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Tubulin-β-III Overexpression by Uterine Serous Carcinomas is a Marker for Poor Overall Survival after Platinum/Taxane Chemotherapy and Sensitivity to Epothilones

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Abstract

BACKGROUND—Uterine serous carcinoma (USC) is a subtype of endometrial cancer associated with chemoresistance and poor outcome. Overexpression of tubulin-β-III and p-glycoprotein has been linked to paclitaxel resistance in many cancers but has been undercharacterized among USC. Epothilones have demonstrated activity in certain paclitaxel-resistant malignancies. In this study, we clarify in USC relative to ovarian serous carcinomas (OSC) the relationships between tubulin-β-III and p-glycoprotein expression, clinical outcome, and *in vitro* chemoresponsiveness to epothilone B, ixabepilone and paclitaxel.

METHODS—Tubulin-β-III and p-glycoprotein were quantified by real time polymerase chain reaction (qRT-PCR) in 48 fresh-frozen tissue samples and 13 cell lines. Copy number was correlated with immunohistochemistry and overall survival. IC_{50} was determined using viability and metabolic assays. Impact of tubulin-β-III knockdown on IC_{50} was assessed with siRNAs.

RESULTS—USC overexpressed tubulin-β-III but not p-glycoprotein relative to OSC in both fresh-frozen tissues (552.9±106.7 versus 202.0±43.99, p=0.01) and cell lines (1701.0±.376.4 versus 645.1±157.9, p=0.02). Tubulin-β-III immunohistochemistry reflected qRT-PCR copy number and overexpression stratified patients by overall survival (copy number ≤400: 615 days; copy number >400: 165 days, p=0.049); p-glycoprotein did not predict clinical outcome. USC remained exquisitely sensitive to patupilone *in vitro* despite tubulin-β-III overexpression ($IC_{50,\ USC}$ 0.245±0.11 nM versus $IC_{50,\ OSC}$ 1.01±0.13 nM, p=0.006).

CONCLUSIONS—Tubulin-β-III overexpression in USC discriminates poor prognosis, serves as a marker for sensitivity to epothilones, and may contribute to paclitaxel resistance. Immunohistochemistry reliably identifies tumors with overexpression of tubulin-β-III, and a subset of individuals likely to respond to patupilone and ixabepilone. Epothilones warrant clinical investigation for treatment of USC.

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INTRODUCTION

In 2012, a projected 29,520 deaths will occur from gynecologic malignancies.[1] Among gynecologic cancers, endometrial cancer is the most common and may be broadly dichotomized into two classes with separate underlying molecular pathogenesis, clinical behavior, and histopathology.[2] *Type I* endometrial cancers comprise 80% of cases, and are associated with endometrioid histology (grade 1 or 2) and favorable prognosis.[3] *Type II* endometrial cancers are characterized by an aggressive clinical course with relatively poor prognosis.[3–4] Uterine serous carcinomas (USC) represent the most common form of *type II* disease and are poorly differentiated by definition. As many as 37–70% of patients with USC demonstrate extrauterine disease at time of diagnosis,[5–6] sometimes in the setting of scant or no myometrial invasion.[7–8] So while USC represents only 10% of all uterine cancers, it accounts for 39% of deaths.[3]

Ovarian cancer remains the leading cause of death from gynecologic malignancy in the United States and most developed countries.[1,9] Among epithelial subtypes, ovarian serous carcinomas (OSC) represent 75% of all cases and can be organized into two tiers.[10] *Low-grade* OSC develop indolently from non-invasive borderline precursors.[11] The origin of *high-grade* OSC is more controversial; genetic analyses suggest that rapid transformation of intraepithelial carcinoma of the fallopian tube may underlie as many as 50% of cases, in addition to those which evolve primarily from peritoneal or ovarian surface epithelium.[7] OSC has one of the highest death-to-incidence ratios, attributable largely to a lack of effective screening modalities with subsequent initial diagnosis at late stages.

High-grade serous carcinomas of the uterus and ovary may be morphologically indistinguishable.[8] Advanced-stage disease is associated with grim 5-year overall survival (OS) rates of 18.5–32.5%[5] and 34%,[11] respectively. Though a growing body of molecular evidence delineates the distinctness of these entities,[12–13] first-line therapy for both begins with surgical staging followed by platinum/taxane combination chemotherapy for advanced disease.[14–17] Initial response rates of advanced USC to this regimen are as low as 60%,[18] which compare unfavorably to rates of 80% for OSC.[19] Responses in USC are generally non-durable.[20] In patients treated with intravenous carboplatin/paclitaxel for advanced disease, median progression-free survival in USC is 13 months,[17] compared to 20.7 months in OSC.[15]

Resistance to paclitaxel has been linked to overexpression of class III β-tubulin,[21] one of 9 β-isoforms[22] capable of heterodimerizing with α subunits to form microtubules critical to cell division. Paclitaxel binds preferentially to the tubulin-β-I isoform,[23] which differs from tubulin-β-III at paclitaxel-binding site positions 275 (Ser→Ala) and 364-5 (Ala-Val→Ser-Ser).[22] High tubulin-β-III expression reduces the rate of microtubule assembly[24] and correlates with poorer OS in ovarian serous,[25] colon,[26] non-small cell lung,[27] and breast[28] cancers. This clinicopathologic association has not yet been demonstrated in endometrial cancers.[29]

Epothilones (EPOxide THIazoLe ketONEs) are novel microtubule-stabilizing macrolides isolated from *Sorangium cellulosum*[30] with activity in paclitaxel-resistant malignancies putatively due to their unique ability to bind class III and I isoforms with at least equal affinity.[23] Epothilones generally do not share with paclitaxel overlapping mechanisms of

resistance,[31] and are not substrates for the drug efflux pump p-glycoprotein, the overexpression of which has been linked to multidrug resistance to cytotoxic agents including taxanes.[32] Despite encouraging *in vitro* data and strong biologic plausibility, p-glycoprotein expression often does not reliably correlate with clinical outcome and trials of p-glycoprotein inhibitors have failed to demonstrate improved survival benefit.[33] This underscores the need to identify novel biomarkers that predict clinical response.

In this study, we sought to (1) quantify by qRT-PCR tubulin-β-III and p-glycoprotein expression in a large number of USC in comparison to high-grade OSC within solid tissues and cell lines, (2) describe the association of tubulin-β-III and p-glycoprotein with *in vitro* chemoresponsiveness to epothilone B (patupilone), ixabepilone and paclitaxel, (3) characterize the contribution of tubulin-β-III to paclitaxel resistance, (4) correlate tubulin-β-III immunohistochemistry with qRT-PCR copy number, and (5) examine the prognostic implications of tubulin-β-III overexpression on clinical outcome.

METHODS

Tissue procurement, establishment of cell lines, and characterization of growth rate

As approved by the institutional review boards at Yale University and the University of Arkansas, fresh-frozen tissues and patient characteristics were obtained at time of primary debulking. Tumors were staged according to criteria established by the AJCC 7th ed./FIGO. As per WHO guidelines for epithelial tumors,[34] only those that contained <10% of a second malignant component were considered 'pure;' those which contained >10% were considered 'mixed.' Carcinoma and normal human ovarian surface epithelial cell lines were established from tissue biopsies and maintained as described previously.[35–36] Cellular growth rate was determined per established protocol. [37] Survival data were obtained through review of electronic medical records and/or public records.

RNA extraction, purification, and reverse-transcription

Total RNA extraction from cell culture lysates and fresh-frozen tissues was performed using AllPrep DNA/RNA/Protein Minikit (Qiagen, Germantown, MD). Quantitative real time polymerase chain reaction (qRT-PCR) was performed with a 7500 RealTime PCR System per the manufacturer's protocol (Applied Biosystems, Foster City, CA). All RNA samples were treated with Turbo DNase enzyme (Applied Biosystems). Total RNA (5μg) was reverse-transcribed using Superscript III (Invitrogen, Carlsbad, CA). Five microliters of reverse-transcribed RNA was amplified using Taqman PCR Master Mix (Applied Biosystems). Tubulin-β-III (Hs00964962_g1) and p-glycoprotein (Hs00184491_m1) specific primers were obtained (Assay-On-Demand, Carlsbad, CA), with GAPDH (Hs99999905_m1) as an internal control. Gene expression was analyzed using the comparative threshold method and normalized to levels observed in lymphoid cell lines.

Chemosensitivity and viability assays

Patupilone (Novartis Pharma, Basel, Switzerland), Ixabepilone (Bristol-Myers Squibb Company, Princeton, NJ) and paclitaxel (T7402: Sigma-Aldrich, St. Louis, MO) were dissolved in dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO) as 10mM stock solutions protected from light exposure and stored at −20°C prior to serial dilution. Metabolic assays were performed after drug exposure using MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assays (Promega Corporation, Madison, WI). Briefly, cells at log phase of growth were seeded in 96-well culture plates at optimum density, incubated, and exposed to drug after 24 hours. Tetrazolium was added after 72 hours of additional incubation. Absorbance at a reference wavelength of 490 nm was measured with a VersaMax microplate reader (Molecular Devices, Sunnyvale, CA). For

cell viability assays, cells at log phase of growth were seeded in 6-well microtiter plates at optimum density and exposed to drug at 24 hours. After 48–72 hours of additional incubation, well contents were harvested in entirety, centrifuged then stained with either trypan blue for manual counts or propidium iodide (2 μL of a 500 μg/mL stock solution in PBS with 0.1% sodium azide and 2% fetal bovine serum) for flow cytometric counts. Unless stated otherwise, all IC_{50} values presented have been determined by flow cytometry.

Immunohistochemistry

All slides were reviewed by a gynecologic pathologist (NB). Formalin-fixed sections were cut at 4 μm and stained with anti-tubulin-β-III monoclonal antibody (TUJ1; Covance, Berkeley, CA) at 1:500 dilution. Staining intensity was assessed using a semi-quantitative scoring system: 0, negative (no staining observed); 1+, focal/weak staining; 2+, diffuse weak/focal moderate staining; 3+, diffuse moderate/focal strong staining; 4+, diffuse strong immunoreactivity. For mixed specimens, only the serous component was examined.

Small-interfering RNA (siRNA)

Tubulin-β-II-specific (5′>3′ GAUCAAUGCUGCAUCCUUAtt) and tubulin-β-III-specific small-interfering RNA oligonucleotides (5′>3′ CCAUUCUGGUGGUGGACCUGGAtt) were purchased from Ambion, Inc. (Silencer Select, Life Technologies, Carlsbad, CA). Cells were seeded in 6-well plates at a concentration of 200,000 per well and transfected with siRNA duplexes at 10 nM with 5 μL Lipofectamine RNAiMAX (Invitrogen) in OptiMem (Life Technologies) and RPMI with 10% fetal bovine serum. Mock transfections with nonspecific siRNA duplexes were used as negative controls. Cells were exposed to drug 24 hours after plating. Cells remained in culture for a total of 72 hours prior to harvest.

Statistical analyses

Dose response curves were analyzed using GraphPad Prism 5.0 (GraphPad Software, Inc., LaJolla, CA). $IC_{50,\ absolute}$ was defined as the concentration of drug required to result in reduction of cell viability to 50% of untreated control. $IC_{50,\ relative}$ was defined as the concentration at 50% between maximum and minimum points of the dose-response curve. IC_{50} was determined through interpolation of sigmoidal curves fit with a standard Hill slope of −1.0. Unless stated otherwise, all values presented reflect $IC_{50,\ absolute}$ and mean ± standard error of the mean (SEM). Kaplan-Meier curves were analyzed with the log-rank test. All student's t-tests and Pearson correlations employed 2-tails and assumed equal variance. Non-parametric Mann-Whitney analyses were applied to examine the distributions of immunohistochemistry scores. P values <0.05 were considered statistically significant.

RESULTS

Uterine serous carcinomas overexpress tubulin-β-III but not p-glycoprotein in fresh-frozen tissues and cell lines by qRT-PCR

Fresh-frozen tissue specimens representing 28 USC and 20 OSC were analyzed. A total of 6 USC and 7 OSC primary cell lines were established. Source patient characteristics are provided in Table 1a/b. Mixed specimens all contained endometrioid in addition to serous components; in one specimen, clear cell features were also present. USC overexpressed tubulin-β-III relative to OSC in both fresh-frozen tissues (Figure 1a; 552.9±106.7 versus 202.0±43.99, p=0.01) and cell lines (Figure 1b; 1701±376.4 versus 645.1±157.9, p=0.02). Tubulin-β-III expression in fresh-frozen tissues was greater for patients with pure versus mixed histology (703.2±196.4 versus 403.4±72.57, p = 0.02; Figure 1c). There was no difference in tubulin-β-III expression among those with early versus advanced stage disease (533.2±134.3 versus 595.8±183.3, p = 0.79; data not shown). Tubulin-β-III was not

expressed at high levels in normal human ovarian surface epithelial cell lines (133.3±22.37, n=3; data not shown).

Compared to OSC, USC exhibited a trend towards higher p-glycoprotein expression in fresh-frozen tissues (Figure 1d; 120.9±31.39 versus 39.22±15.82, p = 0.05) and cell lines (Figure 1e: 1005±455.4 versus 266.5±136.5, p = 0.15). The greater expression of both tubulin-β-III and p-glycoprotein in cell lines compared to fresh-frozen tissues is thought to reflect heterogeneity of the tissue specimens, which might contain vessels and stromal components.

Immunohistochemistry staining for tubulin-β-III correlates with qRT-PCR copy number

Approximately 35% of specimens included in the qRT-PCR analysis were also examined with immunohistochemistry (IHC) (5 pure UPSC, 3 mixed USC, 9 OSC). USC demonstrated stronger staining (median IHC score: 3+) relative to OSC (median IHC score: 0 to 1+) (p = 0.004). qRT-PCR copy number among these specimens was 631.12±193.31 versus 92.98±25.53 (p = 0.01). Normal ovary and endometrium did not express tubulin-β-III by IHC; this reflects qRT-PCR copy number among unmatched normal ovary, endometrium, and myometrium (32.63±6.51, n=3). Representative slides and distribution of IHC scores are shown in Figure 2.

Uterine serous carcinomas are highly sensitive to patupilone relative to ovarian serous carcinomas

Next, we performed chemosensitivity profiling of 4 USC and 3 OSC cell lines, which were selected given their similarity in growth rates as this may influence response to cytotoxic agents (mean doubling time ± SEM USC versus OSC: 24.5±4.25 versus 35.0±8.5 hours, respectively, p=0.28; data not shown). USC were found to be highly sensitive to patupilone. As determined by flow cytometric assays of cell viability, IC_{50} values for patupilone among 4 USC and 3 OSC cell lines were 0.25±0.11 nM [range: 0.02–0.53] and 1.01±0.13 nM [range: 0.81–1.24], respectively (p = 0.006) (Figure 3). Corresponding IC_{50} values for paclitaxel were not significantly different (26.41±27.14 [range: 48.42–182.52] versus 95.64±75.34 [4.89–65.52]; p=0.14). Within these 7 cell lines, tubulin-β-III expression was higher among USC compared to OSC with a trend towards statistical significance (2297±462.2 versus 834.0±289.5, p=0.058) (Figure 3-inset). p-glycoprotein expression was not significantly different (p=0.27; not shown). Flow cytometric findings were robust across both metabolic assays and manual counts by trpyan blue exclusion. IC_{50} values determined by flow cytometry are presented given superior sensitivity and dynamic range of the assay; results of these independent validation studies have been described previously (submitted for publication).

Uterine serous carcinomas are significantly more sensitive to patupilone and ixabepilone when compared to paclitaxel

We next compared the efficacy of ixabepilone (a commercially available and FDA-approved epothilone) to patupilone and paclitaxel in a limited number of USC cell lines (i.e., USC-1 and USC-2). As representatively shown in figure 4 for USC-1, both USC cell lines tested demonstrated significantly higher sensitivity to patupilone and ixabepilone when compared to paclitaxel. Indeed, as determined by flow cytometric assays of cell viability, IC_{50} values were 0.41 nM (range 0.3–0.55 nM) for patupilone and 0.87 nM [range: 0.58–1.27 nM] for ixabepilone, respectively, while IC_{50} values were 2.12 nM [range: 1.44–3.05 nM] for paclitaxel (paclitaxel vs patupilone: p = 0.003, paclitaxel vs ixabepilone p = 0.02, Figure 4). In multiple parallel experiments, we consistently noted higher sensitivity of USC cell lines to patupilone when compared to ixabepilone (p = 0.02, Figure 4).

Chemoresistance of uterine serous carcinomas to paclitaxel and patupilone varies with tubulin-β-III expression

We investigated the relationship of tubulin-β-III as a marker of patupilone sensitivity and paclitaxel resistance in USC by plotting by qRT-PCR copy number against the ratio of $IC_{50,\ paclitaxel}$ to $IC_{50,\ patupilone}$ (Figure 5a; r^2=0.8038, p=0.05). P-glycoprotein expression in the USC (n=4) and OSC (n=3) cell lines did not correlate with patupilone IC_{50} (R^2=0.357, p=0.16, data not shown). To investigate the role of tubulin-β-III in paclitaxel and patupilone resistance, siRNAs were then used to down-regulate expression prior to exposure to drug. Transfection successfully produced reductions in tubulin-β-III of 83–93%, and efficiency was not affected by addition of chemotherapy (Figure 5b). Knockdown of tubulin-β-III enhanced sensitivity of both USC and OSC cell lines to paclitaxel (Figure 5c); based on $IC50_{50,\ relative}$ values, sensitivity in USC-3, USC-7, and OSC-1 increased by 2.14, 1.64, and 2.22-fold, respectively. In contrast, down-regulation of tubulin-β-II (i.e., a tubulin used as additional control in our experiments), did not enhanced sensitivity to paclitaxel (Figure 5d). Knockdown of tubulin-β-III expression sensitized USC cells to patupilone (Figure 5e).

Tubulin-β-III but not p-glycoprotein expression stratifies among patients with advanced stage disease poorer OS after platinum/taxane combination chemotherapy

Kaplan-Meier estimates of OS were generated for patients with stage III/IV USC (67.9% white vs 32.1% black) and OSC (90% white vs 10% black) (Table 1a and Figure 6a). All patients received adjuvant therapy with intravenous carboplatin and paclitaxel with or without radiation therapy. None of the patients received neoadjuvant chemotherapy. Follow-up was available for 16/19 patients with advanced USC and 16/18 patients with advanced OSC. Median OS was much worse for patients with either pure USC (221 days) or mixed component USC (675 days) compared to OSC (1306 days) (p<0.0001). The prognostic value of tubulin-β-III and p-glycoprotein copy number was then assessed in patients with USC. Using a cutoff of 400, median OS among patients with low and high tubulin-β-III was 615 and 165 days, respectively (Figure 6b; p=0.049). Of patients with high tubulin-β-III expression, 71% demonstrated pure histology compared to 44% in those with low expression but this difference was not statistically different (p=0.33). The hazard ratio for death (HR) was 3.929 (95% CI 1.04–14.84). Within this same group, no differences in OS were found between patients with low and high p-glycoprotein expression using a cutoff of 30 (Figure 6c) (median OS: 268 versus 341 days, respectively, p=0.69; HR: 0.79, 95% CI 0.26–2.438). Among patients with high and low p-glycoprotein expression, there was not a difference in distribution of patients with mixed and pure histology (p=0.31). Among the USC cohort, mean tubulin-β-III and p-glycoprotein copy numbers were 643.31 (range: 150–2837) and 72.63 (range: 9–304), respectively (Figure 6d/e). Median follow-up interval in the cohort was only 345 days, but only 2 patients remained alive at time of last follow-up. In the OSC cohort, mean tubulin-β-III and p-glycoprotein copy numbers were 212.59 (range: 12–839) and 32.69 (range: 1–118), respectively; median follow-up was 693 days, and 9 patients were alive at time of censoring.

DISCUSSION

Though uterine and ovarian serous carcinomas represent two morphologically similar diseases often treated with identical chemotherapeutic regimens, they differ in their biologic pathogenesis and clinical behavior. In accordance with this diversity, we have shown for the first time using quantitative methods within a large number of fresh-frozen tissues and cell lines that USC overexpress tubulin-β-III relative to high-grade OSC. We also demonstrate correlation between qRT-PCR copy number and immunostaining. This validation is crucial since mRNA transcript copy number and protein expression may be discordant due to post-transcriptional modifications in mammalian cells.[38]

The pathways leading to chemoresistance in USC are incompletely elucidated. A brisk growth rate likely permits rapid tumor regrowth despite initial intrinsic sensitivity to chemotherapeutic agents.[39] These tumors are also associated with a distinct milieu of autocrine factors including upregulation of IL-6,[40] which has been shown to confer resistance to paclitaxel in gynecologic malignancies.[41] Though p-glycoprotein has been shown *in vitro* to maintain low intracellular concentrations of paclitaxel,[33] expression has not shown to be clinically meaningful for endometrial cancers.[29,42–43] The design of rational therapeutic strategies requires a more thorough understanding of the mechanisms that contribute to resistance as well as isolation of novel prognostic markers for response.

Tubulin-β-III has been proposed as a biomarker of chemoresistance to paclitaxel[44] as well as response to patupilone.[37] In this study, USC exhibited greater tubulin-β-III expression and were 4.1-fold more sensitive (p=0.006) to patupilone relative to OSC cell lines, despite equal sensitivity to paclitaxel. As chemosensitivity is multifactorial, the similar sensitivity to paclitaxel may reflect the concurrent absence of differential p-glycoprotein expression across the uterine and ovarian serous cell lines tested. Chemosensitivities were confirmed rigorously across assays based on both cell viability (trypan blue exclusion and flow cytometry) and metabolism (MTS) since the latter methodology may be greatly influenced by variables such as media composition.[45] Given that heightened sensitivity to paclitaxel in response to tubulin-β-III downregulation had not yet been demonstrated in USC cell lines, we used siRNA to reduce tubulin-β-III expression; consistent with previous studies, we witnessed improved *in vitro* sensitivity of both USC and OSC to paclitaxel by approximately 2-fold. Tubulin-β-III downregulation has been previously shown to improve sensitivity to multiple chemotherapeutic agents,[46] including epothilone B[47] by predisposing cells to apoptosis through mechanisms other than reduction in mitotic block induced by chemotherapeutic agent.[46–47] The results of our siRNA tubulin-β-III knockdown experiments in USC are in agreement with this view and suggest that tubulin-β-III may not only serve as a marker for sensitivity to epothilones but also as a mediator of multiple cell survival pathways. [46–47]

In this study, overexpression of tubulin-β-III had prognostic relevance. Among patients with advanced-stage disease, we have shown for the first time in USC that tubulin-β-III copy number may identify patients who are at risk of poorer OS. Consistent with prior publications, we found reduced OS in uterine cancers with increasing volume of the serous component.[48] Our findings differ from the report by Vandenput et al.[29] and by Zhu et al. [49] who failed to identify a relationship between tubulin-β-III expression and outcome in patients who had received paclitaxel for endometrial cancer, however, their analyses employed IHC alone.

Patupilone has shown encouraging activity in the treatment of gynecologic malignancies, but has not been tested beyond phase II in endometrial cancers.[50,51] Ixabepilone, which differs from patupilone by a single moiety, has been evaluated in GOG-129P, a study of 50 patients with recurrent or persistent endometrial cancer who received one prior line of taxane-based chemotherapy including 40% with serous and 2% with clear cell histology. An overall response rate of 12% was achieved using 40mg/m^2 every 21d; disease stabilization for at least 8 weeks occurred in 60%. Median progression-free and overall survival was 2.9 months and 8.7 months, respectively.[51] Among the various epothilones, patupilone was chosen for most of our work because our institution previously participated in EPO2303, a multi-center phase III trial of patupilone versus pegylated liposomal doxorubicin in taxane- and/or platinum-refractory/resistant recurrent ovarian cancer. In our study, an in vitro comparison alongside of the sensitivity of USC cell lines to patupilone and ixabepilone versus paclitaxel demonstrated a consistent higher sensitivity of USC to Epothilones. Patupilone however had significantly higher potency against USC cell lines when compared

to ixabepilone. These results are consistent with published reports comparing patupilone to ixabepilone activity against human colorectal cell lines.[52]

In conclusion, we have demonstrated that tubulin-β-III overexpression in USC may discriminate poor prognosis among patients with advanced disease, serves as a marker for sensitivity to patupilone, and may contribute to paclitaxel resistance. IHC applied to surgical specimens may be a reliable adjunct to identify tumors with overexpression of tubulin-β-III, and accordingly the subset of individuals at risk of poor outcome in the setting of platinum/taxane chemotherapy who may instead respond to patupilone. Epothilones warrant further clinical investigation in the treatment of these aggressive, chemoresistant tumors, especially in the setting of recurrent or persistent disease and high tubulin-β-III expression.

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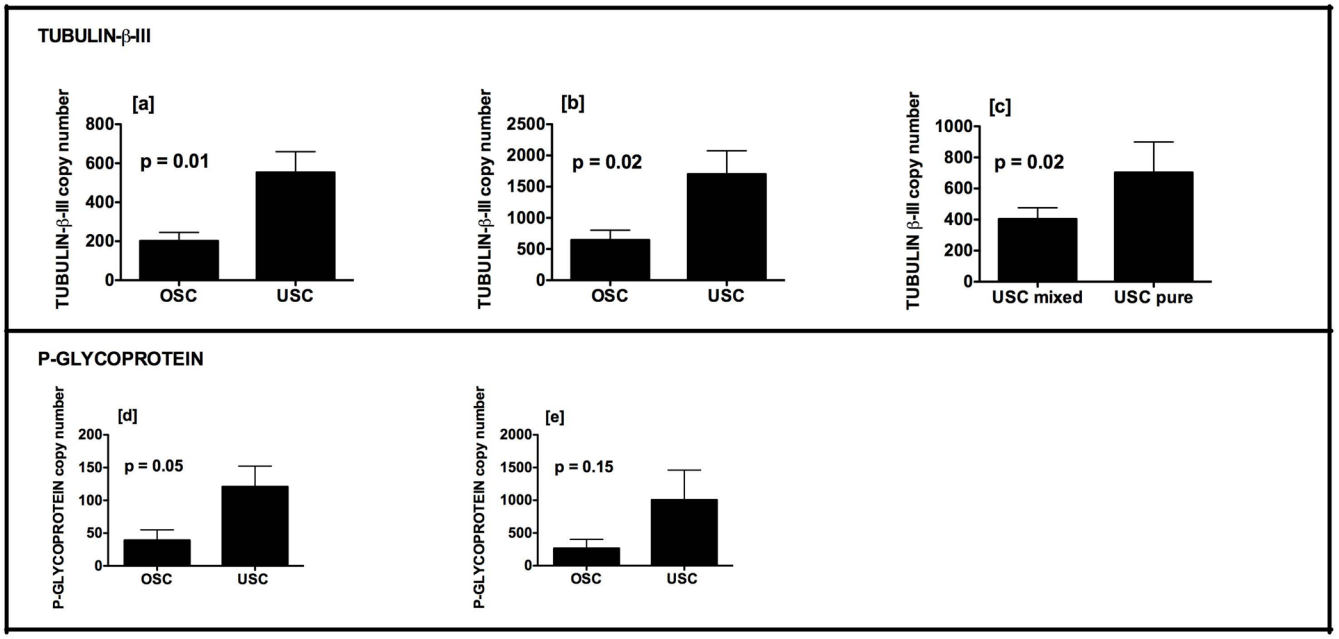

Figure 1.

Expression of tubulin-β-III mRNA (y-axis) is greater for USC relative to OSC in **[a]** fresh-frozen tissue and **[b]** chemo-naive cell lines. Among fresh-frozen tissues, expression is higher in pure compared to mixed serous histology **[c]**. USC exhibit a trend towards overexpression of p-glycoprotein in fresh-frozen tissues **[d]** and cell lines **[e]**. Mean ± SEM are shown.

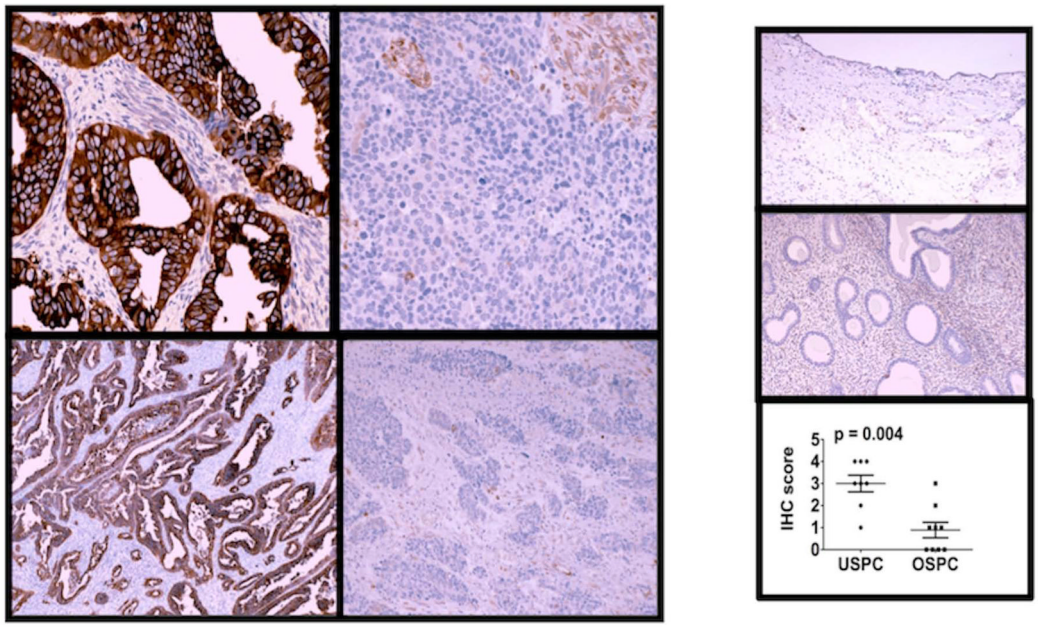

Figure 2.

Immunohistochemistry correlates with tissue expression determined by qRT-PCR. Representative slides are shown at original magnification for USC (score: 4+, **left**) and OSC (score: 0, **right**) at 400x (**top**) and 100x (**bottom**). Corresponding qRT-PCR copy numbers for these specimens are 685.01 and 134.67, respectively. Normal ovary (**inset-top**) and endometrium (**inset-middle**) do not express tubulin-β-III as shown at 100x. Median IHC scores are higher among USC compared to OSC (p=0.004) (**inset-bottom**).

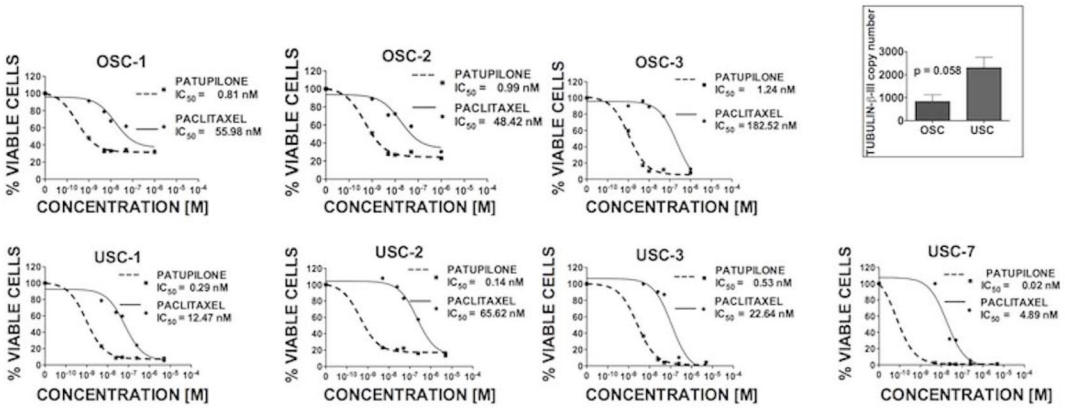

Figure 3.

USC cell lines are highly sensitive to patupilone. Representative dose-response curves and IC_{50} values as determined by flow cytometry after exposure to drug for 72 hours in OSC (**top**) and USC (**bottom**) cell lines are shown. IC_{50} values (mean ± SEM) for patupilone among OSC and USC cell lines are 1.01 ± 0.12 and 0.25 ± 0.11 nM, respectively ($p = 0.006$). There was no statistical difference between IC_{50} values for paclitaxel or p-glycoprotein expression within the cells lines shown. Tubulin-β-III copy number was higher among USC compared to OSC with a trend towards statistical significance (**inset**).

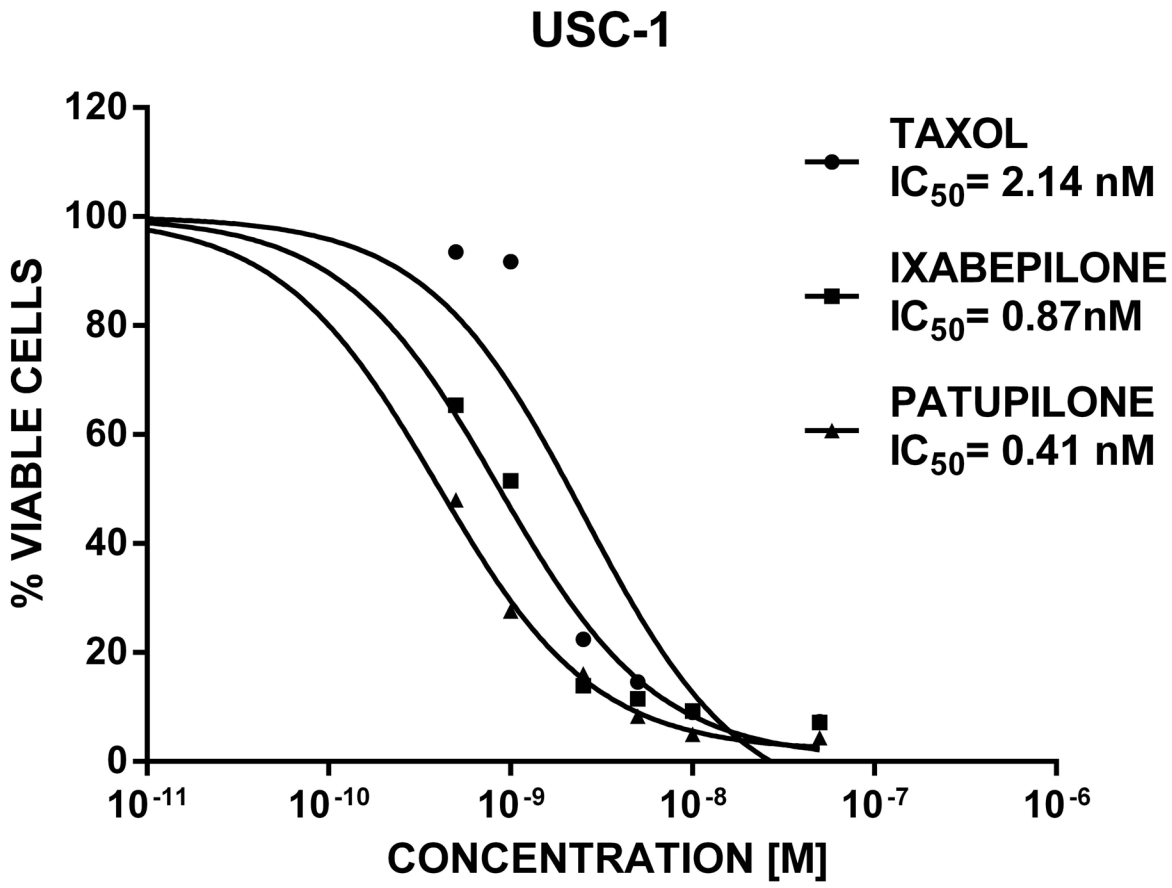

Figure 4.

USC cell lines are significantly more sensitive to epothilones when compared to paclitaxel. Representative dose-response curves and IC50 values as determined by flow cytometry after exposure to patupilone, ixabepilone and paclitaxel for 72 hours in USC-1 cell line are shown. IC50 values (mean) for patupilone, ixabepilone and paclitaxel are 0.41, 0.87 and 2.14 nM, respectively (paclitaxel vs patupilone: $p = 0.003$, paclitaxel vs ixabepilone: $p = 0.02$; patupilone vs ixabepilone: $p = 0.02$).

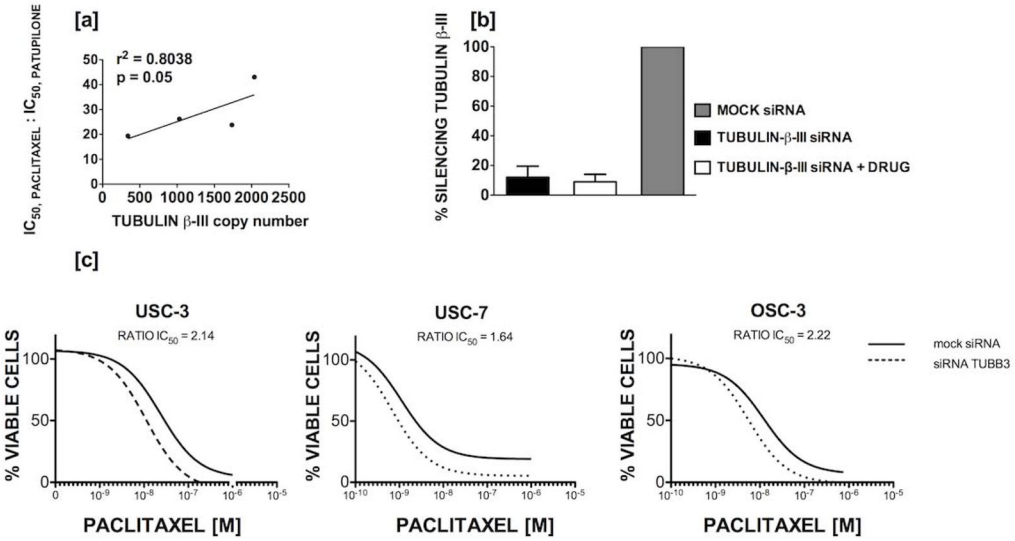

Figure 5.

[a] Tubulin-β-III copy number correlates with paclitaxel resistance and sensitivity to patupilone among uterine serous papillary carcinomas. The ratio of $IC_{50, paclitaxel}$ to $IC_{50, patupilone}$ increases (y-axis) with higher tubulin-β-III copy number (x-axis). Data for 4 cell lines is shown. **[b]** siRNA transfection resulted in reductions in tubulin-β-III expression by 83–93% and transfection efficiency was not affected by addition of drug; data for three cell lines with replicates is shown. Knockdown of tubulin-β-III **[c]** but not tubulin-β-II **[d]** increases sensitivity to paclitaxel in both USC and OSC cell lines. Representative dose-response curves are shown for cells treated with mock (solid line) and tubulin-β-III (dotted line) siRNA. **[e]** Knockdown of tubulin-β-III increases sensitivity to patupilone in a representative USC cell line.

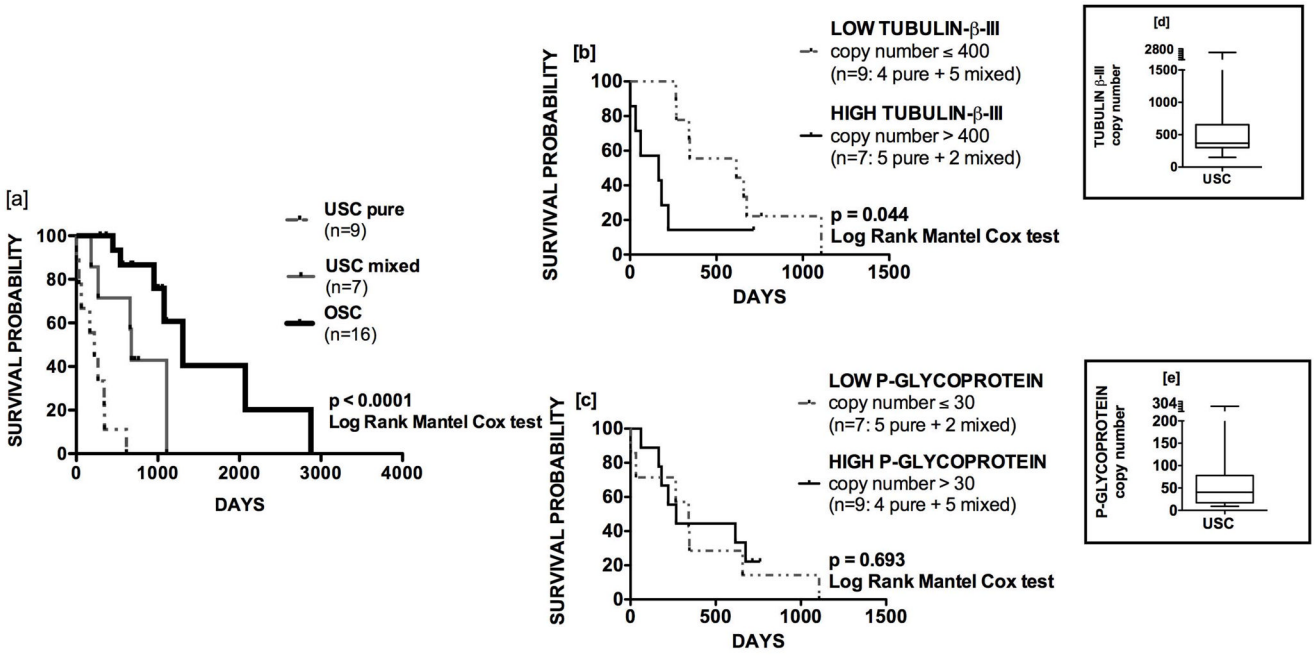

Figure 6.

[a] Median OS of patients with stage III/IV pure USC, mixed USC, and OSC histologies was 221, 675, and 1306 days, respectively ($p<0.0001$). **[b]** Among patients with USC, tubulin-β-III overexpression stratifies poor clinical outcome. Median OS in patients with low and high expression was 615 versus 165 days, respectively ($p=0.044$). **[c]** In this same cohort, p-glycoprotein overexpression had no prognostic utility ($p=0.69$). Box-plots demonstrate median, interquartile range, and overall range of copy number values for **[d]** tubulin-β-III and **[e]** p-glycoprotein.

Table 1a

Source patient characteristics: fresh-frozen tissues.

	USC (n = 28)	%	OSC (n = 20)	%
STAGE				
I	7	25.0	0	0.0
II	2	7.1	2	10.0
III	10	35.7	18	90.0
IV	9	32.1	0	0.0
HISTOLOGY				
Pure	14	50.0	20	100.0
Mixed	14	50.0	0	0.0
RACE				
White	19	67.9	18	90.0
Black	9	32.1	2	10.0
AGE, mean [range] (y)	69.2 [36–88]		60.6 [33–90]	

USC-uterine serous carcinoma; OSC-ovarian serous carcinoma

Table 1b

Source patient characteristics: cell lines.

	RACE	STAGE	CELL LINE HISTOLOGY
OSC-1	WHITE	IIIC	Pure
OSC-2	WHITE	IV	Pure
OSC-3	WHITE	IV	Pure
OSC-6	WHITE	IIIC	Pure
OSC-7	WHITE	IIIC	Pure
OSC-8	WHITE	IC	Pure
OSC-9	WHITE	IIIC	Pure
USC-1	BLACK	IVA	Pure
USC-2	BLACK	IVB	Pure
USC-3	BLACK	IVB	Pure
USC-5	BLACK	IIIC	Pure
USC-7	WHITE	IIC	Pure
USC-12	WHITE	IVB	Pure

USC-uterine serous carcinoma; OSC-ovarian serous carcinoma

Fresh frozen tissue specimens were available for PCR only for USC cell lines 5 and 7.