Gender influences the Class III and V β-tubulin ability to predict poor outcome in colorectal cancer

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Running Title: Linking TUBB3 and gender in colorectal cancer

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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 demonstrated 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 which grow in the most compelling microenvironments will overexpress both proteins and will exhibit an aggressive behavior. In male this correlation is lost since the survival pathway is constitutively active and not dependent on microenvironmental conditioning. This makes that in males the disease is intrinsically more aggressive and less sensitive to treatments, thus explaining the higher mortality. The involvement of AR (androgen receptor) 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.
Abstract

Purpose: Colorectal cancer is one of the deadliest diseases in Western countries. In order 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 (SS), 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 SS 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, since 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 anti-androgens for increasing the efficacy of chemotherapy in male colorectal cancer patients.
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 51,370 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 biological 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 to predict the response to chemotherapy are linked to pathways of cell survival which 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 demonstrated 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 agent or not (7). Looking at the protein sequence, one possible explanation for TUBB3 function is that this protein is able to form a cysteine–disulphide bridge with other proteins involved in the adaptive response to oxidative stress (8), thereby allowing these proteins’ incorporation into microtubules. As compared to the constitutive isotypes, a peculiar feature of TUBB3 is the switch cysteine/serine and alanine/cysteine at positions 124 and 239, respectively. The regions around 124 and 239 are resistant and sensitive to oxidation, respectively (9). Therefore cysteine 124 can form stable complexes even in conditions of oxidative stress, while this does not occur at position 239. Remarkably, this feature is shared also with class V β-tubulin isotype (TUBB6)(10), a protein for which to the best of our knowledge no clinical studies have been reported either in colorectal or other tumors. Translational studies on TUBB6 are lacking due to the absence of a specific commercial antibody available for its analysis in paraffin-embedded specimens.

This work investigates the expression of TUBB3 and TUBB6 in colorectal cancer as a proof of concept that the link between TUBB3 and colorectal cancer is a purely prognostic factor related to biological aggressiveness, rather a marker of sensitivity for a microtubule-targeting agent. The results reveal that both TUBB3 and TUBB6 proteins are overexpressed in cancer specimens with poor outcomes, but surprisingly this phenomenon is restricted to female patients, with a clear-cut difference in the behavior of this factor between genders. Further analysis of this phenomenon reveals that TUBB3 activation is related to the expression of androgen receptors (AR) and that the combination of chemotherapy with anti-androgens can be useful in the clinical management of male colorectal cancer 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 fetal bovine serum and antibiotics. Serum starvation experiments were performed by plating cells at 25,000 cells/ml for 24 h. Thereafter, cells were cultured with serum-free medium for 48 h. Control cells were kept with standard medium. Clonogenic assays were performed with or without drug treatment for a period of 72 h. 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 10nM/L. Oxaliplatin and SN-38 were purchased from Tocris and diluted in DMSO. Growth inhibition effects were performed as previously described (11) and a comprehensive table showing the IC50 values for all the used cell lines is shown in supplemental Table I. 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:

GGATCCCGCTCGATCGTATCATTGCATTTCAAGAGAATGCAATGATACGATCGAGTTTTTTCCAAAAGCTT . The construct was prepared by Genscript. The control vector SiC not targeting any know 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 h, 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 three to four passages, cells were collected and qPCR analysis was performed 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-min template denaturation step at 95ºC followed by 40 cycles of 15 s at 95ºC and 1 min at 60ºC. The following primers were used: TUBB3 forward 5’-CTCCTGTCTCTGTTAT-3’ and reverse 5’-GCAATAGATTTATTAAGTATCCC-3’; TUBB6 forward 5’-GCAAATTAGGAGGAGTTAG-3’ and reverse 5’-GCATATTCATATAAGGCAACAC-3’; AR forward 5’-TCTCAAGAGTTTGGATGG-3’ and AR reverse 5’-TGGAATAATGCTGAAGAGT-3’. Other primers were previously reported (13). To normalize the possible variation in sample concentration, we used 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 Tris buffered saline plus 0.1% Tween-20, Western blots were incubated with horseradish peroxidase-conjugated secondary antibody (Santa Cruz); detection was performed 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 performed following IRB approval of the protocol study at Danbury Hospital and used two 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 overnight. 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 pH6.0. 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-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% bovine serum albumin-PBS. TUBB6 detection was revealed by an anti-chicken secondary antibody diluted 1:1000 in TBS. Negative control for TUBB6 immunostaining was obtained in normal colon tissue, where TUBB6 reaction was absent while positive control was represented by endothelial cells. Representative slides are shown in supplemental Fig. I. 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)

FFPE samples were cut to 10 μm thickness and two 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 min at room temperature. Total RNA was then automatically extracted with the QIAcube using the Qiagen miRNeasy FFPE kit (Valencia, CA) following manufacturers' protocols. The RNA from SW837 cells was automatically extracted with the QIAcube using the Qiagen miRNeasy kit (Valencia, CA) following manufacturer's protocols. RNA quantity and the quality were assessed by Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). 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 five 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, Valencia, CA) following manufacturers' protocols.

Gene Expression analysis (cohort II)

Total RNA was reverse transcribed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystem, Foster City, CA). The 20 μl reverse transcription reaction contained 10 μl of total RNA, 0.8 μl of 100 nM 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 min, 37°C for 120 min and then 85°C for 5 min. For pre-amplification of cDNA, we pooled TaqMan Assays at a final concentration of 0.2X for each assay. The pre-amplification PCR was performed at one cycle 95°C for 10 min, 14 cycles at 95°C for 15 sec and then 60°C for 4 min. After pre-amplification PCR, the product was diluted 1:5 with DNA Suspension Buffer and stored at -20°C until needed. Preparation of the chip was then performed following manufacturers' protocols on a Biomark system (Fluidigm, South San Francisco, CA).

SNP analysis (cohort II)
Genotyping of CYP17A1 (RS743572) was determined using PCR pyrosequencing with the PyroMark PCR Kit (Qiagen, Valencia, CA). The PCR was performed in a 25 μl mixture containing 20–40 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’- CGGCAGGCAAGATAGACAG- 3’ and reverse primer 5’-biotin- TGGGCTCCAGGAGAATTTT- 3’.

Pyrosequencing of 20 μl of the PCR product immobilized onto the beads was performed using the following sequencing primers: 5’- CAGGCAAGATAGACAGC - 3’. All reagents were purchased from Qiagen (Valencia, CA). SNP genotype analysis was performed 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, gender) was done by Cox proportional hazards model and non parametric testing with the Kruskal Wallis test. In order to test correlation, multivariate analysis was performed between quantitative gene expression and protein quantification results using pairwise correlation and Pearson test. In order to test difference in the expression between groups, TUBB3 and TUBB6 data were compared with the Kruskal Wallis test, since data distribution was assumed not normal and required non parametric 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). In order to get information on the role of these antigens as predictive biomarkers, we performed a retrospective analysis in paraffin embedded samples from 180 colorectal cancer patients. The main features of the clinical setting are reported in supplemental Table II. 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). Concerning 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% (32/82) and 20% (13/65), 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 co-express the two antigens in 60 and 59 patients belonging to groups 1 and 4, respectively, thereby making statistically significant the probability of co-expression (Fig. 1B, Pearson’s $\chi^2=18.7$, $p<0.001$). Kaplan-Meier curves for all 4 groups are shown in Fig. 1C and no statistically significant differences were appreciated in the patient population as a whole among the 4 groups. When stratified by gender the results were different. The behavior of the curves in males was similar to that observed in the general
population without any statistically significant change between the 4 groups (Fig. 2A). In contrast, in females double negative patients (n=18) performed very well and all survived, while mortality in the remaining 3 subsets was about 28% (13/47) (Fig. 2B). The difference between double negative patients as compared with the 3 other groups was statistically significant in females (p value=0.022). In order to find an explanation for these findings, we performed 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 TNM 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-B), but not in males (Wilcoxon/Kruskal Wallis test p value =0.67 and 0.73 for TUBB3 and TUBB6, respectively, Fig. 3C-D). This finding suggests that expression of TUBB3 and TUBB6 is associated with events leading to metastasis only in females, thereby suggesting gender-specific regulation of the expression of both TUBB3 and TUBB6.

TUBB3 and TUBB6 expression in 22 colorectal cancer cell lines

In order 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) while 13 were from males (HCT116, SW480, SW837, SW116, SK-CO-1, DLD1, HCT15, LoVo, Colo201, Colo205, SW620, T84 and CACO2). This analysis was performed 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), while 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 to the control kept in serum-supplemented medium, the mean increase in TUBB3 for female cell lines was 1.99±1.11 vs. 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) as compared to 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), while 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 to 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 h having a mean value of 0.79±0.68 while 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, while in males the pathway seems to be activated independent of the serum starvation treatment, as demonstrated 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), while 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 (non-canonical). The first case applies mainly to males who are exposed to testicular androgens while the second may be applicable to both genders. Importantly, canonical signaling may occur also in females with high levels of circulating androgens. In order to assess if androgens are capable to activate this pathway we selected two 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 non-aromatizable androgen homolog of the testicular androgens. Cells were cultured for 72 h and the levels of TUBB3 and TUBB6 assessed at the gene and protein level. Results are shown in the supplemental Fig II. 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 canonic signaling is possible only in cell lines originating from males. In order to switch off both signaling pathways (canonical and non-canonical) 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. In order to check whether silencing of AR was associated with any modulation in the TUBB3 and TUBB6 expression, we performed a qPCR analysis of the 3 genes. AR-silencing was associated with both TUBB3 and TUBB6 downregulation (Fig. 5A-B), confirming that AR is involved upstream in the regulation of the two β-tubulin isotypes in these five 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 which 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 five cell lines, there was a sensitization to both oxaliplatin and SN-38 (Fig. 5C-D).

If silencing targets both canonical and non-canonical 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 anti-androgen bicalutamide (CDX). A representative clonogenic assay is shown in Fig. 6A, while the mean values for 2 independent experiments are shown in Fig. 6B. If CDX increased the effect of chemotherapy in SW480 cells, the same phenomenon was not noticed in COLO-320 cells. This pointed out that anti-androgen could be effective in the presence of a canonical AR-signaling but not when the non-canonical signaling is prevalent. Canonical signaling requires the expression of the long isoform of AR containing the ligand binding domain, while non-canonical 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 (Tab. III supplemental data), a factor that is capable of conferring resistance to anti-androgens in prostate cancer cell lines (16) through androgen-independent non-canonical 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, while in females it seems prevalent an activity connected to non-canonical 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 ($r=0.471, p=0.03$). In order to have the possibility to perform intra-sample 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 IV. 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. In order 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 perform this analysis the obtained values for TUBB3 and TUBB6 were summed and the analysis performed 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 demonstrated 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). In order to link the outcome to the levels of androgens, in the same clinical cohort we phenotyped, through pyrosequencing, the CYP17A1 RS743572 SNP. CYP17A1 is involved in
the androgen production and we chose this SNP since the male carriers of the GG phenotype are featured by elevated androgen levels and a bigger femoral head as compared to males with the AG and AA phenotype (17). If in females no statistically significant differences were detected between GG and the other phenotypes, in males the GG carriers were featured by the worst outcome. These results strongly support the hypothesis that androgens sustain biological aggressiveness in male colorectal cancer patients through canonical signaling, while 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 report 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 biological 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 since 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 two 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 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, while 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 only in five 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’UTR 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 CDX in SW480 cells. This finding opens the way for a novel treatment aimed at inhibiting this pathway of drug Research.
resistance and biological aggressiveness, not by targeting TUBB3 directly but through the inhibition of the androgen activity onto this survival pathway.

When we compare results between males and females, it seems that in females the expression of AR plays the same driving role, but in a ligand-independent way. Throughout the epidemiological studies there is an increased incidence and mortality in male gender for cancer without a clear explanation of this finding (28). This by gender difference is 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 report 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, while 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 anti-androgen 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 CDX was effective in enhancing chemotherapy effects. However, as demonstrated in our work on Colo320 cells, CDX can be effective only if AR signaling is canonical and androgen-dependent, since the shortest AR isoforms are capable of circumventing anti-androgen through non-canonical 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, where this non-canonical signaling is well established and generate a complete resistance to CDX.

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 seems to indicate that reduction of circulating androgens may increase the effectiveness of chemotherapy in male colorectal cancer patients.
Legends to figures

Fig. 1 A) Kaplan-Meier analysis of the clinical cohort I of 180 patients according to clinical stage. Red and green lines are for stage 1 and 2 patients, while blue and orange lines are for stage 3 and 4. Stage of the disease is the most powerful indicator of outcome of colorectal cancer with highest stage exhibiting a statistically significant worst outcome (Wilcoxon test p<0.001). B) Box-square blot reporting the percentage of single positive and double positive for TUBB3 and TUBB6. Tendency to co-express the two markers was statistically significant (Pearson’s $\chi^2=18.7$, p<0.001). C) Kaplan Meier analysis of the clinical cohort I according to TUBB3/TUBB6 staining. Red and Green lines are TUBB3-/TUBB6- double negative and TUBB3+/TUBB6 +, respectively. Yellow and blue lines are TUBB3-/TUBB6+ and TUBB3+/TUBB6-, respectively. No statistically significant difference is noticeable throughout the groups.

Fig. 2 A and B. Kaplan-Meier analysis of the clinical setting according to TUBB3 staining in males (A) and females (B). Red and Green lines are TUBB3-/TUBB6- and TUBB3+/TUBB6 +, respectively. Yellow and blue lines are TUBB3-/TUBB6+ and TUBB3+/TUBB6-, respectively. No statistically significant difference is noticeable throughout the groups in A, while in B double negative patients exhibited the best outcome with no deaths (0/18), while the highest rate of death was noticed in double positive patients (7/22). Difference was statistically significant at a level of p=0.022, (Wilcoxon test).

Fig. 3 Diamond plots of TUBB6 (A, C) and TUBB3 (B, D) expression values as assessed with immunohistochemistry in female (A and B) and male (C and D) colorectal cancer patients. Values are shown in two different columns reporting data for patients without (left column) or with (right column) metastatic disease. The diamond in each plot represents the 95% confidence interval and the line in the middle of the diamond represents the mean value for each column. In metastatic patients it was noticed an increased expression of both TUBB3 and TUBB6 only in females (Kruskal Wallis test p= 0.0073 and 0.0084 for TUBB3 and TUBB6, respectively). In males no statistically significant difference was noticed.
Fig. 4 Dot plot reporting the fold increase of TUBB3 (A, C) and TUBB6 (B, C) at the gene (A and B) and protein level (C and D). Closed and empty dots refer to cell lines coming from males and females, respectively. The line in each chart indicates the average obtained in the same gender. In E a representative Western blot is shown.

Fig. 5 A) Bar chart reporting qPCR analysis of TUBB3, TUBB6, PIM1 and AR in a panel of SiAR colorectal cancer cells. Bar and error bars refer to mean and SD of two independent assessment performed in triplicate. B) Representative Western Blot for the expression of TUBB3, TUBB6 and AR in SW480 cells (SiC and SiAR). Both A) and B) reveal that silencing of AR is accompanied by decrease of TUBB3 and TUBB6 at the gene and protein level. Similar results were obtained for COLO320. C) and D) depict the growth inhibition effects for oxaliplatin and SN-38, respectively. Bar and error bars are mean and SD of two independent experiments performed in triplicate. A relevant sensitization occurred for both oxaliplatin and SN38 when AR was silenced.

Fig. 6 A Representative picture of the clonogenic dishes at a cell density of 450 cells/dish in SW480 cells treated with Oxaliplatin with or without CDX (20µM). B Line Chart showing the effects of CDX on clonogenic assays performed with or without CDX 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 two independent experiments. Oxaliplatin and SN38 effects were enhanced by CDX in SW480 but not in Colo320 cells.

Fig. 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 all the other categories pooled together. In B the blue and red line 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).
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References

Fig. 1
Fig. 2
Fig. 3
Fig. 5

A

IC50 (nM)

0
0.5
1
15
2
15
25
3
15
3

WDR
SW48
SW837
SW480
Colo320

SiC
SiAR

% of Control (SiC cells)

0%
20%
40%
60%
80%

WDR
SW48
SW837
SW480
Colo320

SiC
SiAR

TUBB3
TUBB6
HuR

B

IC50 (µM)

0
10
20
30
40

WDR
SW48
SW837
SW480
Colo320

SiC
SiAR

SiC
SiAR

C

D
Fig. 6

**A**

<table>
<thead>
<tr>
<th>CDX</th>
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**B**

- **SW480**
  - Control
  - CDX

- **COLO320**
  - Control
  - CDX

- **SN38**
  - Control
  - CDX

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**Results**

- Oxaliplatin at different concentrations shows varying effects on cell viability.
- CDX treatment demonstrates a significant reduction in cell survival compared to controls.

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**Conclusion**

- The combination of CDX with oxaliplatin significantly enhances the therapeutic efficacy in cancer cell lines.

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**Fig. 6**

- Graphs illustrating the impact of oxaliplatin and SN38 on cell viability with and without CDX treatment.
Fig. 7

Surviving

TUBB3/TUBB6

A

p=0.006

Survival (Months)

AR

B

p=0.01

p=0.004

GG AG/AA

CYP17A1 (RS743572)

C

Survival (Months)
Gender influences the Class III and V beta-tubulin ability to predict poor outcome in colorectal cancer

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