620 POSTER

Elevated expression of IL-8 and IL-8 receptors in prostate cancer cells correlates with disease progression and resistance to oxaliplatin

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Animal models demonstrate that interleukin-8 (IL-8) expression correlates with the angiogenesis and metastasis of prostate cancer, however, its expression in human disease is poorly characterized. Therefore, we studied the expression of IL-8 and IL-8 receptors (CXCR1 and CXCR2) in normal epithelium and neoplastic prostate tissue from patients with increasing stages of disease. Weak to moderate IL-8 expression was localized strictly to the apical membrane of normal prostate epithelium in all cases examined. In contrast, increased expression of IL-8, CXCR1 and CXCR2 was detected on the apical and basal membranes and also within the cytoplasm of cancer cells in all Basal membrane expression and cytoplasmic expression of IL-8 but not apical membrane expression correlated with markers of cell proliferation (Ki67 and cyclin D1 expression) and microvessel density (CD34 staining), confirming the relationship of IL-8 expression with disease progression in human prostate cancer. *In vitro* models using the androgen-independent metastatic prostate cancer cell line PC3 were established to study the significance of constitutive IL-8 signaling in prostate cancer cells. Stimulation of PC3 cells with IL-8 (i) potentiated the phosphorylation of Akt in immunoblotting experiments (ii) increased NF-κ B binding to DNA and (iii) increased the expression of the anti-apoptotic NF-κ B-regulated genes IAP and Bcl2. To test the hypothesis that IL-8 signaling may confer a survival advantage, PC3 cells were treated with full concentration-response curves to oxaliplatin in the absence and presence of (i) a small molecule inhibitor of CXCR2 and (ii) a monoclonal anti-human IL-8 antibody. Inhibition of IL-8 signaling by either strategy had a marked effect on oxaliplatin cytotoxicity increasing both the sensitivity of PC3 cells to this agent and the potency of the response in these cells. FACS analysis confirmed an increase in the percentage of apoptotic cells detected following combination of oxaliplatin with inhibitors of IL-8 signaling when compared to oxaliplatin treated or untreated PC3 cells. Therefore, attenuation of constitutive IL-8 signaling in prostate cancer cells increases their sensitivity to oxaliplatin and suggests that the elevated expression of IL-8 contributes to the high intrinsic resistance of prostate cancer to chemotherapy.

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Disruption of the serine kinase SKY1 gene in yeast results in hypersensitivity to 5-fluorouracil (5-FU) and low expression of the human SKY1 ortholog SRPK1, in colorectal cancers correlates with prolonged overall survival after 5-FU treatment

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The fluoropyrimidine 5-fluorouracil (5-FU) is one of the most commonly used chemotherapeutic drugs. However, the therapeutic potential of 5-FU is severely limited by the widespread occurrence of intrinsic and acquired resistance. Insight in the molecular mechanisms governing the 5-FU induced cytotoxicity may ultimately lead to ways to overcome unresponsiveness and give rise to better treatment results, especially in colorectal cancer. We initially used the budding yeast *Saccharomyces cerevisiae* as a model organism to identify and characterize novel genes/pathways involved in cell kill provoked by the anticancer agent, cisplatin, and found that disruption of *SKY* – a serine kinase that specifically phosphorylates serine/arginine (SR) rich protein domains – conferred resistance to cisplatin (Cancer Res. **61**: 6982–6986, 2001; Mol. Pharmacol. **61**: 659–666, 2002). In cross-resistance studies, we found that the Δ*sky1* knockout strain was also resistant to the cisplatin analog carboplatin (but not oxaliplatin) and the anthracyclines doxorubicin and daunorubicin. In contrast the Δ*sky1* cells were found to be hypersensitive to 5-FU. The 5-FU hypersensitivity phenotype was associated with an increased incorporation of 5-FU metabolites into DNA and RNA. In addition we observed that Δ*sky1* cells display an increased retention of 5-FU in their DNA suggesting an impaired DNA repair. Currently we are investigating the hypothesis that the Δ*sky1* phenotype (that is a mutator phenotype in combination with resistance to cisplatin and sensitivity to 5-FU and oxaliplatin) is associated with increased activity of an error-prone DNA polymerase with translesion synthesis activity. Because of the similarity of the Δ*sky1* phenotype with a MMR-deficient colorectal cancer phenotype, we performed a large-scale immuno-histochemistry study on colorectal cancer tissue arrays from patients treated with 5-FU as adjuvant chemotherapy. In patients with low expression of SRPK1, the human ortholog of *SKY1*, 5-FU adjuvant chemotherapy led to a significant increase (about 20%) in overall survival. In contrast, patients with a high SRPK1 expression did not benefit from 5-FU treatment. These data suggest that low SRPK1 expression levels might be predictive for a survival benefit of colorectal cancer patients treated with 5-FU.

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Resistance to cytotoxic drugs is associated with changes in telomere length

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We have previously shown that testicular cancer cell lines and tissues resistant to cisplatin have elevated telomerase activity and we found that breast cancer cells expressing the dominant negative catalytic subunit of human telomerase reverse transcriptase (hTERT) were more sensitive to certain cytotoxic agents. This study was therefore aimed to investigate whether changes in telomerase activity and telomere length are a general phenomenon of development of drug resistance to standard agents in tumor cell lines and whether this would have implications for selecting treatment options.

We generated a total of 4 renal, lung and ovarian tumor lines sensitive and resistant to either cisplatin, gemcitabine, or vindesine and determined telomerase activity and telomere length upon occurrence of resistance. Parental cell lines A2780 (ovarian), RXF944L (renal), LXFL529L (lung) and LXFA526L (lung) were maintained for 15–32 passages under continuous exposure to cisplatin, vindesine, or gemcitabine at increasing concentrations and development of resistance was monitored by sulforhodamine B proliferation tests. Resistance factors (RF) obtained for the 3 agents ranged between 25–100.000. Telomerase activity was measured using the telomeric repeat amplification protocol (TRAP assay); mean telomere restriction fragment (TRF) length was analyzed by Southern blotting. We found that cell lines with longer telomeres such as RXF944L (9.1 kb) expressed up to 2-fold higher telomerase levels than those with short TRF length e.g. LXFA526L (3.5 kb). In the isogenic ovarian cancer lines A2780, ADDP (cisplatin resistant) and AG6000 (gemcitabine resistant), appearance of drug resistance led to induction of telomerase activity and marked increase in telomere length (from 4.2 to 9.0 and 6.1 kb respectively). In contrast, gemcitabine treatment of RXF944L renal and LXFL529L lung cancer cells resulted in marked telomere shortening (from 9.1 kb to 3.3 kb and 4.0 to 3.6 kb). Both, LXFL529L and LXFA526L lung cancer lines resistant to vindesine (RF = 25) had also reduced telomere length (from 4 kb to 2.7 and 3.5 to 3.3 kb).

Our findings indicate that telomere length maintenance and telomere dynamics are altered under treatment with cytotoxic drugs, the induction of telomere lengthening or shortening seems to be dependent on the molecular mechanism of a particular agent and tumor type. An arsenal of telomerase inhibitory and telomere interactive compounds are currently in advanced preclinical development, they have been found to be more effective in tumors with short telomeres. The observation that some gemcitabine and vindesine resistant cell lines have shorter telomeres indicates that gemcitabine and vindesine refractory tumors might be more sensitive to telomerase inhibition and this might aid the selection of patients that would qualify for treatment with anti-telomerases in phase I/II clinical trials.

623 POSTER

Gene expression enhancement by inhibitors of methylation: potential role in augmentation of apoptosis induced by interferons (IFNs) in melanoma and renal cell carcinoma (RCC)

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To determine whether methylation-mediated silencing of genes plays a role in resistance of melanoma and RCC to IFNs, the effects of 5-aza-deoxycytidine (5-Aza-dC) and an antisense phosphorothioate oligonucleotide selective for DNMT1 were assessed. In melanoma cells, 5-AZA-dC (1uM) overcame resistance to antiproliferative effects of IFN-β, reducing the ID50 from 1500 to 300 U/ml for A375 melanoma cells and from 900 to 100 U/ml for Minors melanoma, synergistic by combination index analysis. When assessed by RNase protection assay, expression of the interferon-stimulated genes (ISG54 and IRF-1) was augmented in melanoma cells. Similar potentiation of antiproliferative effects occurred

with SK-RC45 clear cell RCC and ACHN papillary RCC. In addition, 6x augmentation of apoptosis as assessed by TUNEL assay resulted for the combination of IFN-α 2 with 5-AZA-dC for ACHN cells. Caspase 3 activity increased 2.5x in ACHN cells with either IFN-α 2 or IFN-β. To further affirm specificity of the effects as being mediated through DNMT-1, transfection of ACHN cells with 40 nM of the antisense (MG98) was evaluated. The antisense for 8d suppressed DNMT-1 protein to undetectable levels for up to 48 hr after withdrawal, as did 5-AZA-dC. Reactivated was RASSF1A, a tumor suppressor gene that is silenced by DNA methylation in ACHN cells. MG98 treatment caused little or no apoptosis (<5% TUNEL positive) but sensitized ACHN cells to apoptosis in response to 50 U/ml of IFN-α 2 or IFN–β over 5 days (25% and 80% TUNEL positive, respectively). Mismatch oligonucleotide (MG207) treatment did not sensitize ACHN cells to IFN-induced apoptosis (< 5% TUNEL positive). To identify responsible genes, RNA of MG98 treated ACHN cells was harvested 16 hr after IFN-α 2b or IFN-β (24 hr after the 8th transfection) for analysis on Affymetrix U133A human genome arrays. Compared to MG207, MG98 reduced DNMT-1 signal by 94%. IFN-stimulated genes associated with apoptosis (TRAIL, XIAP, Caspases 1, 7, 10, IRF1, OAS1 and PKR) were minimally increased by the addition of MG98 to IFN-β. Up-regulated ⩾4-fold in response to MG98 compared to MG207 treated cells were 45 genes scored as present ($p<0.05$), 36 of which were absent ($p>0.06$) in all controls (MG207, Lipofectin, no treatment). Of the 45 MG98 induced genes, 24 had CpG islands (length ⩾200 bp, GC ⩾50%, observed/expected CpG ⩾0.6) within 200 bp 5′ of their transcription start. To confirm the validity of the array results, 8 genes with potential apoptotic activity and CpG islands were assessed by RT-PCR after either the antisense to DNMT-1 or 5-AZA-dC. Although there was variability in absolute effects, all were augmented by both methylation inhibitors but not the mismatch antisense. Results suggest different mechanisms may account for the augmentation by methylation inhibitors of IFN-induced apoptosis in melanoma and renal carcinoma cells.

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Global identification of genes involved in 5-flurouracil resistance

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5-fluorouracil (5-FU) and tomudex (TDX) are thymidylate synthase (TS) inhibitors widely used in colorectal, breast, head and neck and aerodigestive cancer treatment. Apart from inhibition of TS, 5-FU also induces DNA and RNA strand breaks and apoptosis by direct incorporation of fluorinated nucleotides into DNA and RNA. TS overexpression is a common feature of 5-FU and TDX resistant cells and has been widely accepted as a major molecular abnormality responsible for 5-FU and TDX resistance. The influence of TS overexpression on 5-FU and TDX sensitivity was studied in 6 TS-overexpressing resistant cancer cell lines (5-FU resistant: 3; TDX resistant: 3). Compared to relevant parental sensitive cell lines, the 5-FU resistant cell lines were >20000-fold cross-resistant to TDX. In contrast, TDX resistant cell lines were only slightly resistant to 5-FU (0.6- to 1.3-fold). Thymidine (20 μM) rescue induced TDX resistance in sensitive cell lines (>10000-fold) but only very mildly affected 5-FU sensitivity (1.2- to 2.3-fold). These data indicate other molecular events rather than TS overexpression may play more important role in 5-FU resistance.

To identify genes involved in 5-FU resistance, 5 pairs of 5-FU resistant and parental cancer cell lines were analyzed on Affymetrix HG-U133A microarrays. Ninety one 5-FU sensitivity phenotype associated genes were identified and subdivided into several biological pathways. Key genes involved in 5-FU activation were significantly down regulated (TK, 2.9-fold; OPRT, 2.3-fold; UMPK, 3.2-fold; PNP 3.6-fold) in resistant cells. 5-FU induced resistant cell lines manifested reduced expression of genes governing G1-S and S phase transition. Expression of genes involved in DNA replication was also down-regulated in resistant cell lines. These findings were highly consistent with the longer doubling time and S phase time, slower growth rate, higher proportion of G1 and lower proportion of S phase cells in the resistant cell lines. This phenotype may protect resistant cells from cell death induced by incorporation of 5-FU into DNA chains and allowing time to repair 5-FU induced damage.

NFκB p65 mRNA and protein over-expression and high DNA binding activity were detected in resistant cells. NF-κB transfected MCF-7 and p53 knockout HCT116 cells were resistant to 5-FU (5.9- and 2.2-fold respectively) but not to TDX. The TS protein expression in NF-κB transfected and p53 knockout cell lines was comparable to the relevant parental cell lines. Thus, p53 mutations and NF-κB overexpression may be critical TS-independent molecular events mediating 5-FU resistance in cancer cells. Our findings may provide novel targets for tackling 5-FU resistance.

626 POSTER

Targeting Mcl-1 exhibits a strong single agent activity in hepatocellular carcinoma

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Background: Advanced hepatocellular carcinoma (HCC) is highly treatment resistant to various systemic therapeutic modalities. The Bcl-2 family members Bcl-2 and Bcl-xl have been previously implicated to contribute to treatment resistance of HCC. Recently, the antiapoptotic bcl-2 family member Mcl-1 was reported as an even more important treatment resistance factor in various type of cancers. However, there is no data about the significance of Mcl-1 in HCC. In the present study we evaluated the biological role of Mcl-1 as a molecular drug target in HCC by an antisense oligonucleotide (ASO) strategy.

Methods: ASO targeting Mcl-1 were evaluated as single agent and in combination with cisplatin or doxorubicin in the HCC cell lines HepG2 and SNU398. Protein regulation, cell viability and apoptosis were assessed by western blotting, cell count and FACS analysis, respectively.

Results: HCC cell lines display strong endogenous Mcl-1 protein expression. ASO targeting Mcl-1 specifically downregulated Mcl-1 protein expression by up to 80% and decreased cell viability by about 60% in a dose- and time-dependent manner. A moderate increase of apoptosis was observed. Notably, no significant target regulation or cell growth inhibition was observed for control oligonucleotide treatment. Combination of ASO with cisplatin or doxorubicin showed an additive, but not synergistic effect on cell viability and apoptosis.

Conclusions: Mcl-1 protein is expressed in HCC cell lines and appears to be an attractive molecular target. ASO targeting Mcl-1 revealed a powerful single agent activity against HCC *in vitro*. Given the hepatotropic pharmacokinetic properties and low toxicity of ASO *in vivo*, targeting Mcl-1 by ASO might become a promising novel approach in HCC therapy.

625 POSTER

Comparison of gene expression profiles in breast cancer cells treated with 5-fluorouracil, cisplatin or etoposide using cDNA microarray

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Background: Drug resistance in cancer is a major limitation for successful chemotherapy. Mechanisms of development of intrinsic drug resistance are not thoroughly understood and may involve the expression of multiple genes during tumor progression and also the emergence of acquired resistance may be associated with drug selection during chemotherapy. 5-fluorouracil(5-FU), cisplatin, and etoposide are commonly used in the treatment of breast cancer. To identify downstream mediators of tumor cell response to each chemotherapeutic agent, we used cDNA microarray technology to elucidate genes that are regulated by different chemotherapeutic agent treatment in the MCF-7 breast cancer cell line.

Methods: Breast cancer cells, MCF-7 were treated with IC50 concentration of 5-fluorouracil, cisplatin, and etoposide for 24 hour exposure, respectively. The use of cDNA microarrays containing 13,000 genes in our analysis provides a global view of the response of breast cancer cells to each chemotherapeutic agent at the genomic levels.

Results: Of 13,000 genes, 31, 108 and 6 genes were up-regulated (>2-fold) in breast cancer cells treated with 5-FU, cisplatin, or etoposide, respectively. Sixty-two, 137, and 11 genes were down-regulated (>2-fold) in breast cancer cells treated with 5-FU, cisplatin or etoposide, respectively. Thirty-eight and 8 genes were commonly up- or down-regulated by 5-FU and cisplatin. However, only 15 and 6 genes were commonly up- or down-regulated by 5-FU, cisplatin, and etoposide.

Conclusion: Our studies demonstrate that the downstream mediators of tumor cell response to chemotherapeutic agents may be variable according to the individual chemotherapeutic agents. However, several commonly regulated genes may be useful biomarkers of resistance. Our results suggest that cDNA microarray has a potential to identify genes involved in mediating the response of breast cancer cells to chemotherapy.