Class III β-Tubulin Overexpression Is a Prominent Mechanism of Paclitaxel Resistance in Ovarian Cancer Patients

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ABSTRACT

The vast majority of women with advanced ovarian cancer will ultimately relapse and develop a drug-resistant disease with an overall 5-year survival of <50%. Unfortunately, the mechanisms of drug resistance actually operating in patients are still unknown. To address this issue, in 41 patients affected by advanced ovarian cancer the three main mechanisms of paclitaxel resistance were investigated: overexpression of *MDR-1* gene, point mutations at prominently expressed α-*tubulin* and β-*tubulin* genes and selective alterations in the expression of β-tubulin isotypes. MDR-1 and the β-tubulin isotypes expression were evaluated by semiquantitative and real-time PCR. On the same specimens, quantitative immunohistochemistry was also done in the tumor area. No statistically significant changes of MDR-1 expression were noticed between the sensitive and resistant patients either at the mRNA or protein level. The tubulin mutations for the ubiquitous α-*tubulin* and β-*tubulin* genes were evaluated by automated DNA sequencing, and in all patients, no mutations were detected in both resistant and sensitive cases. With regard to the expression of tubulin isoforms, a statistically significant up-regulation of class III β-tubulin was found in the resistant subset. It is worth noting that this statistically significant increase of the expression of class III β-tubulin was detectable at the mRNA and protein level. By a direct comparison of the three main known mechanisms of paclitaxel resistance, this study indicates that overexpression of class III β-tubulin is the most prominent mechanism of paclitaxel resistance in ovarian cancer.

INTRODUCTION

Despite the success obtained with the introduction of taxanes in first line chemotherapy, advanced ovarian cancer still remains one of the leading cause of death. In fact, although clinical response is achieved at a rate of nearly 80% in first line chemotherapy, the vast majority of patients ultimately relapse and do not successfully respond to further treatments. Several mechanisms have been suggested as responsible for taxane resistance, but a critical reading of such reports consistently reduces this list, as a large part of these mechanisms have been described in cellular systems at taxane concentrations greatly exceeding those reported in patients (1). Furthermore, translational research is very limited in this field and there are few studies addressing this issue in clinical settings, so that the actual mechanisms responsible for drug resistance to taxane-including regimens are still unknown. This major problem needs to be solved in order to permit the development of novel strategies able to overcome drug resistance.

The first mechanism reported as a mediator of taxane resistance was the overexpression of the *MDR-1* gene, encoding for an efflux pump (P-glycoprotein, P-gp) able to efflux taxanes and other cationic drugs, thereby hampering drug retention (2). Such a mechanism is easily obtainable in *in vitro* cultured cancer cells, but only scattered evidence has been provided for its actual occurrence in patients affected by solid tumors (3). Nearly a decade later, after the identification of the paclitaxel binding site in β-tubulin, Giannakakou et al. (4) described point mutation in tubulin at the paclitaxel binding site as being responsible for taxane and epothilone resistance. A strong correlation between tubulin point mutation and resistance to taxane-including therapy was detected in lung cancer patients (5). However, other studies have failed to confirm the presence of tubulin point mutations in resistant patients in lung as well as in advanced ovarian cancer (6–8), so that the role of tubulin point mutations as a mechanism for taxane resistance is still uncertain. An additional mechanism of resistance is the selective overexpression of β-tubulin isotypes. In fact, β-tubulin is encoded by a large multigene family, with the most significant differences at the carboxyl terminus region. Cleveland and Sullivan (9) used this region to devise a classification system to distinguish tubulin isotypes in vertebrates. In humans, at least six possible β-tubulin isotypes have been identified and characterized for tissue expression. Classes I and IVb are constitutively expressed in all tissues, whereas classes III, IVa, and II are typical of brain tissues and are expressed only at low levels with a few exceptions in other tissues. Class VI possesses the lowest degree of homology when compared with the others and is specifically expressed in the hematopoietic compartment. In functional terms, the presence of the class III inhibits the assembly of β-tubulin subunits promoted by paclitaxel (10). In 1997, Kavallaris et al. (11) discovered that paclitaxel-resistant cells exhibit class III β-tubulin (TUBB4) overexpression. It is worth noting that in this paper such a mechanism was also detected at the mRNA level in four resistant advanced ovary patients. However, 4 years later in human

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xenografts, no correlation was shown between the isotype expression and paclitaxel sensitivity in a panel of 12 xenografts in mice showing *in vivo* progression upon paclitaxel treatment. The authors concluded that increased expression of specific β-tubulin isotypes is not a determinant of paclitaxel sensitivity and is probably not involved in the clinical occurrence of drug resistance (12).

The present study was done to directly compare the actual incidence of these three mechanisms of drug resistance in a clinical setting of advanced ovarian cancer patients treated at the first surgery with the standard paclitaxel-including chemotherapy. MDR-1 overexpression and tubulin point mutations were not detected in resistant patients, whereas class III β-tubulin overexpression was found and seems to represent the most important mechanism of drug resistance and a possible target to develop novel strategies in drug-resistant ovarian cancer.

MATERIALS AND METHODS

Patients. The study included 41 ovarian cancer patients admitted to the Department of Obstetrics and Gynecology, Division of Gynecologic Oncology, Catholic University of Rome. The median age was 54 years (range = 41–74). Thirty-one (83%) patients were stage III and six (15%) were stage IV disease. Most of the tumors were serous adenocarcinomas (66%) and showed a poor grade of differentiation (56%). All the patients enrolled for this study did not achieve complete cytoreduction at the first surgery. Gynecologic examination, abdominopelvic ultrasonography, CA-125 assay, and radiological investigations, if necessary, were done monthly to obtain the clinical assessment of response which was recorded according to the WHO criteria (13). In particular, complete and partial response were defined as the disappearance or reduction of disease >50%, respectively. Stable disease or progression of disease were defined as no change/reduction <50% of disease dimensions or increase >25%, respectively. Tumor tissue biopsies were obtained at the first surgery and chemotherapy followed surgery in all cases. Patients underwent six cycles of platinum-paclitaxel containing chemotherapy. In this clinical setting, 28 patients responded to the chemotherapy, whereas the remaining 13 patients progressed despite chemotherapy. As a control, five ovary biopsies taken from noncancer patients were included to assess expression of Mdr-1 and class III β-tubulin in normal ovarian tissue. Written informed consent was acquired from all the patients used in this study.

Semiquantitative RT-PCR Analysis. Total cellular RNA was obtained from cells by using the TRI-REAGENT solution according to the manufacturer's directions (Molecular Research Center, Inc., Cincinnati, OH). Five micrograms RNA per sample were separated on a 1% formaldehyde-agarose gels to assess their integrity. A Gene Amp RNA PCR kit was used for all the reverse transcription-PCR (RT-PCR) reactions which were done in the Gene Amp PCR system 9700 (Applied Biosystems, San Diego, CA). After removal of contaminating chromosomal DNA with DNase I treatment, 1 μg of RNA was reverse transcribed with 25 units of Moloney murine leukemia virus reverse transcriptase. Two microliters of cDNA products were used in each PCR reaction. The sequences of the specific primers for β-tubulin isotypes (14) and Mdr-1 (15) have been previously described. The target cDNAs were coamplified with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control using the following pair of primers: 5′-ACCACCATGGA-GAAGGCTGG-3′ and 5′-CTCAGTGTAGCCCAGGATGC-3′.

All oligonucleotide primers were synthesized by Pharmacia Biotech (Uppsala, Sweden). The PCR reactions were carried out using AmpliTaq Gold polymerase with different cycling parameters and reaction conditions. Class III β-tubulin was amplified in 3 mmol/L $MgCl_2$ and a starting denaturation step of 10 minutes at 95°C followed by 28 cycles of 45 seconds 95°C, 45 seconds at 68°C and 1 minute at 72°C, whereas class I β-tubulin was amplified in 5 mmol/L $MgCl_2$ with a first step of 10 minutes at 95°C followed by 25 cycles of 45 seconds 95°C, 45 seconds at 65°C and 1 minute at 72°C. Mdr-1 was amplified in 5 mmol/L $MgCl_2$ as follows: 10 minutes at 95°C, then 45 seconds at 95°C, 45 seconds at 60°C, 1 minute at 72°C for 31 cycles.

The PCR products were loaded onto 1% agarose gels and stained with Ethidium Bromide. Images of the gels were acquired with a Cohu CCD camera (Cohu, Inc., Poway, CA) and quantification was done with Phoretix 1D (Phoretix International Ltd., Newcastle upon Tyne, United Kingdom). The ratio between the sample RNA to be determined and GAPDH was calculated to normalize for initial variations in sample concentration and as a control for reaction efficiency.

Real-Time Quantitative PCR. A real-time quantitative RT-PCR was done using the iCycler iQ system (Bio-Rad, Hercules, CA) on the same RNA samples analyzed by semiquantitative RT-PCR to assess the expression levels of class III β-tubulin isotype and Mdr-1. cDNA was prepared starting from 1 μg of total RNA using the iScrypt cDNA Synthesis Kit according to the manufacture's instructions. Amplifications were carried out using the following primers (class III forward 5′-GCGAGATGTACGAAGACGAC-3′, reverse 5′-TTTAGACACTGCTGGCTTCG-3′; Mdr-1 forward 5′-GGAAGACATGACCAGGTATGC-3′, reverse 5′-GCCAGGCACGAAAATGAAACC-3′; GAPDH forward 5′-CTGACCTGCCGTCTAGAAA-3′, reverse 5′-CCACCATG-GAGAAGGGTGG-3′) 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. A2780wt and A2780-TAX50 cells were used as control cells in each set of reactions. Features of the control cell lines and methods of cell culture have been previously reported (16). Standard curves were generated using a serial dilution of the initial amount of control cDNA to determine the range of template concentrations, which showed a good linearity and efficiency for the different reactions. Melt curves of the reaction products were also generated to assess the specificity of the measured fluorescence. Samples were run in triplicate and the mean of threshold cycles (C_t) for each specimen was used to obtain the fold change of MDR-1 and class III β-tubulin expression level applying the following equation:

Fold change = $2^{-\Delta(\Delta Ct)}$, where $\Delta C_t = C_{t\ \text{specific gene}} - C_{t\ \text{GAPDH}}$ and $\Delta(\Delta C_t) = \Delta C_{t\ \text{specimen}} - \Delta C_{t\ \text{control}}$ (i.e., A2780TAX50 and A2780wt for MDR-1 and class III β-tubulin, respectively). A fold change equal to 1 represents a sample with an expression level equal to the control cell line. This operation was done using the Excel spreadsheet RelQuant (Bio-Rad).

DNA Sequence Analysis. The same cDNAs which were analyzed to evaluate the expression levels of class I and class III β-tubulin were also utilized for the sequencing of the fourth exon of class I β-tubulin and for the full-length cDNA of K-α1 tubulin.

For β-tubulin, the following primers were utilized both for amplification and sequencing: forward 5′-TGGGCACTCTCC-TAATCAGC-3′ and reverse 5′-AAGGGGCAGTTGAGTAA-GACGG-3′. Additional inner primers were selected to perform the sequence analysis: forward 5′-AACCACCTTGTCTCAGG-CAC-3′ and reverse 5′-CTTGAAGAGCTCCTGGATGG-3′. For the sequencing of K-α1 tubulin isotype, four overlapping sets of primers previously described (17) were also used to amplify the gene in the same group of patients.

RT-PCR reactions were done using the AmpliTaq Gold polymerase (Applied Biosystems) and the efficiency was checked in a 1% agarose gel electrophoresis. cDNA sequence analysis was done using an automated DNA sequencer (ABI Prism 3100, Applied Biosystems) and the BigDye Terminator v3.1 staining kit (Applied Biosystems) according to the manufacturer's instructions. The results were analyzed using the SeqScape v2.0 software package (Applied Biosystems) using as a reference sequence the Genbank accession nos. AF141349 and BC00648, for class I β-tubulin and K-α1 tubulin, respectively.

Quantitative Immunohistochemical Analysis. The expression of MDR-1 and class III β-tubulin was immunohistochemically assessed in the same clinical setting. The Envision-peroxidase system (Dako, Glostrup, Denmark) was used. MDR-1 and class III β-tubulin antigens were retrieved by microwave in 1 mmol/L EDTA (pH 8.0) for 10 minutes. Antibodies (polyclonal anti-class III β-tubulin 1:200, Covance (Princeton, NJ); MDR clone H-241 1:50 (Santa Cruz Biotechnology, Santa Cruz, CA) were used in 1% bovine serum albumin-PBS. Negative control for every experiment was done by replacing the primary antibody with albumin-PBS. Positive control was represented by a section taken from the brain and kidney for class III β-tubulin and MDR-1, respectively. For each specimen, five to seven paraffin embedded sections were randomly selected. To quantify results of the immunoreactions image analysis was done, as previously described (18).

RESULTS

In a cohort of 41 patients, affected by advanced ovarian cancer, the three main mechanism of taxane resistance previously reported "in patients" were investigated (i.e., MDR-1 expression, tubulin point mutations, and overexpression of selected β-tubulin isotypes). Twenty-eight of these patients responded to the first-line chemotherapy, which included paclitaxel, whereas the remaining 13 exhibited drug resistance and progressed despite the treatment. In this clinical setting, chemotherapy followed the first surgery in previously untreated patients and samples were obtained at the first surgery. The same clinical setting was used for MDR-1 expression, tubulin gene sequencing, and assessment of expression of β-tubulin isotypes.

The expression of MDR-1 cDNA was measured using semiquantitative RT-PCR and the results are summarized in Fig. 1*A*. As a control, five biopsies from normal ovary tissues were included. In all the patients, regardless of paclitaxel sensitivity, MDR-1 expression was scarcely detectable, without statistically significant differences between sensitive and resistant patients.

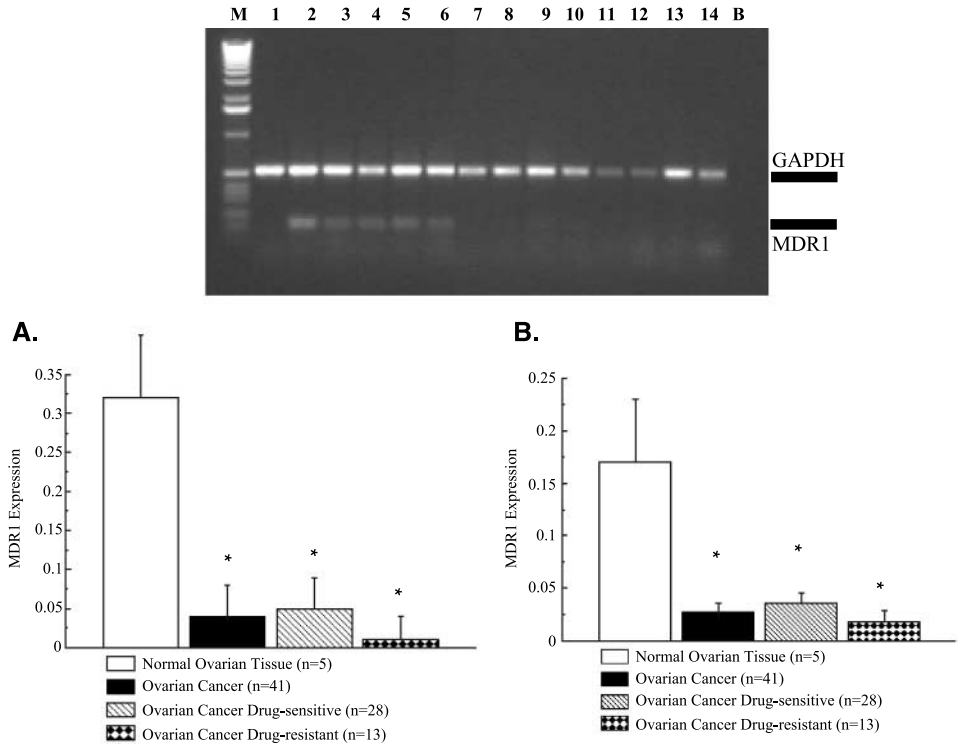

Fig. 1 Semiquantitative RT-PCR for MDR-1. *Top,* images of the agarose-gel electrophoresis of the RT-PCR products. *Lane M,* molecular weights; *lane 1,* negative control (A2780wt cancer cells); *lane 2,* positive control (MDR+ A2780-TAX cells); *lanes 3–6,* normal ovarian biopsies; *lanes 7–10,* drug-sensitive ovarian cancer; *lanes 11–14,* drug-resistant ovarian cancer; *lane B,* water control. *Bottom,* bar chart of MDR-1 expression using semiquantitative RT-PCR in three independent experiments on normal ovarian biopsies, drug-sensitive, and drug-resistant patients (*A*); bar chart of MDR-1 expression on the same samples using real-time PCR (*B*). *Columns,* mean of the three independent experiments; *bars,* SE. *, $P < 0.01$, significant changes with respect to normal ovarian biopsies (Wilcoxon signed rank).

On the other hand, in normal ovary tissue MDR-1 was detectable probably due to the contribution of granulosa cells reportedly expressing MDR-1 (19). This finding suggests that in our clinical setting, drug resistance is not mediated by a modulation in MDR-1 expression in cancer cells. In order to perform a more robust quantitative assay, a real-time PCR was done for the same specimens and results are shown in Fig. 1*B*. Using as reference paclitaxel-resistant MDR+ A2780TAX cells (expression = 1), normal ovarian tissues yielded a mean value of 0.17, whereas sensitive and resistant cases 0.036 and 0.018, respectively. The difference in the expression between normal tissue and cancer is statistically significant ($P < 0.05$), whereas the difference between sensitive and resistant cases did not.

The contribution of tubulin point mutations in inducing drug resistance was ascertained through the DNA sequencing of the tubulin gene. The prominently expressed α-tubulin and β-tubulin isotypes were sequenced, because in cells, made resistant upon *in vitro* treatment, both have been implicated in the drug resistance process. Since both genes are members of a family including several isotypes with a high structural homology, *K-α1 tubulin* (chromosome 12) and *class I β-tubulin* (chromosome 6) genes were chosen, as they represent the isotypes accounting for around 85% to 90% of the total intracellular tubulin pool in the vast majority of somatic cells. K-α1 tubulin was directly sequenced from the full-length cDNA. No missense mutations were detected. Only five single nucleotide polymorphisms (SNP) were noticed: substitution G ⇒ A at nucleotide 572 yielded K164K; this SNP was very common and was found in heterozygosis in 14 patients and in homozygosis in a further four patients; an additional common SNP (substitution A ⇒ G at nucleotide 600 correspondent to P173P) was noticed in 17 patients in heterozygosis and in four patients in homozygosis; an additional SNP (substitution C ⇒ T, Y262Y) was detected in heterozygosis at nucleotide 868 in four patients; substitution T ⇒ C at nucleotide 432 yielded L117L in heterozygosis in one patient; substitution C ⇒ T at nucleotide 792 yielded the SNP S237S in one patient. To our knowledge, SNPs K164K, Y262Y, and S237S are novel and representative electropherograms for these SNP are shown in Fig. 2*A*. P173P and L117L SNPs have been previously reported and are not shown. The distribution of SNP did not vary between sensitive and resistant patients.

Along with K-α1 tubulin, the fourth exon of class I β-tubulin, containing the paclitaxel-binding site, was sequenced at the cDNA level. Also in this case, no mutations were found. Only one polymorphism was present in two patients (substitution G ⇒ A at nucleotide 794 yielding L217L in patients 16 and 28). A representative electropherogram for this SNP is shown in Fig. 2*B*. Taken altogether, these findings show that tubulin point mutations are not involved in paclitaxel resistance in this clinical setting.

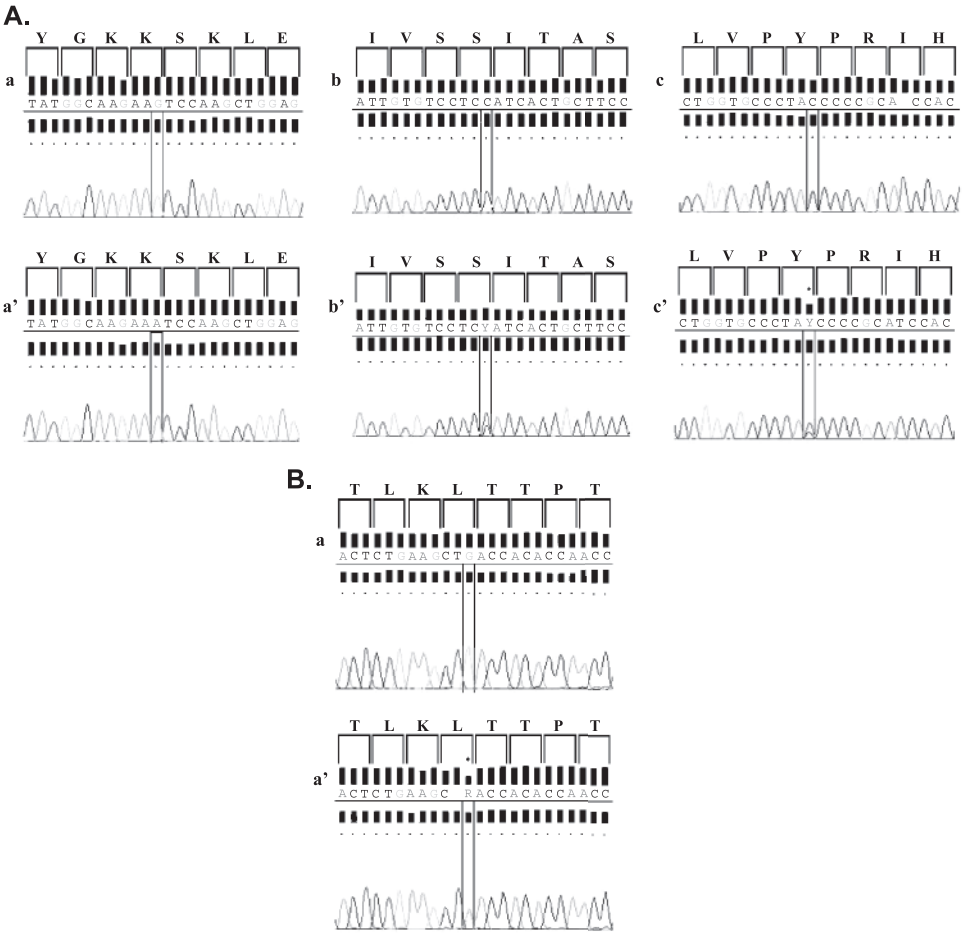

Fig. 2 Representative electropherograms for SNP noticed in *K-α1 tubulin* (*A*) and *class I β-tubulin* gene (*B*) gene. *A, top,* electropherograms of wild-type sequence at loci *164*, *237*, and *262*; *bottom,* electropherograms of SNPs L164L (aag/aaa, homozygosis), S237S (tcc/tct, heterozygosis), and Y262Y (tac/tat, heterozygosis). Reference BC006481.1. *B, top,* electropherogram of wild-type sequence of TUBB at locus *217*; *bottom,* electropherogram of SNP L217L (ctg/cta, heterozygosis). Reference AF141349.

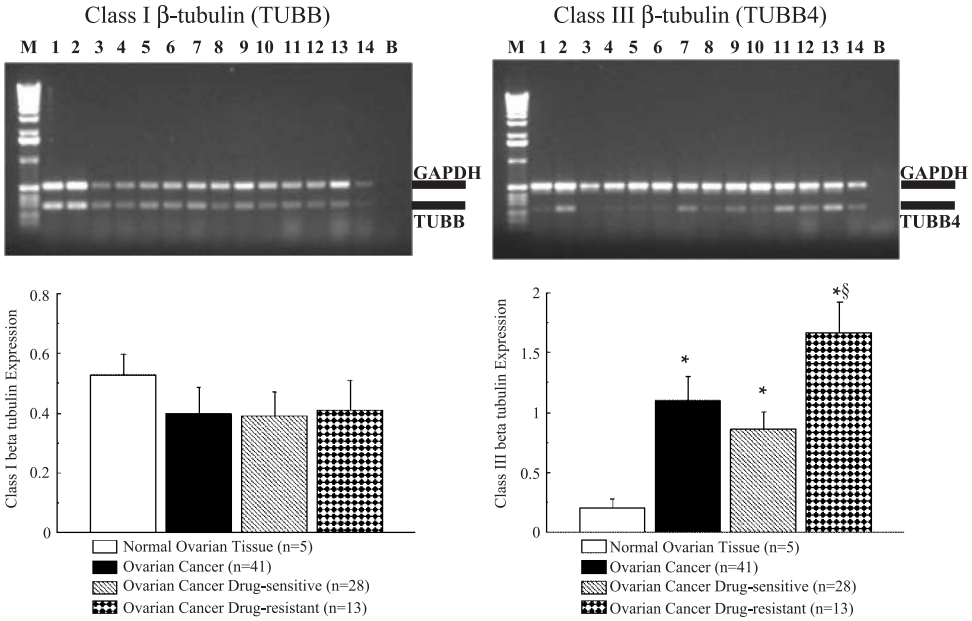

Fig. 3 Semiquantitative RT-PCR for TUBB (*left*) and TUBB4 (*right*). *Top,* images of the agarose-gel electrophoresis of the RT-PCR products. *Lane M,* molecular weights; *lane 1,* negative control (A2780wt cancer cells); *lane 2,* positive control (Class III beta-tubulin + A2780-TC1 cells); *lanes 3-6,* normal ovarian biopsies; *lanes 7-10,* drug-sensitive ovarian cancer; *lanes 11-14,* drug-resistant ovarian cancer; *lane B,* water control. *Bottom,* bar chart of class I and class III β-tubulin expression in three independent experiments on normal ovarian biopsies, drug-sensitive, and drug-resistant patients. *Columns,* mean of the three independent experiments; *bars,* SE. *, $P < 0.01$, significant changes with respect to normal ovarian biopsies (Wilcoxon signed rank). §, $P < 0.05$, statistically significant difference of class III β-tubulin expression between resistant versus sensitive tumors (Wilcoxon signed rank).

The expression of β-tubulin isoforms was ascertained using semiquantitative RT-PCR. The results are summarized in Fig. 3. Our attention was focused on the prominently expressed class I β-tubulin and on class III β-tubulin isotype that was found highly overexpressed in a panel of *in vitro* cultured human ovarian cancer cells with inherent or acquired paclitaxel resistance (data not shown). Class I β-tubulin was detected at similar levels without statistically significant differences in both normal and cancer ovarian tissues, regardless of the drug responsiveness. On the other hand, class III β-tubulin was up-regulated in cancer tissues, where nearly a 5-fold increase (mean = 1.1) was detectable with respect to normal ovarian tissue (mean = 0.2). This difference was statistically significant ($P < 0.01$). An additional difference was detectable when stratifying samples for drug responsiveness. In fact, both groups exhibited statistically significant ($P < 0.01$) higher levels of class III β-tubulin than those noticed in the controls, but a further increase was present when comparing drug sensitive (mean = 0.86) versus drug-resistant samples (mean = 1.67), where these latter exhibited a further increase of class III β-tubulin. Also, the difference between the two groups resulted statistically significant ($P < 0.05$). This data indicates that such a gene is ectopically expressed in ovarian cancer tissues and that a further increase of class III β-tubulin is associated to the paclitaxel-resistant phenotype. In order to perform a more robust quantitative assay, real-time PCR was done for class III β-tubulin (Fig. 4). Using as reference A2780wt cells (expression = 1), normal ovarian tissue exhibited a mean = 1.83, a level nearly 12-fold lower than that related to cancer specimens (22.8). This difference was again statistically significant ($P < 0.01$). After stratification for resistance status, resistant and sensitive tumors yielded values of 8.3 and 37.1, respectively, and this difference was again statistically significant ($P < 0.01$).

In order to assess if the changes noticed at the mRNA expression were actually translated at the protein level, the same clinical setting was analyzed for the expression of MDR-1 and

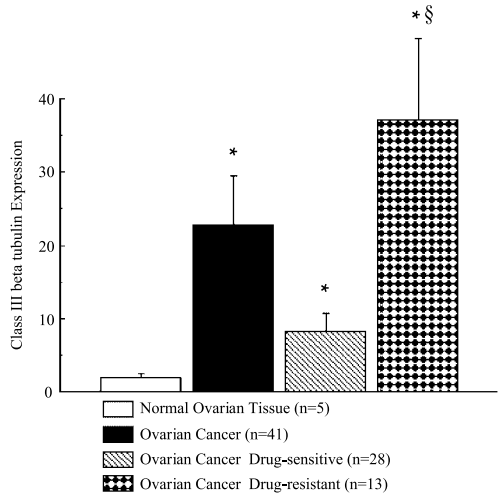

Fig. 4 Bar chart of real-time PCR analysis for class III β-tubulin on the same samples shown in Fig. 3. *Columns,* mean of the three independent replicates; *bars,* SE.

class III β-tubulin using quantitative immunohistochemistry. This analysis was done by selecting only the tumor area in each specimen. A representative picture used for this analysis is shown in Fig. 5. In line with the results obtained at the mRNA level, no statistically significant changes were observed between sensitive and resistant tumors for that concerning MDR-1 expression in the tumor area. Integrated densities were 17.9 ± 13.69 and 20.2 ± 8.64 for resistant and sensitive tumors, respectively (P = NS). Concerning class III β-tubulin, also in this case, the results were in line with those obtained at the mRNA level. IDs in the tumor area were 51.82 ± 8.27 and 9.8 + 3.95 for resistant and sensitive tumors, respectively, and the difference was statistically significant ($P < 0.001$), thereby indicating that class III overexpression occurs in cancer cells at the protein level in the resistant setting.

DISCUSSION

The major obstacle to the successful medical treatment of cancer is represented by drug resistance to currently used chemotherapeutics. The mainstay for the therapy of advanced ovarian cancer is represented by the combination of carboplatin (cisplatin) and paclitaxel. But if several mechanisms underlying platinum resistance have been clarified and shown as effectors of drug resistance in clinical studies (reviewed in refs. 20, 21), to date the mechanism(s) underlying paclitaxel resistance in patients are unknown. This study was addressed in an attempt to clarify this issue. The most prominent mechanisms of paclitaxel resistance previously reported in patients have been here assessed (i.e., MDR-1 overexpression, missense mutations of the tubulin gene, and the overexpression of class III β-tubulin). The clinical setting available for this study was based on 41 patients treated after surgery with the same paclitaxel-containing regimen. Twenty-eight patients responded to the first-line chemotherapy, whereas 13 patients progressed despite the treatment.

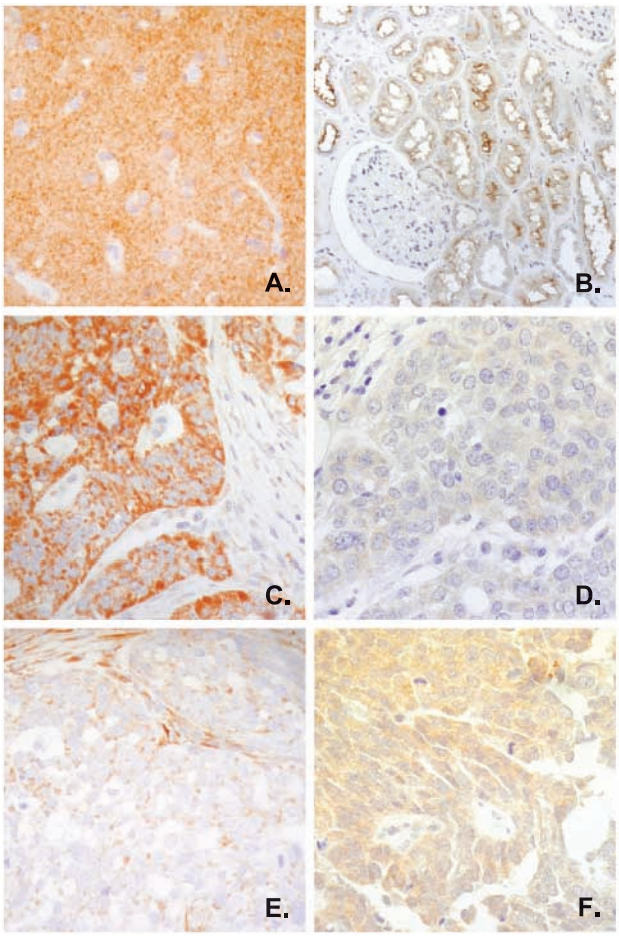

Fig. 5 Immunohistochemistry for class III β-tubulin (*A*, *C*, and *E*) and MDR-1 (*B*, *D*, and *F*). *A* and *B,* brain and kidney normal specimens used as positive control for immunohistochemistry, respectively (magnification ×250). *C* and *E,* representative images coming from drug-resistant and drug-sensitive ovarian cancer patients, respectively (magnification ×500). *D* and *F,* drug-resistant patients with diverse expression of the MDR-1 protein (magnification ×500).

No differences were observed in terms of MDR-1 expression between sensitive and resistant patients either at mRNA or at protein level, indicating that this gene did not affect the drug sensitivity status in our clinical setting. The role of MDR-1 as an actual modulator of drug resistance is still unclear. Some studies support the role of MDR-1 as a potential marker of poor response to chemotherapy (22–24), whereas others do not (25, 26). However, the fact that the combined therapy Paclitaxel and PSC-833, a functional inhibitor of the *MDR-1* gene product, led to a reduction in response rates and no difference in overall survival strongly suggests that MDR-1 does not play a direct role in mediating drug resistance to a paclitaxel-containing regimen therapy (21, 27).

That tubulin point mutations at the paclitaxel binding site underlie drug resistance is also controversial. Since the paclitaxel binding site is located within β-tubulin (28), DNA sequencing of the fourth exon (containing paclitaxel-binding site) of the class I β-tubulin isotype was done. Our findings were in line with previous reports that show tubulin point mutations in the prominently expressed *class I β-tubulin* gene are not involved in primary drug resistance (6, 7, 29).

Since recent reports have suggested that point mutations in α-tubulin are responsible at least *in vitro* for drug resistance (17), the DNA sequence was done also for the K-α1 tubulin. Again in this case, mutations were absent in both sensitive and resistant patients. Only some SNPs were detected but none of these can affect paclitaxel response, since the protein sequence remains unaltered and SNPs are equally distributed in both clinical groups. Therefore, from our findings, it is possible to conclude that tubulin point mutations are not responsible for the emergence of a primary resistance to paclitaxel.

Selective pressure through microtubule-interacting drugs is able to condition *in vitro* the expression of selected tubulin isotypes, thereby generating drug resistance in cell systems of lung and ovary cancer (reviewed in ref. 30). This phenomenon has also been noticed at the mRNA level by Kavallaris et al. (11) in few cases of paclitaxel-resistant ovarian cancer patients. In this study, these previous findings have been confirmed and extended at the protein level. Class III β-tubulin is markedly overexpressed in drug-resistant tumors. Most importantly, the differences noticed at the mRNA level are actually translated at the protein level, so that we have the direct evidence that a strong overexpression of class III β-tubulin is present in resistant tumors.

The exact mechanism by which an increased expression of class III β-tubulin mediates drug resistance is still unknown. Lu and Luduena (10) have shown in cell-free assays that the removal of class III β-tubulin through immunoprecipitation with specific antibodies enhances paclitaxel-induced microtubule polymerization. The physiologic role of this isotype seems associated with the microtubule dynamic instability (31, 32). Class III β-tubulin seems to enhance dynamic instability, thereby overcoming suppression of microtubule dynamicity by paclitaxel. An additional tempting hypothesis has recently been suggested by Hari et al. (33). Using an expression system of class III β-tubulin upon the control of the tetracycline regulatory element, they showed in whole cells that overexpression of such a gene confers drug resistance by reducing the polymerization rate of microtubules. Based on this, the authors theorized that class III β-tubulin acts as a negative regulator of the microtubule sensitivity to paclitaxel, thereby behaving as a natural tubulin mutant inherently resistant to paclitaxel (33). *In silico* promoter analysis of class III β-tubulin promoter[5] reveals the presence of several transcription factors involved in the hypoxic response such as stimulating protein 1 that it is repeated six times in the core promoter region (34–36), Wilms tumor suppressor (ref. 37; repeated four times), p53, and nuclear factor-κB (38). Our working hypothesis is that class III β-tubulin overexpression could be induced by hypoxia-dependent transcription factors and could represent a marker of the so-called hypoxia lethal phenotype (39). This fact may also explain the finding that regardless of drug resistance, class III β tubulin is more expressed in cancer specimens (growing in a hypoxic environment) than in normal ovarian biopsies. This hypothesis is currently under investigation in our laboratory.

To summarize our results, our findings are consistent with a prominent role of class III β-tubulin as mediator of paclitaxel resistance in advanced ovarian cancer. Moreover, class III β-tubulin seems an ideal target for the development of novel agents in ovarian cancer, since the target is ectopically expressed in cancer tissues and far less in normal tissues, and is probably directly involved in the drug-resistant phenotype. Therefore, novel therapeutic strategies targeted against class III β-tubulin could aid in resolving the clinical problem of paclitaxel resistance and enhance paclitaxel-based standard regimens.

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