypol, and Azathioprine, Fig. 2) on the morphology of the cells. As shown in a representative Fig. 3, the reagents had varying effects on cell size, shape and confluency, with some reagents exhibiting a more pronounced impact than the others, such as celastrol. This observation suggests that these reagents, in addition to inhibiting growth, also potentially modulate a cellular pathway that may govern morphology.

One of the pathway's that is dysregulated in a variety of cancers is the insulin/mTOR signaling which controls cellular growth. To determine whether the observed growth inhibition by these reagents was as a result of impact on mTOR signaling, the activation of one of the key proteins in this pathway- S6 kinase (S6K) was analyzed. S6K is phosphorylated upon mTOR activation and a decrease in phospho-S6K levels reflects inhibition of mTOR activity. The InstantOne ELISA assay was utilized to monitor the pS6K levels in the cell lines in response to treatment with the six reagents. Cells were treated with a fixed concentration (5μM) of these reagents for 24h and analyzed. As observed in Fig. 4, the ELISA based assay showed that different reagents affect every cell line differently. In general, celastrol was found to target the mTOR pathway in a majority of the cancer cell lines but not in KLE or the control. Interestingly, miltefosine was found to inhibit only MES-SA cells suggesting a very specific targeting in this cell line. Furthermore, etoposide inhibited mTOR signaling in SKBR and TOV112D cells only. Finally, azathioprine was only found to be effective at inhibiting S6K phosphorylation in HEK 293 cells. From these results, it is possible to conclude that if a decline in cell growth occurs after treatment with a reagent that is not shown to inhibit mTOR signaling in that cell line, the decline in growth is probably due to some other pathway being targeted. These results provide a unique insight into how cell lines from the same tissue respond differently to a specific reagent, although the cell lines

have a different lineage (carcinoma versus sarcoma) and malignancy grade. Thus, we hypothesize that these reagents are likely to target multiple pathways in exerting their growth-inhibitory effects, with mTOR signaling being one of them.

In order to test the effects of the reagents on cell growth overall, a dose-dependent profile was analyzed. All cell lines were treated with the six reagents individually at concentrations ranging from 0.05-5μM and growth was monitored 24h post treatment. Due to the large volume of data gathered only a representative result is shown in figure 5 for celastrol treatment. Celastrol was found to inhibit growth in SKBR, MESA-SA, and TOV 112D cells at a concentration of 2μM and above. However, the control cell line HEK 293 was not inhibited significantly by celastrol. From this observation, it is possible to conclude that celastrol effectively reduces growth in cancer cell lines specifically, but not in a non-cancerous cell line. Celastrol has previously been shown to possess anti-tumor activity (*3*) and target mTOR signaling in prostate cancer cells (*4*) but our data provides the first evidence that this pathway is also impacted in women's cancer cell lines.

Our project is innovative, as metabolic/growth profiling of cancer cell lines using Phenotype MicroArray technology, specifically derived from women has not been reported in literature. Although a similar study has been done for blood, colon, lung, liver and prostate cancer, women's cancers have not been studied. To our knowledge, this project is the first of its kind to examine women cancer cell lines.

Future directions of this study include inclusion of cell lines from cervical cancer. In addition, assessment of phospho-S6K via immunoblotting as well as colony forming and wound healing assays will also be employed. The impact of the reagents will also be analyzed via flow cytometry to carefully assess changes in cell size and shape. Thus, the Phenotype Microarray panels can serve as valuable tools for screening for anti-cancer compounds.

References:

1. mTOR signaling: implications for cancer and anticancer therapy. E Petroulakis, Y Mamane, O Le Bacquer, D Shahbazian, N Sonenberg (2006) Br. J. Cancer 94(2):195-9.

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3. Triterpenoids as new promising anticancer drugs. A Petronelli, G Pannitteri, U Testa (2009) Anticancer Drugs 20(10):880-92.

4. Celastrol suppresses angiogenesis-mediated tumor growth through inhibition of AKT /mammalian target of rapamycin pathway. X Pang, Z Yi, J Zhang, B Lu, B Sung, W Qu, BB Aggarwal, M Liu (2010) Cancer Res. 70(5):1951-9.

Figures and Tables

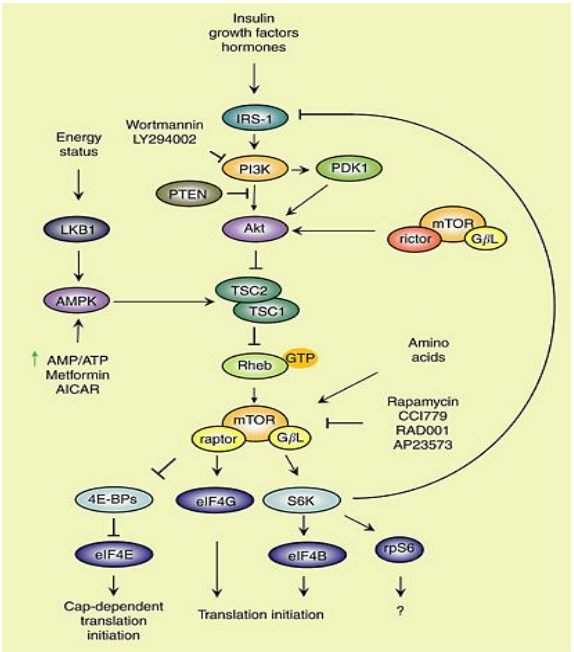

Fig. 1: mTOR signaling (http://www.nature.com/bjc/journal/ v94/n2/fig tab/6602902f1.html)

	HEK293	TOV112D	OV90	MES-SA
4'-DE	-	-	+	+
5-FdU	-	-	-	+
Acivicin	-	+	-	+
Aclarubicin	+	+	-	+
Acriflavinium	+	+	+	+
Aklavine	+	+	-	+
Altretamine	+	-	-	-
Aminolevulinic acid	-	+	-	-
Amygdalin	-	+	-	+
Ancitabine	-	-	-	+
Azaserine	-	-	+	-
Azathioprine	+	-	-	-
Berberine Chloride	-	+	+	+
beta-Peltatin	-	+	-	-
Camptothecin	-	+	+	-
Carmofur	+	+	-	-
Carmustine	-	+	-	-
Celastrol	+	+	+	+

	HEK293	TOV112D	OV90	MES-SA
Cisplatin	-	-	+	+
Colchicine	-	+	-	-
Cycloheximide	+	-	-	+
Dactinomycin	+	+	-	-
Daunorubicin	+	+	-	+
Deguelin(-)	-	+	-	+
Doxorubicin	+	+	-	+
Emetine	+	+	-	+
Emodin	-	-	+	-
Etoposide	-	-	+	+
Floxuridine	-	+	+	-
Gossypol	-	+	-	+
Hydroxyurea	-	+	-	-
Indole-3-Carbinol	-	-	+	-
Mechlorethamine	+	-	-	-
Miltefosine	+	-	-	-
Mitomycin C	+	-	-	+
Mitoxantrone	+	+	+	+
Mycophenolic Acid	-	-	+	+

Table 1: Phenotype Microarray analyses of cancer cell lines
Cells were seeded on four different PM anti-cancer panels and growth was monitored 24-36h post seeding using an MTT assay *(+ indicates growth inhibition, - indicates no growth inhibition)*

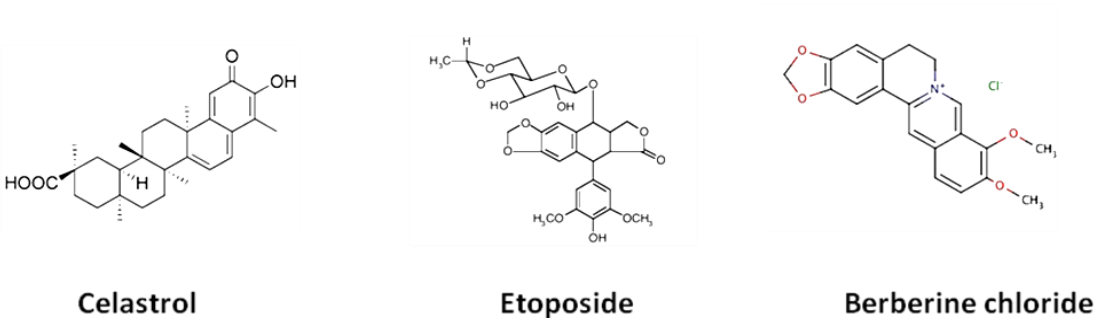

Fig. 2: Structures of anti-cancer compounds

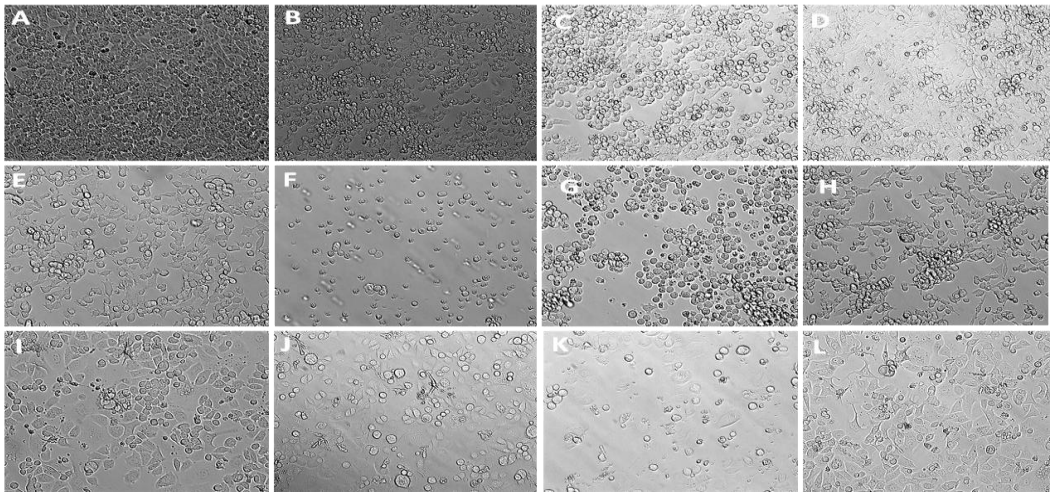

Fig. 3: Impact of anti-cancer compounds on cellular morphology
A, E, I: Control; ***B, F, J***: Celastrol; ***C, G, K***: Etoposide; ***D, H, L***: Berberine chloride
Top panel (MES-SA cells); middle panel (TOV112D cells); bottom panel (SKBR cells)

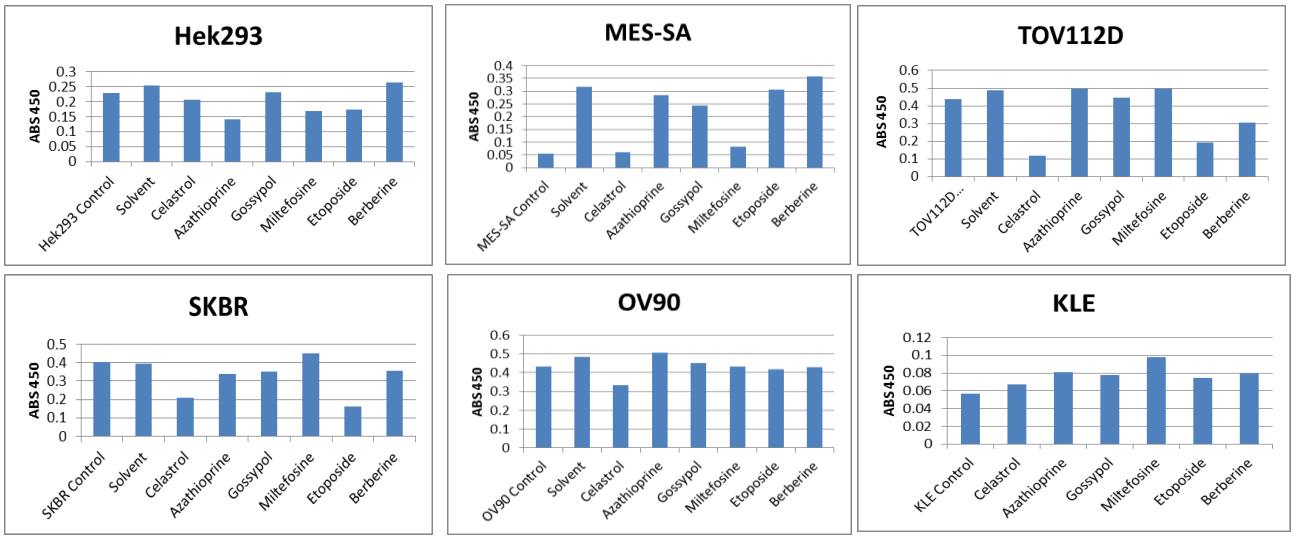

Fig. 4: Impact of anti-cancer compounds on mTOR signaling. Cells were treated with 5µM of the various reagents and after 24 hours, protein lysates were prepared and estimated. An equal quantity of protein was loaded on an ELISA immunological assay with an affinity for phosphorylated S6. Absorbance was measured at 450nm.

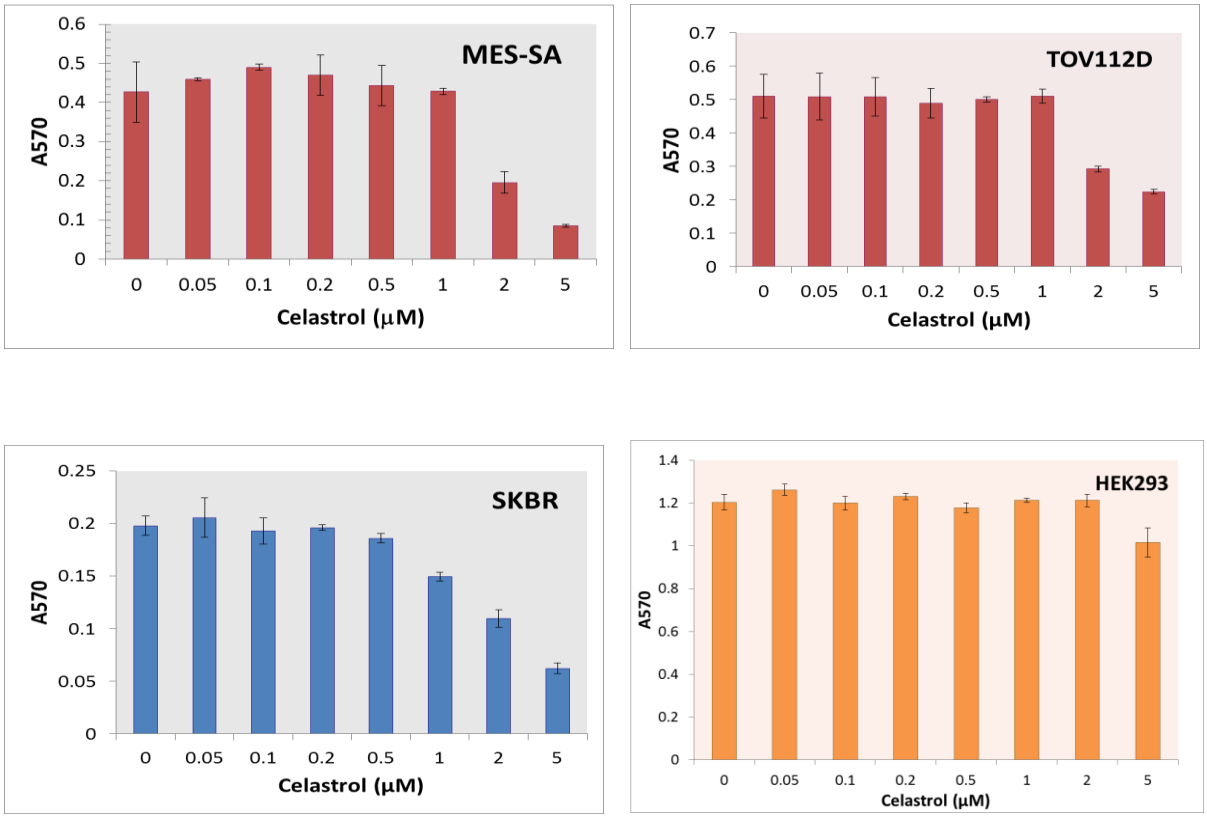

Fig. 5: Dose dependent impact of celastrol on cell growth. Cells were treated with different concentrations of celastrol and growth was monitored 24h post treatment. Each treatment was done in triplicates.