Gender Influences the Class III and V β-Tubulin Ability to Predict Poor Outcome in Colorectal Cancer

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Abstract

Purpose: Colorectal cancer is one of the deadliest diseases in Western countries. To predict the outcome of therapy, we assessed the role of class III (TUBB3) and class V β-tubulin (TUBB6) as predictive biomarkers.

Experimental Design: Using immunohistochemistry and nanofluidics, the expression of TUBB3 and TUBB6 was assessed in two cohorts of 180 and 134 patients, respectively. The CYP17A1 RS743572 was genotyped to identify GG carriers with enhanced androgen levels. TUBB3 and TUBB6 were investigated in 22 colorectal cancer cell lines in basal conditions and after serum starvation, the latter serving as activator of this prosurvival pathway. To ascertain the role of androgen receptor (AR) in such regulation, we silenced AR and checked TUBB3 and TUBB6 expression and sensitivity to chemotherapy.

Results: There was a link between poor survival, the expression of TUBB3/TUBB6, and AR only in females. Conversely, only in males carriers of the GG phenotype exhibited the worst outcome. Importantly, male cell lines were resistant to serum starvation and exhibited higher levels of TUBB6, thereby suggesting that the pathway is activated by androgens. In female cells this phenomenon was absent. In both genders, AR was the main driver of TUBB3/TUBB6 expression, as constitutive silencing of AR was associated with downregulation of TUBB3/TUBB6 expression and increased sensitivity to oxaliplatin and SN-38.

Conclusions: The involvement of androgens in the TUBB3 pathway opens the way for clinical trials to assess the efficacy of antiandrogens for increasing the efficacy of chemotherapy in male colorectal cancer patients. Clin Cancer Res; 18(10); 2964–75. ©2012 AACR.

Introduction

Colorectal cancer is one of the deadliest diseases known in Western countries (1). Its incidence in the United States is about 140,000 novel cases per year, and it is the third leading cause of cancer-related deaths when men and women are considered separately, second when both genders are combined. It is estimated to have caused about 31,370 deaths (26,580 in men and 24,790 in women) during 2010. Although the number of deaths has gone down in the last 2 decades, including the wider use of early surgery and endoscopic techniques combined with screening campaigns, there is an urgent need to improve outcomes for those patients who are diagnosed at an advanced stage. In advanced colorectal cancer, surgery alone is not sufficient and chemotherapy is needed, but its success will be largely dependent on biologic factors whose nature is still elusive. In fact, the main obstacle to successful treatment of this disease, as in the other solid tumors, is presented by drug resistance. Looking at the mechanisms underlying this phenomenon, several reports have described the close relationship between genetic stability of the tumor and the selective pressure of the tumor microenvironment (2). This explains why many biomarkers capable of predicting the response to chemotherapy are linked to pathways of cell survival that are activated as adaptive response in stressing microenvironments (3). These cell survival pathways also allow cancer cells to survive also in conditions characterized by poor nutrient supply and low oxygen levels. Unfortunately, the same pathways can also be responsible for resistance to radio- and chemotherapy, thus explaining the failure of these therapies using conventional treatments in colorectal cancer as well as in other tumors.

Among these survival pathways, a prominent role is played by the overexpression of class III β-tubulin (TUBB3). Although originally identified as a mechanism of drug resistance to taxanes (4), recent studies have shown that TUBB3 is involved in an adaptive response to low oxygen levels and poor nutrient supply in a growing number of solid tumors (5, 6). This explains the involvement of TUBB3 in drug resistance independent of whether the disease is treated with a regimen that includes a microtubule targeting...
Translational Relevance
Mortality for colorectal cancer is different by gender and higher in males. The molecular basis for such difference is unknown. In this study, we have shown that only in female patients TUBB3 and TUBB6 are predictive biomarkers for the outcome. Activation of the survival pathway mediated by TUBB3 and TUBB6 is mainly driven by microenvironmental stress. Those tumors that grow in the most compelling microenvironments will overexpress both proteins and will exhibit an aggressive behavior. In males this correlation is lost because the survival pathway is constitutively active and not dependent on microenvironmental conditioning. This indicates that in males the disease is intrinsically more aggressive and less sensitive to treatments, thus explaining the higher mortality. The involvement of androgen receptor (AR) in the activation of this survival pathway opens the avenue to clinical trials assessing the efficacy of targeting AR in the management of advanced colorectal cancer in male patients.

Materials and Methods

Cell cultures and reagents
All the 22 colorectal cancer cell lines used in this study were generously donated by John M. Mariadason, Ludwig Institute for Cancer Research, Melbourne, Australia. Cells were grown in a fully humidified atmosphere of 5% CO2/95% air, in MEM (Sigma) medium complemented with FBS and antibiotics. Serum starvation experiments were conducted by plating cells at 25,000 cells/mL for 24 hours. Thereafter, cells were cultured with serum-free medium for 48 hours. Control cells were kept with standard medium. Clonogenic assays were carried out with or without drug treatment for a period of 72 hours. Cells were plated at a density of 450 cells/mL and dishes were stained with Giemsa to count colonies (more than 50 cells) after additional 14 days. R1881 was diluted in ethanol and used at the final concentration of 10 nmol/L. Oxaliplatin and SN-38 were purchased from Tocris and diluted in dimethyl sulfoxide. Growth inhibition effects were carried out as previously described (11), and a comprehensive table showing the IC50 values for all the used cell lines is shown in Supplementary Table SI. All other chemicals were purchased from Sigma-Aldrich if not otherwise specified.

Stable silencing of AR
A construct to stably silencing AR (SiAR) was developed by cloning the following sequence into pRNA-U6.1/Neo vector: GGATCCCGCTCGATCGTATCATTGCATTTCAAGA-GAATGCAATGATACGATCGAGTTTTTTCCAAAAGCTT. The construct was prepared by Genscript. The control vector SiC not targeting any known gene was obtained as previously described using the same vector (12). Colo320 and SW480 cells were transfected using 2 µg DNA per 1 × 106 cells. DNA was electroporated using a Gene PulseR (Bio-Rad) at 160 V, 500 µF. Cells were allowed to recover for 48 hours, after which the medium was removed and replaced with fresh medium containing 1.5 mg/mL G418 (Sigma). Fresh G418 containing medium was added every 3 days until visible colonies appeared. After 3 to 4 passages, cells were collected and qPCR analysis was done to evaluate the mRNA expression of AR.

Quantitative PCR and Western blot
Total RNA was obtained from cultured cells using RNeasy mini kit (Qiagen) according to the manufacturer’s directions. cDNA was prepared using iScript cDNA Synthesis Kit (Bio-Rad). Real-time quantitative PCR was done using the iCycler iQ System (Bio-Rad) and the iQ SYBR Green Supermix (Bio-Rad) in a final volume of 25 µL, starting with a 3-minute template denaturation step at 95°C followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. The following primers were used: TUBB3 forward 5'-CTCCGTCTCCTCTCATAT-3' and reverse 5'-GCTAATAGT TTAATAGTGATCC-3'; TUBB6 forward 5'-CCAAATAGG GAGATAGTTAG-3' and reverse 5'-GCTAATCTATAAGGCCAACCAC-3'; AR forward 5'-TCTAAGAAGTTGGATGG-3'.
and AR reverse 5'-TGGAATAATGCTGAAGAGT-3'. Other primers were previously reported (13). To normalize the possible variation in sample concentration, we used glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as housekeeping control, as previously described (12). The results were analyzed using the REST software.

The proteins were run by SDS/PAGE and transferred to poly (vinylidene fluoride) membranes for incubation with primary antibodies. TUBB3 primary antibody was from Covance (TuJ1 clone), instead TUBB6 was developed in house as previously described and validated (11). AR (441 clone) was from Santa Cruz. HuR served as loading control and was from Santa Cruz. After overnight incubation with primary antibodies in 5% nonfat milk in TBS plus 0.1% Tween-20, Western blots were incubated with horseradish peroxidase–conjugated secondary antibody (Santa Cruz); detection was done using the enhanced chemiluminescence system (GE Healthcare Life Sciences). Images were acquired by the Image Station 4000R PRO (Carestream) and quantified by the Image Pro software.

**Immunohistochemistry**

This retrospective clinical analysis was carried out following Institutional Review Board approval of the protocol study at Danbury Hospital and used 2 clinical settings of 180 (cohort I) and 134 (cohort II) patients. Enrolled patients were untreated previously and at the first surgery. Immunostaining was done on 3-μm tissue sections mounted on poly-L-lysine–coated slides and dried at 37°C wave oven heating in 1 mmol/L citrate buffer pH 6.0. After the slides were deparaffinized in xylene and rehydrated conventionally, the endogenous peroxidase activity was blocked with 3% H2O2 in TBS for 5 minutes. For TUBB3, antigen retrieval procedure was done by microwave oven heating in 1 mmol/L citrate buffer pH 6.0. Sections were incubated with 20% normal goat serum for 30 minutes at room temperature to reduce nonspecific binding, then with the monoclonal anti-TUBB3 TUJ1 antibody (diluted 1:350) in 1% bovine serum albumin (BSA)–PBS. Positive control for TUBB3 was inside the slide represented by the nerves. Results were expressed as the proportion of immunostained tumor cells. For TUBB6 staining sections were incubated with 20% normal rabbit serum for 30 minutes at room temperature to reduce nonspecific binding, then with the in house developed chicken anti-TUBB6 antibody (diluted 1:100) in 1% BSA–PBS. TUBB6 detection was revealed by an anti-chicken secondary antibody diluted 1:1,000 in TBS. Negative control for TUBB6 immunostaining was obtained in normal colon tissue, in which TUBB6 reaction was absent whereas positive control was represented by endothelial cells. Representative slides are shown in Supplementary Fig. S1. The analysis of all tissue sections was done without any prior knowledge of the clinical variables by certified pathologists by means of light microscopy. The proportion of immunostained tumor cells was scored at low magnification (5× objective lens) by evaluating the entire tumor area.

**RNA and DNA extraction from FFPE and colon cancer cell line (cohort II)**

Formalin-fixed, paraffin-embedded (FFPE) samples were cut to 10-μm thickness and 2 tissue slices were put into a 1.5-ml tube. For RNA one milliliter of xylene was added for deparaffinization followed by mixing twice with a high-speed vortex for 3 minutes at room temperature. Total RNA was then automatically extracted with the QIAcube using the Qiagen miRNeasy FFPE kit following manufacturers’ protocols. The RNA from SW837 cells was automatically extracted with the QIAcube using the Qiagen miRNeasy kit following manufacturer’s protocols. RNA quantity and the quality were assessed by Agilent 2100 Bioanalyzer (Agilent Technologies). For DNA, FFPE samples were obtained from the same patients using lymph nodes exempt from disease, or if not available, slices containing minimal or no amount of cancer cells at the pathologist’s review. FFPE samples were cut to 10-μm thickness and 5 tissue slices were put into a 1.5-ml tube. Buffer G2 and Proteinase K were added, followed by an overnight incubation at 56°C in shaking. Genomic DNA was then automatically extracted using the EZ1 instrument with the EZ1 DNA Tissue Kit (Qiagen) following manufacturers’ protocols.

**Gene expression analysis (cohort II)**

Total RNA was reverse transcribed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystem). The 20-μl reverse transcription reaction contained 10 μl of total RNA, 0.8 μl of 100 nmol/L dNTP, 1 μl of RNase inhibitor 20 U/μl, 1 μl of reverse transcriptase (50 U/μl), 2 μl of 10X RT random primers, 2 μl of 10X RT buffer, and 3.2 μl of H2O. The reaction mixture was mixed with RNA and incubated as follows: 25°C for 10 minutes, 37°C for 120 minutes, and then 85°C for 5 minutes. For preamplification of cDNA, we pooled TaqMan Assays at a final concentration of 0.2X for each assay. The preamplification PCR was done at one cycle at 95°C for 10 minutes, 14 cycles at 95°C for 15 seconds, and then 60°C for 4 minutes. After preamplification PCR, the product was diluted 1:5 with DNA Suspension Buffer and stored at −20°C until needed. Preparation of the chip was then carried out following manufacturers’ protocols on a Biomark system (Fluidigm).

**SNP analysis (cohort II)**

Genotyping of CYP17A1 (RS743572) was determined using PCR pyrosequencing with the PyroMark PCR Kit (Qiagen). The PCR was done in a 25-μl mixture containing 20 to 40 ng of genomic DNA template, 2X PyroMark Master Mix, 10X CoralLoad, 10X Primer Set. The PCR began with a denaturation step at 95°C for 15 minutes, followed by 45 cycles of denaturing at 94°C for 30 seconds, annealing at 60°C for 30 seconds, plus extension at 72°C for 30 seconds, and ended with a final elongation step at 72°C for 10 minutes. We used the following primers: forward primer 5’-CGGCGAGGCGACAATAGACAG-3’ and reverse primer 5’-biontin-TGGGCTCCAGGAGAATCTTT-3’. Pyrosequencing of 20 μl of the PCR product immobilized onto the beads was done using the following sequencing primers: 5’-
CAGCAGAATGACAGC-3’. All reagents were purchased from Qiagen. Single-nucleotide polymorphism (SNP) genotype analysis was done using the PyroMark Q24 software.

Statistical analysis
Overall survival was calculated from the date of diagnosis to the date of progression/death or date last seen. Medians and life tables were computed using the product-limit estimate by the Kaplan and Meier method, and the Wilcoxon test was employed only to assess the statistical significance. Statistical analysis was carried out using JMP9 (SAS). Multivariate analysis assessed the clinical role of TUBB3/TUBB6 expression matched with other clinical variables (age, stage, grading, type of tumor, and gender) was done by Cox proportional hazards model and nonparametric testing with the Kruskal–Wallis test. To test correlation, multivariate analysis was carried out between quantitative gene expression and protein quantification results using pairwise correlation and Pearson test. To test difference in the expression between groups, TUBB3 and TUBB6 data were compared with the Kruskal–Wallis test, as data distribution was assumed not normal and required nonparametric test.

Results
Immunohistochemical analysis of TUBB3 and TUBB6 expression in a cohort of 180 colorectal cancer patients
Characterization of the anti-TUBB6 antibody used in this study has been reported previously (11). To get information on the role of these antigens as predictive biomarkers, we carried out a retrospective analysis in paraffin-embedded samples from 180 colorectal cancer patients. The main features of the clinical setting are reported in Supplementary Table SII. In keeping with previous findings (14), the most powerful prognostic factor for disease outcome was the stage of disease. Risk of death at 5 years (60 months) was 100% in patients with stage 4 (Fig. 1A). With regard to TUBB3 and TUBB6 immunostaining, median expression value for the 2 antigens was 40% and 67%, respectively. As previously reported (15), the median served as the cutoff value to identify negative and positive groups of patients. The rationale to use median was based on the fact that these proteins exhibit a bimodal expression with the vast majority closed to the values of 0% and 100%. In a similar distribution, median is capable of stratifying reliably patients with high and low level of expression. Follow-up data were available for 147 patients. Median follow-up was 36 months and the mortality rate differed by gender [39% (32 of 82) and 20% (13 of 65), for males and females, respectively]. Four groups were categorized according to TUBB3 and TUBB6 immunostaining. Groups 1, 2, 3, and 4 were TUBB3+/TUBB6+, TUBB3+/TUBB6-, TUBB3-/TUBB6+, and TUBB3-/TUBB6-, respectively. There was a tendency to coexpress the 2 antigens in 60 and 59 patients belonging to groups 1 and 4, respectively, thereby making statistically significant the probability of coexpression (Fig. 1B, Pearson χ² = 18.7, P < 0.001). Kaplan–Meier curves for all 4 groups are shown in Fig. 1C and no statistically significant differences were appreciated in the patient
A level of \( P \) the presence of metastasis in females (Wilcoxon/Kruskal Wallis test) between the expression of both TUBB3 and TUBB6 and age groups, histotype, and tumor–node–metastasis values. A statistically significant correlation was noticed only with the expression of TUBB3 and TUBB6 in both genders. To find an explanation for these findings, we carried out a multivariate analysis to find clinical factors correlated with the expression of TUBB3 and TUBB6 in both genders. We assessed as variables by gender grading, clinical stage, age groups, histotype, and tumor–node–metastasis values. A statistically significant correlation was noticed only between the expression of both TUBB3 and TUBB6 and the presence of metastasis in females (Wilcoxon/Kruskal Wallis test \( P \) value = 0.0073 and 0.0084 for TUBB3 and TUBB6, respectively; Fig. 3A and B), but not in males (Wilcoxon/Kruskal Wallis test \( P \) value = 0.67 and 0.73 for TUBB3 and TUBB6, respectively, Fig. 3C and D). This finding suggested that expression of TUBB3 and TUBB6 is associated with events leading to metastasis only in females, thereby indicating gender-specific regulation of the expression of both TUBB3 and TUBB6.

**TUBB3 and TUBB6 expression in 22 colorectal cancer cell lines**

To gain insight into the gender-specific regulation of this survival pathway, we used a panel of 22 colorectal cancer cell lines to analyze the expression of both TUBB3 and TUBB6 in basal conditions and after serum starvation, which is a stressor capable of activating the TUBB3 pathway. Nine cell lines came from female patients (WiDR, HT29, LS-174, SW48, RKO, CO115, SW403, Colo-320, and KM12), whereas 13 were from males (HCT116, SW480, SW837, SW116, SK-CO-1, DLD1, HCT15, LoVo, Colo201, Colo205, SW620, T84, and Caco2). This analysis was done at the gene and protein level. In basal conditions in females, there was a statistically significant correlation between the expression of TUBB3 and TUBB6 (\( R = 0.8806, P = 0.0017 \)). This correlation was not present in male cancer cell lines. When pooled together, the expression of TUBB3 did not reveal any statistical significance between cell lines from males (mean 11.6) and females (11.3), whereas at the gene level TUBB6 expression was higher in males (mean 0.4 ± 0.55) than in females (mean 0.18 ± 0.37, \( P = 0.0488 \)). Subjection to serum starvation was always able to induce a TUBB3 increase at the gene level in cell lines from females (Fig. 4). Compared with the control kept in serum-supplemented medium, the mean increase in TUBB3 for female cell lines was 1.99 ± 1.11 versus 1.07 ± 0.43 recorded in male cell lines (Fig. 4A), and the difference was statistically significant (\( P = 0.0033 \)). TUBB6 also (Fig. 4B) exhibited a tendency to increase in female cell lines upon serum starvation (mean 1.59 ± 0.96) compared with males (mean 1.03 ± 0.30), but the difference was not statistically significant (\( P = 0.109 \)). At the protein level, we revealed the same trend. TUBB3 expression (Fig. 4C) increased significantly upon serum starvation in female cell lines (mean 1.95 ± 1.54), whereas in male cells remained essentially stable (mean 1.09 ± 0.42) and again the difference was statistically significant (\( P = 0.003 \)). At the protein level, we noticed an increased expression of TUBB6 (Fig. 4D) in female cell lines (mean 1.28 ± 0.47) as compared with male cell lines (mean 0.89 ± 0.25), but the difference was not significant. Activation of the survival pathway mirrored the sensitivity to serum starvation. In male cell lines, featured by higher levels of TUBB6, we observed a relative resistance to serum starvation with the number of live cells (assuming 1 as control) after 72 hours having a mean value of 0.79 ± 0.68, whereas in female cell lines the mean value was 0.36 ± 0.16. The statistical significance of these results was borderline (\( P = 0.056 \)).
The functional connection between AR and TUBB3/TUBB6

Altogether these findings seem to indicate that the TUBB3/TUBB6 pathway is inducible only in cell lines coming from females, whereas in males the pathway seems to be activated independent of the serum starvation treatment, as shown by constitutively high TUBB6 expression levels and relative resistance to serum starvation. This hypothesis is supported by gene expression analysis of AR in the panel of cell lines. Indeed, in female cell lines, there was a strong correlation between AR expression and TUBB3 (r = 0.893; P = 0.0012) and TUBB6 (r = 0.9858; P < 0.0001), whereas in male cell lines there was no correlation between TUBB3 and AR and a borderline significant connection between TUBB6 and AR (r = 0.4978; P = 0.052). AR signaling can be ligand dependent (canonical) and ligand independent (noncanonical). The first case applies mainly to males who are exposed to testicular androgens, whereas the second may be applicable to both genders. Importantly, canonical signaling may occur also in females with high levels of circulating androgens. To assess whether androgens are capable to activate this pathway we selected 2 cell lines with low TUBB3/TUBB6 expression, one coming from a male patient (LoVo) and the other from a female (RKO). Cells were cultured in the presence of R1881, a nonaromatizable androgen homolog of the testicular androgens. Cells were cultured for 72 hours and the levels of TUBB3 and TUBB6 assessed at the gene and protein level. Results are shown in the Supplementary Fig. SII. If in LoVo cells there was a modest increase of TUBB3 and TUBB6, in RKO cells R1881 treatment did not affect TUBB3/TUBB6 expression, thus suggesting that canonical signaling is possible only in cell lines originating from males. To switch off both signaling pathways (canonical and noncanonical), we designed a specific strategy to knock down the level of AR. Using this approach we addressed the hypothesis that AR signaling is related to the TUBB3/TUBB6 pathway in both genders. As cellular models, we chose the cells with the highest expression of AR and the putative target genes TUBB3/TUBB6 in the male (SW480) and female subset (COLO-320). As additional models, we successfully silenced also SW837, SW48, and WiDr cells. Concomitantly, we prepared control cells in which a scrambling sequence not targeting any gene was cloned. The 2 constructs were named SiAR and SiC, respectively. To check whether silencing of AR was associated with any modulation in the TUBB3 and TUBB6 expression, we conducted a qPCR analysis of the 3 genes. AR silencing was associated with both TUBB3 and TUBB6 downregulation (Fig. 5A and B), confirming that AR is involved upstream in the regulation of the 2 β-tubulin isotypes in these 5 cellular models. The silencing approach was attempted also in the additional 17 cell lines. Although in these cells we were unable to obtain the AR silencing, we did not observe any relevant change in terms of TUBB3/TUBB6 expression (data not shown). In parallel with the silencing of TUBB3/TUBB6, we also measured the expression of PIM1, a protein kinase that is intimately bound to the TUBB3 function (13). Also this gene exhibited the same trend of downregulation. Most importantly, when drug sensitivity was assessed in the 5 cell lines, there was a sensitization to both oxaliplatin and SN-38 (Fig. 5C and D).
If silencing targets both canonical and noncanonical AR signaling, treatment with pure antiandrogens interferes only with the canonical one. Thereafter, we treated SW480 cells and COLO-320 with the same drugs with and without the antiandrogen bicalutamide. A representative clonogenic assay is shown in Fig. 6A, whereas the mean values for 2 independent experiments are shown in Fig. 6B. If bicalutamide increased the effect of chemotherapy in SW480 cells, the same phenomenon was not noticed in COLO-320 cells. This pointed out that antiandrogen could be effective in the presence of a canonical AR signaling but not when the noncanonical signaling is prevalent. Canonical signaling requires the expression of the long isoform of AR containing the ligand binding domain, whereas noncanonical is mainly related to shortest AR isoforms devoid of the C-terminus and encoding for a protein incapable to bind testicular androgens (16). In keeping with this hypothesis, COLO-320 cells originated from a female patient and it was the cell line with the highest expression of the short AR-V7 isoform (Supplementary Table SIII), a factor that is capable of conferring resistance to antiandrogens in prostate cancer cell lines (16) through androgen-independent noncanonical signaling.

Multidimensional analysis of TUBB3/TUBB6 expression, AR expression, and SNP CYP17A1 RS743572 in an independent cohort of additional 134 colorectal cancer patients

The above presented data suggested us a model in which there is a difference in the regulation of TUBB3/TUBB6 by gender. In males the TUBB3/TUBB6 pathway is constitutively activated by testicular androgens through the activity of a canonical signaling, whereas in females it seems prevalent an activity connected to noncanonical signaling and directly linked to the expression levels of AR.

We took advantage from the fact that we noticed a significant correlation between gene and protein levels in the above analyzed panel of cell lines for both TUBB3 ($r = 0.785; P < 0.0001$) and TUBB6 ($r = 0.471; P = 0.03$). To have the possibility to carry out intrasample measures of TUBB3/TUBB6 and AR, we used a nanofluidic genetic analyzer and a chip 48.48 array. Analysis was conducted on an independent additional cohort of 134 colorectal cancer patients (cohort II). Clinical features are summarized in Supplementary Table SIV. Analysis was assessed at the gene level with GAPDH as housekeeping. All the results were normalized with the expression levels measured in the
same chip in SW837 cells (\(= 1\)). As criteria to set the cutoff, we used the same approach described above, and patients were categorized as positive if the expression value exceeded their median. As depicted in Fig. 7A and in keeping with the results obtained at the protein level in the cohort I, female patients expressing TUBB3/TUBB6 exhibited the worst outcome as compared with the other patients. This difference was completely absent in males. To avoid any possible interference due to the cutoff procedure, we also analyzed the gene expression values as continuous variable using the Cox’s proportional hazard method. To carry out this analysis, the obtained values for TUBB3 and TUBB6 were summed and the analysis done by gender. In males, this analysis revealed that TUBB3/TUBB6 values were not a predictor of the outcome (\(\chi^2 = 0.11, P = 0.74\)). On the other hand in females the same analysis showed that TUBB3/TUBB6 values were capable to act as predictor also if used as continuous variable (\(\chi^2 = 4.8, P = 0.028\)).

In the same chips we also analyzed the expression of AR. In keeping with our hypothesis, high expression levels of AR were correlated with a poor outcome in females, but this correlation was completely missing in males (Fig. 7B). To link the outcome to the levels of androgens, in the same clinical cohort we phenotyped through pyrosequencing, the CYP17A1 SNP. CYP17A1 is involved in the activity of taxanes which in turn induce tubulin polymerization (4). This study began by challenging this traditional hypothesis in female patients. Patients whose tumors expressed high levels of TUBB3 and TUBB6 exhibited the worst outcomes in 2 independent cohorts of colorectal cancer patients. In females the expression of both TUBB3 carriers were featured by the worst outcome. These results strongly support the hypothesis that androgens sustain biologic aggressiveness in male colorectal cancer patients through canonical signaling, whereas in female the aggressiveness seems mediated by the levels of AR, possibly through an activity independent on androgen levels.

Discussion

TUBB3 is a prognostic biomarker in several solid malignancies including ovary, lung, stomach, pancreas, and others (18). To our knowledge this is the first article addressing the role of TUBB3 in colorectal cancer. Recent findings support the notion that the TUBB3 pathway is an adaptive response to exposure to microenvironmental stressors, such as hypoxia and poor nutrient supply (5, 6). For this reason TUBB3 is a biomarker of biologic aggressiveness and the tendency toward metastasis (7). In contrast with this view, the traditional hypothesis links TUBB3 expression to resistance to microtubule interacting drugs, pure and simple, and attributes its role owing to the fact that TUBB3 enhances the depolymerization of microtubules, thus counteracting the activity of taxanes which in turn induce tubulin polymerization (4). This study began by challenging this traditional hypothesis and we chose colorectal cancer because it is a disease that is not treated with microtubule targeting agents in the clinical setting. Our results confirmed our hypothesis in female patients. Patients whose tumors expressed high levels of TUBB3 and TUBB6 exhibited the worst outcomes in 2 independent cohorts of colorectal cancer patients. In females the expression of both TUBB3
and TUBB6 was higher in patients with metastatic disease. Therefore in females, the same pathway that we previously reported in ovarian cancer (6, 15, 19) is implicated. On the other hand, if these 2 biomarkers are not expressed, the disease seems less aggressive and the outcome better. In males the scenario was completely different and there was no relationship between the expression of TUBB3/TUBB6 and the outcome in both clinical cohorts. How is it possible to explain this finding? We used a panel of 22 colorectal cancer cell lines to gain insight into this gender difference in colorectal cancer. We used 13 cell lines from males and 9 from females. In terms of TUBB3 expression, there was no difference in expression at the gene and protein level. Indeed, a statistically significant difference in expression levels in males and females was present only for TUBB6, with the male cell lines expressing more of this factor. When cells were exposed to a stressing stimulus such as serum starvation, the TUBB3/TUBB6 pathway was induced in female cell lines, whereas in males this pathway seemed constitutively active and independent of exposure to a conditioning microenvironment. Although our data are weakened by the fact that we obtained a successful silencing

Figure 6. A, representative picture of the clonogenic dishes at a cell density of 450 cells per dish in SW480 cells treated with oxaliplatin with or without bicalutamide (20 μmol/L). B, line chart showing the effects of bicalutamide on clonogenic assays done with or without bicalutamide in the presence of oxaliplatin and SN38 in SW480 (top) and Colo320 (bottom) cells. Each data point and error bar corresponds to the mean and SD of 2 independent experiments. Oxaliplatin and SN38 effects were enhanced by bicalutamide in SW480 but not in Colo320 cells.
only in 5 of the 22 analyzed cell lines, this idea is supported by the relationship between AR and both TUBB3/TUBB6 expression we noticed both in cells and in patients. AR seems capable of orchestrating and regulating the expression of TUBB3, as reported recently in relation to murine Sertoli cells (20). Remarkably, stable AR silencing in cell lines was capable of decreasing TUBB3/TUBB6 expression in 5 cell lines. In this context, we feel that it is extremely important what we discovered through the genotyping of CYP17A1. This enzyme mediates the steroid 17α-hydroxylase and 17,20 lyase activities at key points in testosterone biosynthesis in the gonads and adrenal glands (21), and a drug targeting this gene (abiraterone) is now in clinical use for the treatment of prostate cancer because of its activity in decreasing the circulating levels of testosterone. Previous studies implicated that patients carrying the G phenotype at RS743572 at the 5'-untranslated region of CYP17A1 have an additional binding site for the Sp-1 transcription factor, which leads to increased transcription of the gene and enhanced levels of circulating steroids (22). This finding was questioned by later studies reporting that the same SNP was not associated with increased circulating steroid levels (23, 24). The conclusion of these studies may be biased by the fact that androgen levels exhibit a high variability, related also to environmental factors such as diet, circadian rhythm, and physical/sexual activity (25, 26). Therefore, it is possible that an effect related to the genotype is masked by these confounding factors. At support of this interpretation, male patients carrying the GG allele at RS743572 have indirect signs of high androgen activity as measured on the diameter of the femoral head and increased bone density (17, 27). In this study, for the first time we reported that the same allele is responsible for poor prognosis in male colorectal cancer patients, strongly suggesting that in males circulating steroids can be linked to the worst outcome. As additional support, we were able to get a remarkable sensitization to chemotherapy using the antiandrogen bicalutamide in SW480 cells. This finding opens the way for a novel treatment aimed at inhibiting this pathway of drug resistance and biologic aggressiveness, not by targeting TUBB3 directly but through the inhibition of the androgen activity onto this survival pathway.

Figure 7. Kaplan–Maier analysis of the clinical cohort II of 134 patients according to the analysis of TUBB3/TUBB6 expression (A), AR expression (B), and CYP17A1 genotyping (RS743572; C). On the left and right columns, the data are reported for females and males, respectively. In A, the blue line identifies the patients with high expression of TUBB3/TUBB6 and the red one the other categories pooled together. In B, the blue and red lines identify patients with high and low AR expression, respectively. In C, blue and red lines identify patients with GG phenotype and AG/AA phenotype at RS743572 of the CYP17A1 gene. Statistical significance is reported inside the graphs when below 0.05 (Wilcoxon test).
commonly noticed not only in colorectal cancer but also in other tissues such as lung, pancreas, bladder, and kidney. In some cases such differences may be related to the fact that occupational exposure is more commonly found in males. In a old report it was found that androgens are involved in chemically induced colorectal cancer (29). In this article, we provided an additional explanation and a by-gender difference directly related to the enhancement of aggressiveness exerted through AR signaling. Our findings support the notion that the status of the survival pathway dependent on TUBB3 is activated by the microenvironment in females, whereas it is constitutively active in males. Male colorectal cancer cell lines are more resistant to serum starvation and constitutively express high levels of TUBB6. Female cell lines, in contrast, are able to overexpress TUBB3 as a response to a stressing microenvironment and with a strong correlation between AR levels. This key difference could be responsible for the fact that TUBB3 behaves as a prognostic biomarker only in female patients. As a result of this observation, male colorectal cancer patients could receive antiandrogen treatment to enhance the efficacy of chemotherapy, as strongly suggested by the results obtained in SW480 cells, a cell line derived from a male patient in which the antiandrogen bicalutamide was effective in enhancing chemotherapy effects. However, as shown in our work on Colo320 cells, bicalutamide can be effective only if AR signaling is canonical and androgen dependent, as the shortest AR isoforms are capable of circumventing antiandrogen through noncanonical signaling, a phenomenon that is probably also present in female patients. It is worth noting that Colo320, the cell line with the highest level of AR and TUBB3 in this study, is derived from a female patient, in whom this noncanonical signaling is well established and generate a complete resistance to bicalutamide.

In summary, this work suggests that TUBB3 predictive ability of poor outcome is influenced by gender in colorectal cancer. Along with TUBB3, TUBB6 cooperates in this pathway, although its exact role needs to be further clarified.

Unexpectedly, we discovered that such pathway is strongly influenced by androgens and AR. Although these findings need to be further confirmed in additional cellular models and clinical studies, these results seem to indicate that reduction of circulating androgens may increase the effectiveness of chemotherapy in male colorectal cancer patients.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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