Thursday, 22 March 2012 12:30–13:30

POSTER SESSION

Pharmacology, New Drug Development

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Combining Everolimus with a PI3K Inhibitor Mitigate Cross-Talk and Improves Response to Endocrine Therapy in a Pre-Clinical Breast Cancer Model

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Background: The mechanism of endocrine therapy (ET) resistance in breast cancer remains unclear. Everolimus (EVE), an oral mTOR inhibitor, can reverse ET resistance concurrent treatment with tamoxifen (TAM), letrozole or exemestane. However, increasing evidence suggests that EVE may cause a cross-talk pathway activation, which induced Akt phosphorylation, and therefore decreased the treatment efficacy. In this study, we assessed whether EVE combination with a PI3K inhibitor (LY294002) can inhibit this pathway cross-talk activation in breast cancer.

Material and Methods: Breast cancer lines (MCF-7 and BT474) were treated with TAM alone, TAM+EVE, TAM+LY294002, TAM+EVE+LY294002, or control in vitro. Cell viability, division cycle and apoptosis were analyzed by flow cytomentry. PI3K/Akt/mTOR signaling pathway and a potential downstream target (HIF-1a) status was evaluated by western blot assay. VEGF level was detected by ELISA.

Results: EVE had synergistic effects with TAM. TAM+EVE+LY294002 treatment had best antitumor effect compared with TAM alone or other two agents combination group (TAM+EVE; TAM+ LY294002), and more tumor cell apoptosis was observed in this three agents group compared with others. In addition, we found cross-talk phosphorylation of Akt (pAkt) expression increased in EVE alone or TAM+EVE group, even though pathway downstream biomarkers (P70S6k and 4EBP1) expression decreased. However, adding LY294002 to the TAM+EVE treatment can significantly decreased pAKT expression as well as the phosphorylation level of P70S6K and 4EBP1. Moreover, in hypoxic condition, HIF-1a expression increased in TAM alone treatment group, which can significantly inhibited by adding EVE treatment, especially in combination with LY294002. In addition, MMP-3 level can also be decreased in this three agents group, and VEGF level followed the same trend.

Conclusion: Combining EVE with a PI3K inhibitor (LY294002) can reverse the pathway related cross-talk pAkt activation, resulting with enhancement of ET efficacy. These promising results warrant further study in breast cancer treatment.

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Feasibility Examination of Prior Administration of Cyclophosphamide in TC Combination

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Background: TC (docetaxel 75 mg/m^2 and cyclophosphamide 600 mg/m^2 q3w) combination is used for neoadjuvant/adjuvant chemotherapy in primary breast cancer. The incidence of allergic reaction is reportedly more common in patients who receive docetaxel before cyclophosphamide. This study aimed to determine the significance cyclophosphamide and docetaxel sequence.

Methods: A prospective analysis was performed in 49 consecutive patients treated with TC for stage I-IIB breast cancer from March 2010 to June 2011. Premedication was administered with granisetron, dexamethasone and chlorpheniramine. Patient charts were reviewed for completion rate and adverse events. Two-tailed Fisher exact test was used to evaluate the adverse events between cyclophosphamide and docetaxel sequence.

Results: Of 49 patients, 26 received docetaxel prior to cyclophosphamide, and 23 received cyclophosphamide before docetaxel. There were no differences in patient characteristics between the two groups. Completion rates were 95.6% in the prior cyclophosphamide group, and 100% in the prior docetaxel group. The relative dose intensities of docetaxel and cyclophosphamide were 94.5% and 94.8% in the prior cyclophosphamide group, and 98.5% and 98.7% in the prior docetaxel group ($p < 0.01$). In the prior cyclophosphamide group, severe neutropenia occurred in 96% of patients, but in only 46% of patients in the prior docetaxel group ($p < 0.01$). Significantly fewer cases of eczema (27% vs. 61%), nausea (8% vs. 48%), stomatitis (23% vs. 61%), and diarrhea (4% vs. 30%) were observed in the prior docetaxel group as compared with the prior cyclophosphamide group ($p < 0.01$). Decreased incidences of fatigue (50% vs. 65%) and edema (19% vs. 35%) were found in the prior docetaxel group ($p < 0.05$). No difference was observed in allergic reaction or neuropathy between the two groups.

Conclusion: Patients receiving cyclophosphamide prior to docetaxel were at increased risk of several toxicities as compared with patients receiving docetaxel prior to cyclophosphamide in TC combination therapy.

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Regulator of G Protein Signaling 6 (RGS6) Suppresses Mammary Tumorigenesis by Enhancing DNA Damage Signaling and Blocking Oncogenic Transformation

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RGS6 is a member of the RGS protein family that function as negative regulators of G protein signaling by virtue of their GTPase-activating protein (GAP) activity toward Ga subunits. Given the link between persistent G protein activity and cancer and a reduced risk of bladder tumor formation in humans expressing an RGS6 SNP that increases its translation, we hypothesized that RGS6 might function as a tumor suppressor. Here we show that RGS6 was expressed exclusively in ductal epithelial cells in mammary tissue and its expression is markedly down-regulated in human breast cancer with loss of RGS6 correlating with malignancy. Expression of RGS6 in human breast cancer cells provoked impressive anti-proliferative actions and induction of apoptosis by p53-independent mechanisms. RGS6 activated the intrinsic pathway, involving regulation of Bax/Bcl-2, cytochrome C release and activation of caspase-3 and -9. RGS6 promoted loss of mitochondrial membrane potential ($\Delta\Psi$m) and increases in reactive oxygen species (ROS). RGS6-induced ROS mediated its ability to promote caspase activation and loss of $\Delta\Psi$m, suggesting a feed forward amplification mechanism of ROS in RGS6-induced apoptosis. The pro-apoptotic actions of RGS6 are independent of its GAP activity, thus defining an entirely novel signaling activity of RGS6. We next employed RGS6$^{-/-}$ mice to interrogate the role of RGS6 in breast tumor progression. Mice lacking RGS6 exhibited accelerated tumor formation and increased tumor size compared to WT mice in response to the carcinogen DMBA, providing new evidence that RGS6 functions as a suppressor of breast tumorigenesis. Also, nearly 20% of virgin female RGS6$^{-/-}$ mice over 1 year of age developed spontaneous breast tumors in the absence of DMBA treatment. We recently reported that RGS6 is required for doxorubicin-induced ATM and p53 activation. RGS6$^{-/-}$ mice also exhibit increased DMBA-induced DNA damage due to loss of ATM/p53 mediated DNA repair, underscoring the importance of RGS6 in DMBA-induced DNA damage signaling. Further, we found that RGS6 dramatically suppresses oncogene-induced cellular transformation of MEFs mediated by combined expression of constitutively active Ras and dominant negative p53. These results demonstrate that RGS6 is a critical regulator of both the cellular responses to DNA damage and oncogene activation and that RGS6 functions as a tumor suppressor *in vivo*. Thus, RGS6 represents a novel target in the treatment of breast cancer.

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Efficacy and Safety of Fetal Human Estrogen Estetrol (E4) in Women with Estrogen-receptor Positive Early Breast Cancer

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Background: Estetrol (E4) is a natural fetal estrogen which exerts estrogenic effects on reproductive organs and on the bone, and effectively reduces menopausal smyptoms. In contrast to other estrogens, however, E4 has estrogen-antagonistic effects on breast cancer cell lines *in vitro* and in the rat DMBA model, which would make it a suitable Hormone Replacement Therapy (HRT) in breast cancer patients, particularly in women who are being treated with aromatase inhibitors.

Patients and Methods: We have investigated the effect of 14 days pre-operative treatment with 20 mg E4 per day on tumor proliferation, apoptosis,

ER-receptors, PgR receptor and several endocrine parameters in a prospective, randomised, double-blind, placebo-controlled, neo-adjuvant study in 15 pre- and 14 postmenopausal women with estrogen-receptor positive early breast cancer.

Results: Estetrol induced a significant increase of SHBG, a significant decrease of FSH in postmenopausal women and no increase of gonadotrophins in premenopausal women. Estetrol had no effect on Ki67 expression and on apoptosis-related Bax and Bcl-2, but the apoptosis index in tumor tissue increased significantly. Systemic IGF-1 levels decreased significantly. Surprisingly the intratumoral epithelial ER-alpha expression decreased significantly, whereas the ER-beta expression showed a trend to increase.

Conclusion: This data show that E4 has estrogenic endocrine effects. The data support the hypothesis that E4, may be suitable and safe for HRT in women with spontaneous or induced menopausal symptoms, since apoptosis increases, IGF-1 decreases and no unfavourable effects are observed on Ki67, Bax and Bcl-2. The decrease of ER-alpha and the increase of ER-beta suggest a mechanism of action, explaining why the natural fetal estrogen E4 has estrogen-antagonistic effects on breast cancer tissue.

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Investigating the Effect of Extremely Low Frequency Electromagnetic Field On Recombinant Monoclonal Antibody Overall Expression in *E. coli*

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Background: In recent years, recombinant monoclonal antibodies and their derivatives have emerged as important targeted therapy agents and as the fastest growing group within pharmaceutical industry research. Despite benefits of these therapeutic agents, the cost of treatment is drastically high and many patients could not afford their prescriptions. The majority of therapeutic monoclonal antibodies are produced in mammalian cells such as Chinese hamster ovary (CHO). This is while the low yield in expression of active protein, high media costs, the complexity of mammalian production system, costly viral inactivation validation steps, and extremely long production time of mammalian cells increase imbursements. In this regard, we decided to use an alternative method in combination with classic antibody reproduction. Recently, Extremely Low Frequency Electromagnetic Fields (ELF-EMF), which has been known as a potential mutagen agent and in some cases a carcinogen agent, used as manipulating agent in cellular metabolism and signaling. Cooperation of an ELF-EMF generator with an unconventional bioreactor, results in yield improvement in expression of a recombinant protein cloned in *E. coli.* Therefore, we designed an observation in order to investigate the effect of ELF-EMF on overall expression of a recombinant monoclonal antibody in *E. coli.* expressing the protein under exposure of ELF-EMF.

Material and Method: A Helmholtz coil has been used in order to generate 50 Hz. electromagnetic field during 12 hr with the power of 10 to 100 mT. cDNA of monoclonal antibody cloned to the Origami and expression level measured by densitometry. Recombinant cells divided into two groups of test and control. Test group exposed to the field during the expression stage after induction and control was isolated from exposure. Also dried weight of cell plates measured in order to compare proliferation in same time.

Results: As it has been shown in previous studies, recombinant gp41 expression level in *E. coli* increased about 20 percent after exposing to the ELF. Therefore we propose that the expression level of recombinant monoclonal antibody would be increased in this system significantly.

Discussion: expression of recombinant monoclonal antibodies in bacterial host such as *E. coli* and also exposing the host cells during the expression under ELF-EMF eligibly reduces the extraordinary costs of mammalian cells. Also, because of proof reading enhancement effect of ELF-EMF, post translational properties of expressed protein, such as correct folding and bond formations, might become more reliable than mammalian expression system such as CHO cell. At last enhancing vitality of host cells and what mentioned before makes our new method as an economic procedure in order to produce anti cancer monoclonal antibodies with affordable cost for patients.

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Predictive and Prognostic Factors

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Biomarker Discovery and Evaluation of Response to Anti-cancer Therapeutics in Breast Cancer Using a Novel Nanofluidic Immunoassay

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Our research efforts focus on the identification and detection of fundamental molecular differences between normal and tumor cells in breast, as well as differences among distinct breast cancer subtypes, especially in terms of signal transduction pathways that control cell cycle, apoptosis and cell growth. Cancer subtype specific molecular variations dramatically affect patient responses to already existing treatments. For example, the phosphorylation status of many proteins that are involved in signal transduction pathways perturbed in cancer cells is extremely important in determining whether these cells are susceptible to killing by available cancer therapeutics. Therefore, differentially phosphorylated protein isoforms can be a particularly useful prognostic biomarker of drug response in the clinic. However, accurate detection and quantitative analysis of cancer-related phosphoproteins in tumors is limited by current technologies.

Using a novel, fully automated nanocapillary electrophoresis technology (CB1000^{TM}) designed to separate protein molecules based on their isoelectric point (pI), we are currently developing highly sensitive assays for reliable assessment of the phosphorylation status of cancer-related phosphoproteins in tumors, before and during drug treatment.

We have developed and optimized assays measuring AKT1, AKT2, AKT3, ERK1 and ERK2, and their respective phosphoisoforms. Using these assays, we were able to measure levels of activated ERK1/2 and AKT1/2/3 in a breast cancer cell line panel developed in our lab, using protein extracted from as few as 50 cells. Based on RNA expression data, cell lines in this panel have previously been categorized in two distinct subtypes (Basal and Luminal) and their molecular phenotypes closely resemble the respective profiles of tumors obtained from breast cancer patients. This cell line panel is extensively used to measure cellular responses to breast cancer therapeutics, including drugs that target MEK, ERK, PI3K and AKT. Using CB1000 assays, we are currently measuring changes in the phosphorylation states of these targets during drug treatment, in order to completely characterize pharmacodynamic changes in these cells during treatment, and develop molecular profiles that predict response in breast cancer. We have also extended theses studies to include xenografts from *in vivo* experiments.

Since this technology enables accurate detection and quantification of protein isoforms and post-translational modifications from only very small amounts of tumor samples or serum, it promises to propel cancer biomarker discovery and enable the development of clinically useful prognostic and diagnostic assays that predict responses to drugs targeting cancer-specific molecular networks.

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Comparison of Frequencies and Prognostic Effect of Molecular Subtypes Between Young and Elderly Breast Cancer Patients

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Background: To compare the distribution and prognostic effect of the breast cancer molecular subtypes in young and elderly breast cancer patients.

Materials and Methods: Our study population (n = 822) consisted of all early breast cancer patients primarily treated with surgery in our center between 1985 and 1996. A total of 142/822 fresh frozen tissues were available with good quality RNA and analyzed by gene expression microarray. Gene expression molecular subtypes were determined by hierarchical clustering based on patterns of expression of 534 'intrinsic' classifications. Sections of a tissue micro array containing formalin-fixed paraffin-embedded tumor tissue of 714/822 patients were immunohistochemically (IHC) stained for Ki67, EGFR, CK5/6. Tumor expression of