1047 POSTER

Annexin-A1 Mediates Chemosensitivity to PPAR-gamma Ligands in Mammary Carcinoma: a Novel Biomarker for Effective Tailoring of Patients to PPAR-gamma Ligand Therapy

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Background: Annexin-A1 (ANXA1), a calcium-dependent phospholipids-binding protein, has been implicated in the development of breast tumorigenesis. However, evidence on the exact role of ANXA1 in breast cancer is limited. Peroxisome proliferator-activated receptor gamma (PPARg) offers a molecular target for drugs aimed to treat type II diabetes mellitus, while its therapeutic potency against cancer disease, including breast cancer is well established. Despite achievements on improved current clinical therapies and medical treatments on breast cancers have been continuously reported for the past decade, a good biomarker is needed for better screening and selection of cohort of patients for breast cancer therapy in future clinical trials.
Material and Methods: Crystal violet staining and AnnexinV-PI staining were used to measure percentage of cell death. Luciferase reporter assay were used to measure promoter activities of PPARg and ANXA1, and NF-kB transcription activity. Protein expression and mRNA expression levels were measured by Western blotting and real time PCR respectively.
Results: A screen on a panel of breast cancer cell lines reveals ANXA1 is highly expressed in invasive breast cancer cell lines such as MDA-MB-231 and BT549, while non-invasive cell lines such as MCF-7 and T47D show much lower expression of ANXA1. This corroborates our published study where high ANXA1 expression was observed in breast cancer patients with high grade tumour while patients with low grade tumour express low ANXA1 protein. Exposure of breast cancer cells MDA-MB-231 and BT549 to PPARg ligands resulted in upregulation of ANXA1 transcription as well as protein expression with corresponding inhibition in cells' viability through a PPARg-NFkB-ANXA1 axis. Suppression of ANXA1 levels by small-interfering RNA in these cells conferred resistance to PPARg ligand treatment. On the other hand, expression of ANXA1 in MCF-7 and T47D cells and viability remained unchanged with the same dose treatment. Interestingly, sensitivity to PPARg ligands could be achieved in MCF-7 and T47D cells by forced over-expression of ANXA1. Further, MDA-MB-231 xenograft model in nude mice treated with PPARg ligands, showed a significant reduction in tumour size, and tumour tissues stained by immunohistochemistry showed increased ANXA1 protein levels.
Conclusion: Despite the numerous preclinical data supporting for the use of PPARg ligands as anti-cancer drugs, none of the clinical trials have been successful. Herein, we propose that perhaps we could use expression levels of ANXA1 as a biomarker to better select cohort of patients for PPARg ligand therapy in clinical trials.

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Ligand Activation of PPAR Gamma Enhances Cytotoxicity of Chemotherapeutic Drugs in Breast Cancer Cells: the Mechanism Involving Tumour-specific Suppression of Mitochondrial MnSOD in Vitro and in Vivo

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Background: Ligand induced peroxisome proliferator-activated receptor gamma (PPARγ) activation has been reported to inhibit the proliferation of malignant cells, possibly through reactive oxygen species (ROS) production. An increasing number of studies have demonstrated that not only do various therapeutic approaches depend on ROS, but that further elevation of cellular ROS can indeed kill cancer cells more effectively. Manganese superoxide dismutase (MnSOD) is one of the major antioxidant enzymes overexpressed in many cancers and could regulate ROS-mediated cell death induced by PPARγ activation.
Materials and Methods: NoShift Transcription factor assay was used to measure binding affinities of PPARγ to MnSOD promoter. Luciferase reporter assay was used to measure PPRE promoter activity. Real-time PCR and western blot analysis were used to determine MnSOD mRNA and protein levels. MitoSOX Red assay was used to measure mitochondrial superoxide levels. Cell viability assays were used to determine sensitivity of cells upon drug treatment. Immunohistochemistry was used to stain for MnSOD levels in mouse and human tumour tissues.
Results: We report the identification of PPARγ response elements within the human MnSOD promoter region and that activation by low "sensitizing doses" of PPARγ agonists led to significant downregulation of MnSOD mRNA and protein levels. Also, a corresponding receptor-dependent increase in intracellular superoxide production in breast cancer cells was observed. Importantly, normal breast cells were completely refractory to this effect due to low levels of PPARγ protein. Suppression of MnSOD levels by small-interfering RNA or PPARγ agonists in breast cancer cells reduced their colonogenic ability and enhanced chemo-sensitivity to ROS-promoting drugs such as docetaxel and doxorubicin, which could be abrogated by MnSOD overexpression. Furthermore, MDA-MB-231 xenograft model in nude mice treated with PPARγ ligands showed a significant reduction in tumour size, and tumour tissues stained by immunohistochemistry showed a decrease in MnSOD protein levels. Finally, histopathologic analysis of breast cancer biopsies obtained from patients with type II diabetes treated with synthetic PPARg agonists showed significant repression of MnSOD in the tumour tissues.
Conclusion: Together, our data not only identifies MnSOD as a novel target of PPARγ but also provides a molecular mechanism for ROS-manipulation therapy in the clinic through the intelligent use of PPARγ ligands in combination with ROS-promoting drugs such as doxorubin or docetaxel.

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Increased Amplification of HER2 in a Cell Line Model of Acquired Lapatinib Resistance

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Background: Lapatinib is a reversible small molecule inhibitor of HER2 and EGFR, which is approved for the treatment of trastuzumab refractory metastatic breast cancer. However, resistance to lapatinib is a significant clinical problem and to date the mechanisms of acquired lapatinib resistance remain largely unknown. We have developed an *in vitro* model of acquired lapatinib resistance by conditioning the HER2 positive, lapatinib sensitive HCC1954 cells with lapatinib for 6 months.
Materials and Methods: HCC1954 cells were conditioned with 1 mM lapatinib for 6 months and the resulting cell line is referred to as HCC1954-L. Proliferation assays were performed across a range of lapatinib concentrations and measured by acid phosphatase assays. Doubling times were measured over 7 days using Guava viacounts (Millipore). Array CGH analysis was performed; briefly DNA was extracted from cell pellets, fragmented and labeled with Cy5 and Cy3 dyes, hybridized to arrays, scanned by a microarray scanner and analyzed using Agilent analytics software. Western blotting was performed with a HER2 specific antibody (Calbiochem) on parental and resistant cell protein lysates.
Results: HCC1954-L cells are resistant to lapatinib (IC_{50} 2.574±0.251 mM) compared to parental cells (IC_{50} 0.424±0.018 mM; $p = 0.004$). The resistant phenotype is stable in the absence of lapatinib for 3 months. Lapatinib treatment significantly increases the doubling time of the parental cells (83.0±3.6 compared to 29.8±1.0 hours; $p = 0.0007$). Lapatinib does not significantly affect the doubling time of HCC1954-L cells (57.2±7.1 compared to 48.5±2.6 hours; $p = 0.2$).
Array CGH (comparitive genomic hybridization) analysis revealed a significant amplification (1.64 fold; $p < 0.0001$) of the HER2 gene in HCC1954-L cells compared to the parental cells. Increased HER2 protein expression was observed in the HCC1954-L cells compared to the parental HCC1954 cells, by western blot analysis.
Conclusions: HCC1954-L cells represent a stable *in vitro* model of acquired lapatinib resistance. CGH analysis revealed further amplification of HER2 at the gene level and an increase in total HER2 protein expression was confirmed by western blotting. Increased expression of HER2 represents a possible mechanism of acquired resistance to lapatinib in this cell line.

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Copy-neutral Loss of Heterozygosity at the P53 Locus in Esophageal Squamous Cell Carcinomas Associated With p53 Mutations

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Purpose: The elucidation of the mechanisms causing loss of heterozygosity (LOH) in esophageal squamous cell carcinoma (ESCC) will give us further understanding of its carcinogenesis and will also have preventive, diagnostic, and therapeutic implications for this aggressive disease. Loss of tumour suppressor gene function is generally thought to occur in two steps, the first being mutation in one allele followed by the somatic loss or inactivation of the second allele, or LOH. The aim of this study is to

identify potential mechanisms resulting in LOH around the *p53* locus in its carcinogenesis.
Materials and Methods: We investigated ten esophageal cancer cell lines and 91 surgically-resected specimens, examining them for LOH at the *p53* locus on chromosome 17. LOH was screened using microsatellite markers by DNA sequencing. To test whether copy number loss was seen at the *p53* locus, we examined the *p53* gene using comparative genomic hybridization (CGH) and fluorescence in situ hybridization (FISH). Finally, we performed single-nucleotide polymorphism (SNP) − CGH analysis to clarify potential mechanisms of disruption of the intact allele in *p53* mutant ESCCs.
Results: In an analysis of specimens using microsatellite markers, a close positive correlation was found between *p53* mutations and LOH at the *p53* locus ($p < 0.01$). Although four cell lines were found to be homozygous for *p53* mutations, LOH at the *p53* locus was not detected by CGH. Among two *p53* mutant cancer cell lines and five *p53* mutant/LOH cancer specimens analyzed by FISH, both of the cell lines and four of the specimens exhibited no obvious copy number loss at the *p53* locus. SNP-CGH analysis, which allows both determination of DNA copy number and detection of copy-neutral LOH, demonstrated no chromosomal alterations in the *p53* wild type/retention of heterozygosity ESCC specimen and all normal samples. On the other hand, data from the *p53* mutant/LOH cancer specimens indicated that LOHs without copy number change were caused by whole or large chromosomal alteration.
Conclusions: LOH without copy number change at the *p53* locus was frequently observed in *p53* mutant ESCC. Our data suggest that copy-neutral LOH occurring as a result of chromosomal instability might be the major mechanism for inactivation of the intact allele in esophageal squamous cell carcinogenesis associated with *p53* mutation.

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High Level of MiR-21, MiR-10b and MiR-31 Expression in Bilateral Versus Unilateral Breast Carcinomas

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Bilateral breast cancer (biBC) represents approximately 5% of total breast cancer (BC) incidence. Patients with bilateral breast cancer (biBC) develop the same disease twice therefore they are likely to accumulate women with particularly elevated genetic or non-genetic susceptibility to BC. We analyzed the expression of several microRNAs implicated in BC pathogenesis (miR-21, miR-10b, miR17−5p, mir-31, miR-155, miR-200c, miR-18a, miR-205, miR-27a) in 80 breast carcinomas obtained from biBC patients and 40 cases of unilateral BC (uBC). Unexpectedly, 3 miRs (miR-21, miR-10b and miR-31) demonstrated significantly higher level of expression in biBC *versus* uBC ($p = 0.0001$, 0.00004 and 0.0002, respectively). Increased content of miR-21, miR-10b and miR-31 was observed in all categories of biBC tumours, i.e. in synchronous biBC as well as in 1st and 2nd tumours from metachronous biBC cases. Synchronous biBC showed more similarity of miR expression profiles within pairs that the metachronous doublets. This study suggests that bilateral breast tumours have somewhat distinct pattern of molecular events as compared to the unilateral disease.

1052 POSTER

MicroRNA-mediated Restriction of Cell Proliferation and Tumour Growth

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Background: Tumour growth is tightly associated with regular shifts in microRNA (miRNA) expression pattern as well as with amplification, translocation and other damages in fragile chromosome regions containing more than 50% of miRNA genes. Usually, expression of miRNAs miR-15a, miR-16, miR-17−5p, miR-31, miR-125a/b, miR-143 and miR-145 is down-regulated in cancer cells whereas expression of miRNAs miR-21, miR-155, miR-206, miR-221 and miR-222 is up-regulated. This investigation aims to identify how abnormalities in miRNA network contribute to the excessive proliferation of transformed cells.
Material and Methods: miRNA targets within gene transcripts were predicted *in silico* using TargetScan software.
Results: miRNA miR-17−5p can target transcripts of genes coding proliferative signal pathway components E2F1, E2F2, STAT3, Rb, p107, p130 and ErbB3. miR-221 and miR-222 silence genes encoding cell cycle inhibitors p27 and p57 as well as receptor c-Kit. Nevertheless, miR-17−5p targets also transcript of gene coding another cell cycle inhibitor, p21, whereas miR-155, miR-221 and miR-222 silence *E2F2* gene. miR-15a and miR-16 can suppress genes encoding transcription factors E2F3 and E2F7 as well as gene of cyclin-dependent kinase CDK6 and main antiapoptotic gene *bcl-2*. *E2F2*, *STAT3, erbB2* and *bcl-2* gene transcripts carry miR-125a/b binding sites. Also, miR-31 silences *E2F2* gene. miR-143 can suppress *abl2*, *erbB3* and *bcl-2* genes. miR-145 targets transcripts *of RASA1*, *RASA2, erbB3* and *bcl-2* genes. miR-320 can suppress *E2F1*, *E2F3, RASA1*, *CDK6* and *p57* genes. miR-21 as well as miR-155 target transcript of gene encoding TGF-β receptor that can induce cell-cycle arrest through suppression c-Myc-regulated genes. miR-205 silences *E2F1*, *erbB3*, *erbB4* genes. miR-206 targets *RARB* gene encoding retinoic acid b-receptor. miR-181, which is responsible for differentiation of some cells, e.g. lymphopoiesis, can suppress *E2F5*, *E2F7*, *bcl-6* and *bcl-2* genes. miR-150, another differentiation hallmark, targets transcript of gene encoding transcription factor Elk1.
Conclusions: Cell miRNA network is intertwined with signal transduction pathways. Cancer cells down-regulate expression of miRNAs that silence proliferative and antiapoptotic genes and thus can prevent from abnormal cell proliferation and surviving. Up-regulated miRNAs suppress genes encoding cell cycle inhibitors as well as genes responsible for cell differentiation. Therefore, shifts in miRNA expression pattern can themselves cause reactivation of cell oncogenes and antiapoptotic genes as well as repression of cell cycle inhibitor genes. Such alterations facilitate proliferation of transformed cells. Moreover, as each miRNA impairs the expression of many genes, including genes of other miRNAs, illegitimate activation or repression of some miRNA genes can be the first event in carcinogenesis, leading to the reorganization of epigenetic pattern in transforming cells through the RNAi-dependent DNA methylation.

1053 POSTER

Snail1 and Estrogen Receptor Signaling: Two Crossing Pathways in Breast Cancer

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Epithelial-to-mesenchymal transition (EMT) is one of key steps in breast cancer progression. The transcription factor Snail1 helps the breast cancer cells to lose their epithelial morphology (in particular E-cadherin adhesive contacts) and to acquire a fibroblast-like phenotype and a high expression of mesenchymal markers. Estrogen receptor (ERalpha, ER) signaling plays the significant role in breast cancer development and treatment, but relations between Snail1 and ER pathways remain unclear. The goal of this study was to investigate the relationship between ER and Snail1 signaling in breast cancer cells.
Methods: MCF-7 (ER+, hormone responsive), MCF-7/LS (ER+, hormone resistant) and HBL-100 (ER-) cells was cultured in standard DMEM medium supplemented with 7% fetal calf serum. Estrogen receptors and Snail1 expression was assessed by immunoblotting. The transcriptional activity of ER and Snail1 was determined by luciferase assay.
Results: The inverse relationship between Snail1 and estrogen receptors has been demonstrated: MCF-7 and MCF-7/LS cells were characterized by high ER expression and low Snail1 activity/expression, on the contrary, HBL-100 cells were ER-negative and had high level of Snail1 activity/expression. The study of the expression of the Snail-regulated epithelial marker, E-cadherin, has shown high E-cadherin expression in ER-positive MCF-7 and MCF-7/LS cells and a loss of E-cadherin in ER-negative HBL-100 cells. The wild type Snail transfection into ER-positive cells has caused no changes in ER expression, but significantly decreased ER transcriptional activity determined by luciferase assay. Contrariwise wild type Snail transfection into ER-negative HBL-100 cells was not accompanied with the alterations in the reporter gene activity. The transfection of siRNA Snail into MCF-7 and MCF-7/LS cells has caused stimulation of ER activity, totally supporting the involvement of Snail1 in ER down-regulation. Thereby the results show the inverse relationship between ER and Snail1 content/ activity in breast cancer lines demonstrating the possible Snail involvement in the regulation of hormonal signaling.

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17beta-estradiol Signaling via Estrogen Receptor Alpha-36 Enhances Survival and Invasion of ERalpha-negative Breast Cancer Cells

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Background: While several factors associated with tumour metastasis are known to be upregulated in breast carcinomas, the molecular mechanisms of metastasis are not well understood. ERα36, a variant of ERα that we found in ERα-negative HCC38 breast cancer cells, is responsible for 17β-estradiol(E2)-dependent protein kinase C (PKC) activation from the