**SUPPLEMENTAL METHODS**

**Immunohistochemical Analysis of GTPase Targets**

Specimens were deparaffinized and hydrated using xylene and graded ethanol solutions finishing with phosphate buffered saline. Epitopes were retrieved using a Tissue Tek decloaking chamber (Biocare Medical) at 120.5°C for 15 min and allowed to cool slowly to room temperature. Pretreatment with hydrogen peroxide (3%) in phosphate buffered saline was used to reduce background.

To optimize staining conditions, selected cases representing residual, deidentified specimens from patients who had undergone diagnostic and therapeutic surgery for malignant (primary ovarian cancer, or prostate, breast or colorectal cancer as case controls) and non-malignant diseases (benign ovarian neoplasms, or benign prostate, breast or colorectal neplasms as case controls) were obtained through the UNM Human Tissue Repository under an approved IRB protocol (SRC001-10). Colon cancers overexpressing Rac1 and Cdc42 served as a positive control and ovarian tumor tissue was comparatively evaluated against benign ovarian tissue.

**qPCR of Ovarian Cancer cDNA Arrays**

qPCR was performed using Origene SYBR Green I master mix solution diluted to a final concentration of 1X with primers at a concentration of 0.33μM. PCR mix was added to each well of the microarray plate and incubated on ice for 15m to dissolve the cDNA. qPCR was conducted using a Bio-Rad iCycler (Hercules, CA) under the following conditions; 95° for 5m, 30 cycles of (95° for 15s, 60° for 30s, 72° for 1m), followed by melt curve analysis, hold at 4°. Rac1b levels were measured using custom primers synthesized by Invitrogen. Rac1b forward primer, 5’-TCCGCAAACAGTTGGAGA-3’, was coupled with Rac1 reverse primer, 5’-CTACATGTTTGCGGATAGGATAGGG-3’. The identity of the PCR product as Rac1b was confirmed by sequence analysis.

**High Performance Liquid Chromatography**

HPLC analysis was performed with a 50mm long 5μm silica guard column (Phenomenex, 03B-4053-N0) attached to a Partisil® 5 µm ODS(3) 85 Å LC Column 150 x 4.6 mm (Phenomenex, 00F-0120-E0) followed by a Lux 5 µm Cellulose-3 50 x 4.6mm in reverse phase ( Phenomenex, 00G-4493-E0). The columns are equilibrated with acetonitrile/0.1% Formic acid in water (25:75) at a flow rate of 2ml/min. A standard curve of racemic ketorolac in water was generated based on an injection volume of 10μl and ketorolac was detected on a UV310 detector. 500μl serum (or ascites) samples were mixed with 200μl of 600mM sulfuric acid then diluted and mixed in 3ml diethyl ether. The organic layer was separated by centrifugation at 2500 rpm for 5 min using a Hermle Z440K. The organic layer was removed and evaporated to dryness, then reconstituted in 200μl mobile phase. Retention times for R-ketorolac and S-ketorolac were 5.1 min and 6.1 min respectively, and were validated against each individual enantiomer.

**Analysis of Patient Derived Cells**

To purify ovarian cancer cells from ascites, cells were recovered from ascites samples by low speed centrifugation at 1000 rpm. The cell pellets were gently resuspended and overlaid on a Ficoll (density 1.077±0.001 g/ml; GE Helathcare 17-5442-02) to separate red blood cells from lymphocytes and tumor cells per manufacturer's instructions. Anti-CD45 beads (Life Technologies) were used to deplete lymphocytes and the resulting tumor cell fraction was analyzed visually and by flow cytometry. For flow cytometric analysis, cells suspended in phosphate buffered saline (PBS) were fixed with formaldehyde (2-4% final concentration) for 10 min, chilled for 20 min and then processed for immunostaining as described by Cell Signaling Technology {http://www.cellsignal.com/contents/resources-protocols/flow-cytometry-protocol-(flow)/flow; webpage Accessed July 22, 2014}. Briefly, non-specific mAb binding to human Fc receptor was blocked by pretreatment for 10 min at room temperature with human Fc receptor binding inhibitor (14-916, Affymetrix eBioscience). Antibody staining was performed in PBS containing 0.5% BSA using 0.5-1 x106 cells/assay for one hour at room temperature.

Tumor cell fractions were positive for EpCAM (detected with mAb against EpCAM clone Ber-EP4, Dako) and MUC16/CA125 (detected with a Cy5-labeled rabbit pAb directed against human MUC16/CA125; bs-0091R-Cy5, Bioss Antibodies) and negative for CD45 (detected with a PE-labeled rabbit pAb directed against human CD45/LCA, 12-9459, Affymetrix eBioscience) (supplemental Figure S2).

For analysis of GTPase activity in patient-derived cells, active RhoA was quantified based on binding to GST-Rhotekin (RT01, Cytoskeleton, Inc.). Antibodies specific for Cdc42 (sc-8401, Santa Cruz Biotechnology, Inc.), Rac1 (610650, BD Transduction Labs) or RhoA (26C4, sc-418, Santa Cruz Biotechnology, Inc.) and Alexa Fluor 488 donkey anti-mouse lgG (A21202, Life Technologies) were also used.

**SUPPLEMENTAL TABLES**

**Supplemental Table S1.**

|  |  |  |
| --- | --- | --- |
| Table S1. Patient Characteristics for IHC Microarrays OV1005 and OV8010 | | |
| Age# | 46.6 +/- 13.7 (std. dev) | |
| Stage I# |  | 12 |
|  | A | 5 |
|  | B | 5 |
|  | C | 4 |
| Stage II# |  | 21 |
|  | A | 13 |
|  | B | 6 |
|  | C | 3 |
| Stage III# |  | 1 |
|  | C | 31 |
| Stage IV# |  | 11 |
| Stage undetermined# |  | 51 |
| Normal stroma |  | 20 |
| Benign/borderline |  | 25 |
| Grade - Low\* (1#) |  | 13 |
| Grade - Intermediate\* (1-2, 2#) |  | 7 |
| Grade - High\* (2, 2-3, 3#) |  | 98 |
| \*Diagnoses by board certified pathologist with gynecologic pathology specialization were as follows: Clear cell carcinoma (4); endometrioid carcinoma (13); endometrioid neoplasm (5); mucinous carcinoma (4); mucinous neoplasm (9); mucinous cystadenoma (5); normal stroma only-no epithelia (20); papillary serous carcinoma (78); serous neoplasm (5); serous cystadenoma (4); undifferentiated carcinoma (16). There was 77% agreement with #BioMax specification sheet, differences were confined to ID of rarer subtypes (endometrioid, clear cell, mucinous, transitional and serous). Samples included in analysis: 163 of 180 total (9% were excluded due to poor staining at edge of slide or no neoplasm in the section). | | |

**Supplemental Table S2.**

|  |  |  |
| --- | --- | --- |
| Table S2. Patient Characteristics for cDNA Microarray | | |
| Age | 57.26 -/+ 14.85 (st. dev) | |
| Stage I | A | 20 |
|  | B | 8 |
|  | C | 10 |
| Stage II | A | 4 |
|  | B | 9 |
|  | C | 4 |
| Stage III | A | 15 |
|  | B | 17 |
|  | C | 26 |
| Stage IV | A | 10 |
| Grade Not Reported |  | 10 |
| Grade I |  | 9 |
| Grade II |  | 32 |
| Grade III |  | 60 |
| Grade IV |  | 9 |
| Diagnoses were as follows: Carcinoma of Ovary (2); Carcinoma of ovary, endometrioid (6); Carcinoma of ovary, papillary serous (8);  Carcinoma of ovary, clear cell (1); Adenocarcinoma of ovary, endometrioid (23); Adenocarcinoma of ovary, papillary serous (32); Adenocarcinoma of ovary, serous (28); Adenocarcinoma of ovary, metastatic (2); Adenocarcinoma of ovary, mucinous (4); Adenocarcinoma of ovary, clear cell (5); Tumor of ovary, borderline (2); Tumor of ovary, papillary serous, borderline (2); Tumor of ovary, serous, borderline (5)  These microarray plates consisted of 19 normal, 9 Grade I, 32 Grade II, 60 Grade III, 9 Grade IV, and 10 grade not reported patients. Patients with grade not reported were not included in grade analysis. | | |

**Supplemental Table S3.**

|  |  |  |
| --- | --- | --- |
| Table S3. Ketorolac enantiomer concentration in serum or peritoneal fluids. | | |
| Sample | [R] μM | [S] μM |
| Serum 1h | 3.97 | 1.65 |
| Serum 6h | 1.44 | 0.33 |
| Serum 24h | 0.40 | 0.02 |
| Ascites 1h | 0.5 | 0.33 |
| Ascites 6h | 0.98 | 0.32 |
| Ascites 24h | 0.27 | 0.03 |

**SUPPLEMENTAL FIGURES**

**Supplemental Figure S1. Expression of Rho-family GTPases in primary patient cDNA samples analyzed by grade for serous cancer only.**

**Supplemental Figure S1:** Tissuescan ovarian cancer cDNA microarrays (Origene) were amplified using primers against Rac1, Rac1b, Cdc42, RhoA, and β-actin as described in methods. This analysis does not include endometrioid tissue. As per the manufacturer description, patients with leiomyoma of myometrium, follicular cysts, abscesses, or secretory endometrium, but otherwise healthy ovarian tissue, were considered normal (n=11). Data from two normal patients were not included due to Grade 3 carcinomas of adjacent tissues. Tissues are grade I (n=2), grade II (n=24), grade III (n=48), or grade IV (n=9). The cDNA of 10 patients were excluded due to a lack of grade information. Groups were compared to normal cDNA using a two-tailed t-test, \* indicates significance is p<0.05.

**Supplemental Figure S2. Purified Ovarian Tumor Cells Express EpCam and MUC16/CA125.**

**Supplemental Figure S2.** Ovarian tumor cell identification by flow cytometry using patient ascites samples from three individuals (designated 29, 30, and 35). Tumor cells were purified from ascites using Ficoll gradients to remove red blood cells and CD45 microbead negative selection to remove leukocytes. The resulting cells (gated as R3) were negative for CD45 and positive for EpCAM and MUC16/CA125. Marker analyses were tracked by flow cytometry on a Becton Dickinson FACScalibur Flow Cytometer.

**Supplemental Figure S3. Racemic distribution of clinical drug.**

**Supplemental Figure S3.** To confirm that this apparent shift in racemic ratio was not due to either 1) unequal distribution of the racemates in the administered drug or 2) differential extraction of each racemate from serum proteins, we conducted control experiments. A) Racemic distribution of ketorolac enantiomers of the clinically administered drug was confirmed by HPLC. B) Clinical grade ketorolac incubated with human serum (shown) and ascites fluid (not shown, but identical results) at 37°C for 1 hr and the samples were then processed as for samples obtained from patients. The equal distribution of R- and S-ketorolac in the extracted control sample indicates that the difference in enantiomer composition in patient samples is not due to differential extraction from serum proteins during sample processing and more likely represents a pharmacokinetic parameter such as preferential binding of S-ketorolac to tissue proteins.

**Supplemental Figure S4. Survival estimates based on Cox-regression for Stage I (AJCC) with completion of chemotherapy.**

**Supplemental Figure S4.** Cox proportional hazards regression was used to estimate ovarian cancer specific survival probabilities for women who did (dashed line) and did not (solid line) receive ketorolac among ovarian cancer cases with AJCC Stage I cancer, sorted by age group and -/+ neoadjuvant chemotherapy.

**Supplemental Figure S5. Survival estimates based on Cox-regression for Stage II (AJCC) with completion of chemotherapy.**

**Supplemental Figure S5.** Cox proportional hazards regression was used to estimate ovarian cancer specific survival probabilities for women who did (dashed line) and did not (solid line) receive ketorolac among ovarian cancer cases with AJCC Stage II cancer, sorted by age group and -/+ neoadjuvant chemotherapy.

**Supplemental Figure S6. Survival estimates based on Cox-regression for Stage III (AJCC) with completion of chemotherapy.**

**Supplemental Figure S6.** Cox proportional hazards regression was used to estimate ovarian cancer specific survival probabilities for women who did (dashed line) and did not (solid line) receive ketorolac among ovarian cancer cases with AJCC Stage III cancer, sorted by age group and -/+ neoadjuvant chemotherapy.

**Supplemental Figure S7. Survival estimates based on Cox-regression for Stage IV (AJCC) with completion of chemotherapy.**

**Supplemental Figure S7.** Cox proportional hazards regression was used to estimate ovarian cancer specific survival probabilities for women who did (dashed line) and did not (solid line) receive ketorolac among ovarian cancer cases with AJCC Stage IV cancer, sorted by age group and -/+ neoadjuvant chemotherapy.

**Supplemental Figure S8. RhoA activity is insensitive to ketorolac treatment.**

**Supplemental Figure S8.** (A-B) RhoA GTPase target inhibition assayed by quantification of active RhoA using a flow based Rhotekin effector binding assay. (A) Patient derived tumor cells purified from ascites fluids at the time of debulking surgery were treated *in vitro* for 1 h with 10 µM R-ketorolac, S-ketorolac or CID2950007–a Cdc42 selective inhibitor (19, 20). Tumor cells incubated with 0.1% DMSO served as a negative control. (B) Results of administration of racemic ketorolac to ovarian cancer patients post-surgery. Cells isolated from patient ascites samples post-surgery were assayed as in (A). Patient diagnoses were stage II-III, high grade ovarian serous or papillary serous carcinoma, with one suspected primary peritoneal carcinoma (Pt 20, 21, 43). Fluorescence readings were normalized to 0.1% DMSO control or the 0 h time point drawn immediately prior to ketorolac administration. One way non-parametric ANOVA and Bonferroni multiple comparison test showed no statistically significant differences between either the *ex vivo* or the *in vivo* samples.