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MP-09.01
Knockdown of Integrin-linked Kinase Reduces Invasive and Metastatic Potential of Renal Cell Carcinoma by Impeding Epithelial to Mesenchymal Transition

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Introduction and Objectives: Integrin-linked kinase (ILK) is a serine/threonine kinase implicated in the regulation of cell growth and survival, cell cycle progression, epithelial-mesenchymal transition (EMT), invasion and migration, angiogenesis. However, the role of ILK has not been evaluated in renal cell carcinoma (RCC). We investigated the role of ILK on cancer progression and metastasis and therapeutic potential of ILK inhibition in RCC.

Methods: Non-cancerous renal tubular cells (HK-2) and RCC cells (UMRC-6, UMRC-3 and Caki-1) were used to examine baseline expression of ILK and EMT markers in RCC. RNAi using siRNA was used to knock down ILK in vitro. After transient transfection, crystal violet assay and cell cycle analysis using FACS were performed to check the effect of ILK on tumor growth. We examined changes of stress fibers and focal adhesion with phalloidin-rhodamine and vinculin antibody. Scratch assay and invasion assay were performed to evaluate EMT phenotypic behavior after ILK inhibition.

Results: ILK is less expressed in normal cells (HK-2) and low stage RCC cells (UMRC-6) but highly expressed in advanced and metastatic RCC cells (UMRC-3 and Caki-1). Advanced RCC cells also showed high expression or increased activity of molecular EMT markers including Snail, Zeb1 and decreased activity of E-cadherin and increased degradation of β-catenin. ILK knockdown inhibited tumor proliferation but the inhibition was moderate and cell cycle progression was not significantly affected. Knockdown of ILK reduced stress fiber formation and focal adhesions and also effectively impeded phenotypic EMT markers such as cell migration and invasion in Caki-1 and UMRC-3 cells.

Conclusions: ILK is highly expressed in advanced RCC and its high expression is related to EMT markers in RCC. Knockdown of ILK inhibited molecular and phenotypic EMT markers. These results suggest the therapeutic potential of ILK inhibition on invasion and metastasis of advanced RCC.

MP-09.02
Enhanced Tumor Oxygenation Following Short-term Sunitinib Therapy in a Renal Cell Carcinoma Model: An Unexpected Finding

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Introduction and Objectives: It has been shown in vivo that tyrosine kinase inhibitors such as sunitinib exert antiangiogenic effects directly on endothelial cells, but also by blocking compensatory changes in the activity of hypoxia-inducible factors (HIF), mainly HIF-1α. The present study utilized positron-emission tomography (PET) to elucidate the effect of short-term sunitinib therapy on tumor hypoxia.

Methods: Caki-1 tumors were grown for 4 weeks in Balb c/nu-nu mice both subcutaneously and within renal capsule. Both models were analyzed first with dynamic [18F]FAZA PET up to 3h post-injection (p.i.). In a second setup, mice bearing 2 subcutaneous Caki-1 tumors were sorted into 2 groups: a) receiving 40mg/kg/d sunitinib i.p. for 5 days prior to analysis with [18F]FAZA PET, b) vehicle control injections. Tumor uptake of [18F]FAZA and immunohistochemical tissue staining with pimonidazole and CD-31 were determined.

Results: Functional analysis of subcutaneous Caki-1 tumors with PET revealed a mean standardized uptake value (SUV) after 3h p.i. of 0.32±0.02 (n=6/3) for [18F]FAZA. Based on the clearance patterns, orthotopically grown tumors were not detectable with PET alone. The SUV3h for [18F]FAZA was significantly lower in sunitinib group compared to controls in the subcutaneous model: 0.23±0.02 vs. 0.42±0.05 (n=4/2). The time-activity curve indicated significant [18F]FAZA washout from treated tumors as compared to the control. This was confirmed by the biodistribution of [18F]FAZA, resulting in SUV3h of 0.29±0.03 in treated vs. 0.45±0.06 (n=8/4) in control mice. CD31 binding (%) was reduced in sunitinib treated groups: 1.8±0.1% vs. 0.6±0.1% (n=80/4).

Conclusions: A reduction of bound [18F]FAZA in this RCC tumor model following short-term TKI-therapy suggests improved tumor oxygenation. This was observed despite sunitinib causing a significant reduction in vascular density. The mechanism of this paradoxical observation warrants further investigation.

MP-09.03
Evasive Resistance to VEGF-targeted Anti-angiogenic Therapy Is Acquired by Activation Angiogenesis Pathways Independent of VEGF in Renal Cell Carcinoma

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Introduction and Objectives: Anti-angiogenic therapy provides significant growth inhibition in clear cell type renal cell carcinoma (CCRCC). However, evasive resistance develops in most responding cases due to unclear mechanisms. We investigated the mechanism of resistance to VEGF-targeted therapy in CCRCC both in vitro and in vivo.

Methods: Two different conditioned cell lines were developed from wild type Caki-1. Sunitinib-conditioned Caki-1 was developed by chronic exposures to sunitinib and hypoxia-conditioned Caki-1 was developed by chronic exposures to 1% hypoxia. We characterized these conditioned cells in vitro and response patterns to sunitinib were evaluated using subcutaneous xenograft models with parental and conditioned cells. In vivo angiogenesis assays were performed to characterize angiogenesis potentials. Finally, mRNA microarray was performed to find pathways that induce resistance to anti-angiogenic therapy.

Results: Sunitinib inhibited proliferation of HUVEC cells, but did not inhibit tumor proliferation in CCRCC cells at pharmacologically relevant doses. In vitro sunitinib-conditioned Caki-1 cells did not show obvious resistance to sunitinib compared to parental cells, but when tested in vivo these cells appeared to be highly resistant to sunitinib therapy. In contrast, hypoxia-conditioned Caki-1, although more resistant to hypoxia and showing increased vascularity by upregulating VEGF production, did not develop sunitinib resistance either in vitro or in vivo. Matrigel plug assay confirmed that tumor angiogenesis was relatively intact and less affected by sunitinib in xenografts of sunitinib-conditioned cells.

Conclusions: Resistance to VEGF-targeted therapy is acquired by activation of VEGF-independent angiogenesis pathways induced by interactions with VEGF-targeted drug but not by hypoxia. Our results suggest that more broad inhibitions of tumor angiogenesis are required to prevent development of resistance to anti-angiogenic therapy in CCRCC.

MP-09.04

Quantitative Proteomic Analysis of Exosome-enriched Extracellular Microvesicles Obtained Following an *in vitro* Model of Urinary Tract Obstruction

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Introduction and Objectives: An *in vitro* model of urinary traction obstruction (UTO) using renal proximal tubule cells (NRK-52E) subjected to mechanical stretch has helped to elucidate the molecular pathology of UTO. Because renal proximal tubule cells have been suggested to release protein-containing extracellular microvesicles (exosomes) into the urinary space, the goal of this study was to confirm production of exosomes by NRK-52E cells and characterize changes in protein abundance that result from UTO simulation using quantitative proteomics.

Methods: NRK-52E cells were subjected to 24 hours of stretch-relaxation, with a maximum of 20% maximal biaxial stretch on a FX-4000 Flexercell Strain Unit. The efficacy of UTO simulation was measured by cell death ELISA, and extracellular microvesicles were isolated by ultracentrifugation of the cell culture media. Relative protein abundance was measured by spectral counting following mass spectrometry using a ThermoFisher LTQ linear ion trap mass spectrometer with 5 replicate analyses.

Results: Microvesicle production was confirmed by electron microscopy and immunoblotting for known the exosomal proteins Annexin 1 and CD81. Stretched cells showed an average of a 3 fold increase in cell death. Histone proteins had the most significant changes in abundance, but other proteins, gelsolin, nebulin, haptoglobin, an integral membrane serine 2 protease, Fibronectin 1 and polyubiquitin were also noted to be increased in abundance in stretch cell extracellular microvesicles. Significance testing was conducted using a student's t-test.

Conclusions: We show that renal proximal tubule cells generate extracellular microvesicles whose protein profile is altered following an *in vitro* model of UTO. Quantitative proteomic analysis demonstrates proteins important in apoptosis, the cytoskeleton, and cellular response to oxidative stress are altered in abundance after mechanical stretch, providing insight into the pathophysiology of UTO.

MP-09.05

Rat Bone Marrow Derived Mesenchymal Stem Cell Therapy in a Parkinsonian Animal Model of Detrusor Overactivity

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Introduction and Objectives: We have previously shown that cellular therapy can induce a transient urodynamic improvement in a rat model of Parkinson's disease where bladder dysfunction is induced by a unilateral injection of 6-hydroxydopamine (6-OHDA) into the medial forebrain bundle (MFB). Our goal was to test the hypothesis that transplantation of rat bone marrow mesenchymal stem cells (rBMSC) in substantia nigra (SN) can produce longer lasting improvements of cystometric bladder dysfunction if the cells are protected by microencapsulation (ErBMSC).

Methods: Female Sprague-Dawley rats underwent a unilateral stereotactic injection of 6-OHDA in the MFB. Two weeks following the lesion, a treatment injection was performed in the ipsilateral SN of vehicle, 100,000 GFP positive rBMSC, or 100,000 ErBMSC. Animals were evaluated by cystometry at four different time points after treatment: 7, 14, 28, and 42 days. Urodynamic parameters were compared using a two-way ANOVA.

Results: Overall, transplantation of ErBMSC resulted in lower bladder capacity than vehicle treatment and rBMSC (0.65 vs. 0.95 and 0.87 mL respectively, $p<0.01$), but also a lower residual volume than rBMSC (0.078 vs. 0.160 mL, $p<0.05$). The ErBMSC animals had a lower threshold pressure (TP) at 28 days and a lower level of spontaneous activity (SA) at 42 days compared to vehicle-treated animals (TP: 20.85 vs. 43.84 cm H2O; SA: 7.71 vs. 22.43 cm H2O, $p<0.05$). At 42 days, the rBMSC group had a lower TP (16.65 vs. 37.10 cm H2O, $p<0.05$), intermicturition pressure (11.70 vs. 39.57 cm H2O, $p<0.01$), SA (3.57 vs. 22.42 cm H2O, $p<0.01$) and area under the curve (12.46 vs. 39.91, $p<0.01$) than vehicle-treated animals.

Conclusions: We confirmed persistent urodynamic effects of the 6-OHDA lesion up to 42 days after vehicle injection. Lasting urodynamic improvements at 42 days after treatment was observed more markedly in animals treated with rBMSC alone. Microencapsulation of the rBMSC did not improve these effects.

MP-09.06

Intrathecal Cannabinoid Agonist Effects in Cystometric Evaluation of Normal Rats

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Introduction and Objectives: Systemic administration of cannabinoid receptor agonists affects bladder function possibly by binding to peripherally located cannabinoid receptors. Intrathecal cannabinoids have been shown to produce antinociception in several neuropathic pain animal models. Our goal was to determine the effects of cannabinoid receptor agonists administered intrathecally on the cystometric bladder function of normal rats.

Methods: Female Sprague-Dawley rats underwent a bladder catheter and a polypropylene intrathecal catheter insertion prior to cystometric evaluation. Urodynamic parameters were recorded in awake animals at baseline, and after sequentially administering vehicle and incremental dosages of drug. The drugs delivered in two different groups were methanandamide (5, 10, 20, 40 µg), a selective CB1 agonist, and WIN 55212-2 (10, 20, 40 µg), a non-selective cannabinoid agonist. Urodynamic parameters were compared using one-way ANOVA.

Results: The micturition pressures did not change after vehicle or drug administration in either group. In the methanandamide group (n=7), bladder capacity significantly increased from baseline after 40 µg administration (0.62 vs. 0.91 mL, $p<0.05$), and frequency decreased (16.1 v.s 11 voids/hour, $p<0.05$). Bladder capacity also significantly increased after 40 µg administration when compared to baseline (0.80 vs. 1.04 mL, $p<0.01$) and to vehicle (0.82 vs. 1.04 mL, $p<0.05$) in the WIN 55212-2 group (n=8). Frequency decreased after 40 µg was administered when compared to baseline (12.5 vs. 9.6 voids/hour, $p<0.01$) and to vehicle (12.2 vs. 9.6 voids/hour, $p<0.05$). Micturition volume increased from baseline after 20 µg administration (0.76 vs. 1.05 mL, $p<0.05$).

Conclusions: We demonstrated that intrathecal cannabinoid agonist administration increases bladder capacity and decreases micturition frequency in normal rats. These effects may be mediated by central cannabinoid receptors activated by these drugs.

MP-09.07

Rational Targeting of Fibroblast Growth Factor Receptor (FGFR)-3 in Bladder Cancer

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Introduction and Objectives: Mutations in the FGFR3 gene are found in up to 80% of low grade papillary bladder cancers, and can lead to constitutive, ligand independent activation of signaling. Overexpression of FGFR3 is found in approximately 50% of high grade and invasive tumors. In this study we evaluated mutation and expression of FGFR-3 in a North American cohort and studied the effect of targeted FGFR3 inhibition in pre-clinical models of bladder cancer.

Methods: DNA was extracted from 170 bladder cancer specimens and PCR analysis with direct sequencing was performed to detect mutations in exons 7 and 10. FGFR3 expression was analyzed by immunohistochemistry on a tissue microarray derived from 143 patients with bladder can-

cer. FGFR3 expression, phosphorylation and downstream signaling were measured in a panel of bladder cancer cell lines, and efficacy of a small molecule inhibitor (TKI258) was tested on signaling and 3H-thymidine incorporation. Systemic treatment with an inhibitory anti-FGFR3 monoclonal antibody was evaluated in orthotopic xenografts. Tumor burden was measured with bioluminescent imaging.
Results: FGFR3 mutations were identified in 26% of bladder tumors and 56% of low grade papillary lesions. Overexpression of FGFR3 was noted in 53% of all bladder tumors. FGFR3 inhibition effectively abrogated constitutive phosphorylation of FGFR3 and p42/44MAPK in a subset of bladder cancer cell lines, resulting in growth inhibition. The growth of UM-UC14, RT112 and UM-UC1 orthotopic xenografts in nu/nu mice was inhibited by 40%, 66% and 85%.
Conclusions: These studies underline the clinical relevance of FGFR3 mutation and expression in bladder cancer. Furthermore, the results offer proof of principle for rational targeting of FGFR3 in patients with bladder cancer.

MP-09.08

Circulating Interlukin-6 and Nerve Growth Factor Are Associated with Periprostatic Fat Length and Cancer Detection among Non-obese Men Presenting for Prostate Biopsies

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Introduction and Objectives: We previously demonstrated an association between circulating adipokine levels and prostate cancer. Visceral fat (i.e. periprostatic fat) has greater metabolic activity than peripheral fat, however the association of serum adipokine levels with periprostatic fat has not been characterized. We aimed to correlate adipokine levels with BMI (a measure of peripheral fat) and periprostatic fat (visceral fat) in a population of men who present for prostate biopsy.
Methods: Cohort consisted of 200 subjects initially stratified by BMI (100: BMI >27 vs. 100: BMI ≤27). Of the obese subjects 50 prostate cancers were age matched 1:1 with 50 controls. The same process was used for the non-obese subjects. Clinical data (age, PSA, DRE, BMI) and serum collected prior to biopsy were used to measure adipokines (adiponectin, leptin, PAI, Resistin, HGF, IL-1β, IL-6, IL-8, MCP-1, NGF and TNF-α)using Milliplex Multi-Analyte Profiling kits. Periprostatic fat (shortest distance between the pubic bone and the prostate) was measured on a sagital trans rectal ultrasound image. Sample analysis, clinical data recording and fat measurements were done blinded to pathology results. We used Pearson correlation to associate serum adipokine levels with periprostatic fat and BMI.
Results: Periprostatic fat was correlated with NGF (r=0.65, p=0.002) and IL-6 (r=0.54, p=0.004) among non-obese subjects with prostate cancer. Conversely periprostatic fat was not correlated with serum adipokine levels among the obese subjects. BMI did not correlate with serum adipokine levels among subjects diagnosed with prostate cancer.
Conclusions: We demonstrated a correlation between periprostatic fat and serum levels of NGF and IL-6 among non-obese prostate cancer patients. These findings suggest that adipokines may be differentially secreted from visceral fat. Direct measurement of these molecules in the periprostatic fat would further our knowledge of the role of adipokines in prostate cancer.

MP-09.09

Heat Shock Protein 70 (HSP70) and FGFR3 in Non-muscle-invasive Bladder Cancer Treated with BCG: Gene and Protein Expression Analysis in Two Validation Cohorts

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Introduction and Objectives: We have shown that FGFR3 mutation and protein overexpression identifies pT1 bladder cancer (BC) patients with favorable disease characteristics. However, FGFR3 protein overexpression in the absence of a FGFR3 mutation was not associated with favourable disease characteristics. Heat shock proteins (HSPs) have been implicated in BC prognosis. Here, we evaluated FGFR3 and HSP70 expression at the gene and protein level and their relationship to pathological and clinical parameters using 2 cohorts of non muscle invasive BC treated with BCG.
Methods: 66 primary T1 and 12 Ta BC tumors treated at the University Health Network, Toronto were studied. Microarrays were built and HSP70 expression was determined by immunohistochemistry. Slides were co-reviewed with an experienced uro-pathologist with scores dependent on expression and intensity of the marker. Mutation status was examined by multiplex PCR-SNaPshot analysis. In the Seoul, South Korea cohort, 68 patients without tumor, 23 primary Ta, and 80 T1 BC were analyzed using microarray gene expression profiling and RT-PCR analysis.
Results: In Toronto, FGFR3 was mutated in 80% of Ta BC, 42% of T1LG and 21% of T1HG (p<0.05). Over 90% of Ta/T1 mutant FGFR3 overexpressed the protein, but also 50% of wild type overexpressed FGFR3. In Seoul, FGFR3 gene expression was significantly increased in normal vs. Ta BC (p=0.039) and normal vs. T1LG (p=0.0001). For HSP70, Kaplan-Meier analyses showed that the lack of HSP70 expression in Toronto and gene expression levels in Seoul were significant predictors for disease recurrence (p<0.05 and 0.015, respectively). HSP70 was shown to correlate with FGFR3 expression and mutation (p<0.05). FGFR3 and HSP70 had no influence on tumor progression.
Conclusions: In 2 BC cohorts, analyzing gene and protein expression status, both HSP70 and FGFR3 were shown to play an important prognostic role in T1BC identifying a group at lower risk of recurrence.

MP-09.10

NF-kappa B p65 as Prognostic Tool in Prostate Cancer: an Immunohistochemical Study from Biopsy Samples

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Introduction and Objectives: Our previous immunohistochemical studies of NF-κB p65 in prostate cancer (PCa) highlight its clinical potential as a prognostic marker in different cohorts of Canadian and European men. However these studies are essentially based on tissue microarrays built from samples obtained from radical prostatectomy specimens where other prognostic parameters, such as pathologic stage, Gleason score, and margin status are also available. However, limited prognostic parameters are available to clinicians at the time of diagnosis. The current study aims to assess whether the immunohistochemical staining of NF-κB p65 could offer prognostic information at the time of diagnosis and help risk stratify patients.
Methods: Prostate biopsies were obtained from a cohort of 328 PCa patients who were further treated by radical prostatectomy. NF-κB p65 was stained by immunochemistry on biopsy samples containing malignant tissue. The nuclear frequency was quantified as a percentage of positive NF-κB p65 nuclear cells.

Results: Our first statistical analyses revealed that the nuclear frequency of NF-κB p65 on biopsy samples was correlated with clinical paramaters as final Gleason score and BCR. Patients with Gleason score post-prostatectomy of 7 or more had a higher NF-κB p65 nuclear frequency in biopsy samples ($p<0.05$, Student test). NF-κB p65 nuclear frequency was also higher in patients developing a BCR and bone metastasis ($p<0.05$, Student test).
Conclusions: These preliminary results show an association between NF-κB p65 nuclear distribution in biopsy samples and PCa aggressivity. We showed that NF-κB p65 immunohistochemical staining on biopsies was predictive of BCR. NF-κB p65 may be prognostic marker at the time of diagnosis and could eventually be useful in clinical decision making.

MP-09.11

A New Molecular Imaging System Based on Both Transcriptional and Genomic Amplification to Detect Prostate Cancer Cells in vivo

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Introduction and Objectives: The ability to locate intraprostatic prostate cancer (PCa) is a clinical challenge. One technique to increase the specificity of PCa imaging is to interrogate the in vivo transcriptional activity of tumor-specific gene promoters associated with PCa by placing reporter genes under their control. Towards this end, our group has developed a transcriptional amplification system, termed TSTA,that has shown the ability to amplify the transcriptional activity of the PSA promoter 800-fold. In this study, we further improve the sensitivity of TSTA by combining two separate approaches: a transcriptional and a genomic amplification methods.
Methods: Two non-replicative reporter adenoviruses (Ad), and a conditionally-replicating Ad (CRAd) were constructed. The two non-replicative Ad expressed the firefly luciferase (FL) reporter gene using the TSTA system, under control of either the MUC-1 or the PSA promoter (MUC1-TSTA-FL or TSTA-FL). The CRAd was designed to express the viral early genes E1A and E1B under control of the PSA promoter (TSTA-E1) enabling PSA-dependent Ad replication.
Results: In vivo and in vitro,co-infection of PCa cells by TSTA-E1 and TSTA-FL (DTSTA) enhanced PSA-dependent bioluminescence by 25-fold when compared to TSTA-FL alone. This translates to approximately 20 000-fold amplification over native PSA promoter activity. Co-infection of the MUC1 promoter-driven TSTA (MUC1-TSTA-FL) with TSTA-E1, amplified FL signal exclusively in the AR+ LNCaP and LAPC4 PCa cells but not in the breast or liver carcinoma cell lines.
Conclusions: The DTSTA served to significantly enhance the transcriptional amplification of the TSTA system. A considerable advantage of this system lies with the idea that its specificity is directed by the transcriptional activity of two separate promoters, giving rise to the concept of a customizable gene amplification system for in vivo molecular imaging.