Resistance to the Tubulin-Binding Agents in Renal Cell Carcinoma: No Mutations in the Class I *β-Tubulin* Gene but Changes in Tubulin Isotype Protein Expression

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Abstract

Purpose: The primary purpose of this study was to determine whether mutations of the class I *β-tubulin* gene may be implicated in the inherent resistance to tubulin-binding agents (TBA) in renal cancer, with a small number of samples and cell lines also being examined for class I and III β-tubulin isotype protein expression.

Experimental Design: DNA was extracted from 90 renal tumors and the class I *β-tubulin* gene analyzed for mutations. For each sample, eight PCRs were used to cover the complete coding sequence with intronic primers ensuring highly homologous pseudogenes were not coamplified. Additionally, expression levels of class I and III β-tubulin isotypes in 17 matched normal and malignant renal samples and a panel of renal cell carcinoma cell lines with differing intrinsic resistance to the TBAs was examined by Western blotting.

Results: Four polymorphic sequence changes of the class I *β-tubulin* gene were identified with no mutations. Class I protein expression levels were higher in tumor tissue versus normal tissue, whereas class III expression showed no consistent change. In renal cancer cell lines, a significant correlation between class III isotype expression and vinblastine sensitivity was observed.

Conclusions: These results do not support a role for mutations in the class I *β-tubulin* gene in the intrinsic resistance of renal cancer to TBAs. Class III isotype expression may be implicated in resistance *in vitro* but *in vivo*, changes in class I isotype expression in renal cell carcinoma tissue may support a role in resistance to the TBAs and warrants further investigation.

Renal cancer represents ~3% of adult malignancies with a male to female ratio of 2:1 (1), causing 95,000 deaths worldwide annually (2). However, the incidence of this cancer is increasing with a 126% increase in incidence since the 1950s in the United States (3) and a reported 18% increase in incidence rate in the last 10 years in females in the United Kingdom, one of the largest rises in incidence rate of any cancer in women (4). Surgical resection of the primary tumor remains the only effective therapy for organ-confined disease as renal cell carcinoma (RCC) represents one of the most chemoresistant of tumors with none of the commonly used cytotoxic agents exhibiting appreciable activity (5). The reasons for this inherent resistance are not clear but are probably multifactorial. Tubulin-binding agents (TBA) are among the most successful and commonly prescribed anticancer therapies generally, but in RCC response rates of only 3% to single-agent vinblastine have been reported (5).

The cellular target of these agents is the β-tubulin subunit of the microtubules. Formation of microtubules involves polymerization of heterodimeric α/β-tubulin subunits with multiple isoforms of both α-tubulin and β-tubulin present in human cells (6). Six β-tubulin isotypes have been reported (classes I, II, III, IVa, IVb, and VI; reviewed ref. 6). Class I is the predominant isotype expressed in many tissues and is encoded by the *TUBB* gene (6p21.3 AP00512), which has four exons encoding a predicted protein of 444 amino acids. These drugs, which classically include the taxanes and the *Vinca* alkaloids but also now the novel epothilone analogues, function by perturbing the microtubule polymerization dynamics in different ways (7). Several potential mechanisms of TBA drug resistance in cancers have been suggested including increased drug efflux due to overexpression of the membrane transporter, P-glycoprotein (MDR-1). However, our previous studies did not find any relationship between expression of P-glycoprotein and response to paclitaxel, vinblastine, and epothilone-B in a panel of RCC cell lines and P-glycoprotein expression in RCC tissue was decreased compared with normal kidney tissue (8). Other possible mechanisms may include alterations of the β-tubulin target (9) resulting in changes in microtubule dynamics, such as may arise from altered expression of tubulin isotypes (10–12), tubulin mutations (13), and altered expression or binding of microtubule-regulatory proteins (14).

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Numerous studies *in vitro* involving several cancer types have shown correlations between the presence of class I β-tubulin mutations and resistance to the TBAs. Resistance to paclitaxel (15) and epothilone (16) has been associated with mutations that directly affect the drug binding site, for example. Using two paclitaxel-resistant sublines derived from a human ovarian carcinoma cell line (1A9), two different point mutations within the intermediate domain of the protein were identified which directly affected the paclitaxel-binding site and inhibited paclitaxel-induced tubulin polymerization. Similarly, epothilone-resistant sublines derived from the 1A9 ovarian cancer cell line (15), the non–small cell lung cancer (NSCLC) cell line A549, and the cervical cancer cell line HeLa (16) were also shown to acquire various mutations in β-tubulin. Mutations have also been reported in vincristine-resistant but not in vinblastine-resistant sublines from the CEM leukemia cell line (17) and also in an indanocine-resistant subline (18). Such findings however, have not been universal. Mutation analysis of 25 NSCLC cell lines that differed in their sensitivity to paclitaxel showed variant sequences, none of which resulted in changes in the encoded amino acids (19). Similarly, analysis of 10 NSCLC and SCLC cell lines revealed silent mutations only (20).

The significance of β-tubulin mutations in clinical drug resistance *in vivo* remains unclear. β-Tubulin mutations in clinical samples were first described in tumor samples from NSCLC patients where a link between the presence of β-tubulin mutations and a lack of patient response to paclitaxel-based chemotherapy was shown (21). The interpretation of these results however is complicated by the demonstration that the use of exonic primers can lead to the coamplification of highly homologous pseudogenes (19, 22, 23). At least nine class I β-tubulin pseudogenes have been reported to date. Further difficulty arises as there is disparity in the literature as to which gene sequence represents the correct wild-type sequence for the *β-tubulin* gene (22). This is particularly important because some reported mutations are possibly attributable to mismatches between these reference genes.

Modification of the microtubule target by differential expression of β-tubulin isotypes has also been proposed as a mechanism of intrinsic or acquired resistance with microtubules enriched for class III and IV isoforms being significantly more resistant to paclitaxel suppression of microtubule dynamics for example compared with unfractionated tubulin (24). High class I β-tubulin expression has been shown associated with paclitaxel resistance in the A549 NSCLC cell line (12), with high class II expression showing a similar association in a number of human cancer cell lines, including lung, ovarian, prostate, and breast (10). Clinically, evidence is limited but paired samples from patients with advanced ovarian cancer with acquired resistance to paclitaxel showed increases in class I, III, and IVa β-tubulin isoforms (12). More recently, expression levels of classes I and III isotypes have been suggested as useful predictors of response to docetaxel in breast cancer (25).

There has been no previous report examining the *β-tubulin* gene in RCC, and in this study, our primary aim was to do a mutational analysis of the class I *β-tubulin* gene (cDNA, Genbank accession no. AF070600) in RCC patients, taking into account the confounding factors such as highly homologous pseudogenes described above, to determine if tumor-associated mutations could explain the lack of response of RCC to the TBAs. In addition, we also examined the expression levels of class I and III β-tubulin isotypes in a small number of patients as well as in a panel of RCC cell lines with differing intrinsic paclitaxel resistance. These particular isotypes were selected based on class I being the predominant isotype and both, particularly class III, having been implicated in resistance to the TBAs in other cancers.

Materials and Methods

Tissue samples. All samples were obtained with informed patient consent and approval by the Local Research Ethics Committee. Tumor tissues were obtained from 90 patients who underwent surgical resection for renal cancer at St. James's University Hospital, Leeds between 1998 and 2002. Clinical features of patients are shown in Table 1. Clinical stages of patients were determined according to the 1997 tumor-node-metastasis classification (26) and the Fuhrman grading system was used (27). No patients had received any prior treatment for their cancer.

Tumor specimens were obtained immediately upon surgical resection and placed in 20 mL ice-cold RPMI 1640 containing a Complete protease inhibitor cocktail tablet (Boehringer, Lewes, United Kingdom). Each sample was dissected to remove fibrous and necrotic portions of tumor, cut into blocks of ~10 to 20 mm^3 and washed briefly in ice-cold PBS (pH 7.2) containing Complete protease inhibitor. Tissue blocks were touched against tissue paper to remove excess PBS, embedded in ornithine carbamyl transferase, wrapped in aluminium foil, snap-frozen in liquid nitrogen, and stored in liquid nitrogen. Sample processing was carried out rapidly, with tissue being dissected and frozen within 30 to 45 minutes of removal from the patient. In parallel, peripheral blood buffy coats were obtained from venous blood samples collected into Vacuette EDTA tubes following centrifugation and stored frozen at −80°C.

DNA extraction and PCR. Total cellular genomic DNA was extracted from 20-μm cryostat tissue sections (50 sections per block) and the corresponding peripheral blood buffy coats. Tissue sections were cut directly into a 1.5-mL Eppendorf tube and DNA extracted using the QIAmp DNA mini kit (Qiagen, Crawley, United Kingdom) according to the manufacturer's instructions. DNA was extracted from 2 mL of peripheral blood buffy coats using the Nucleon BACC2 genomic DNA extraction kit (Amersham Biosciences, Little Chalfont, United Kingdom). DNA was quantified using the PicoGreen dsDNA quantification kit (Molecular Probes, Leiden, Netherlands).

Table 1. Clinicopathologic characteristics of the renal cancer patients

	Renal cancer type		
	Conventional	Papillary	Others
Total no.	75	9	6
Males	48	7	4
Females	27	2	2
Median age (range)	64 (28-86)	58 (53-79)	62.5 (53-86)
Grade			
1	6	1	0
2	24	1	3
3	31	7	0
4	14	0	3
pT1	28	5	1
pT2	7	3	2
pT3	35	1	3
pT4	5	0	0

Table 2. PCR and sequencing primer sets for mutational analysis of the class I *β-tubulin* gene (22)

Exon	Forward	Reverse	Amplicon (bp)
1	5′-AACCTTCCAGCCTGCGAC-3′	5′-ACTTACCTGGATTTTTCCTTG-3′	203
2	5′-TAGTTGGGGACATAGTTGGC-3′	5′-TAAGGCGTGCCCAGAAATGG-3′	255
3	5′-AATGACAAGTCTCTGATCCC-3′	5′-TCCAATACAACAATCATCTCC-3′	308
4	5′-TGTATTGGAGTGCTAATATACAG-3′	5′-CTCCCTTGAAGCTGAGATGG-3′	1,809
4-1	5′-CATGTATCTTCCATACCCTG-3′	5′-CTGAAGGTATTCATGATGCG-3′	258
4-2	5′-GAATGGGCACTCTCCTTATC-3′	5′-GGACCATGTTGACTGCCAAC-3′	338
4-3	5′-ATGAGTGGTGTCACCACCTG-3′	5′-GACTGCCATCTTGAGGCCAC-3′	398
4-4	5′-CCCAACAATGTCAAGACAGC-3′	5′-CAAGATAGAGGCAGCAAACAC-3′	400

PCR amplification was carried out using Amplitaq Gold polymerase (Applied Biosystems, Warrington, United Kingdom) in the buffer supplied by the manufacturer containing 2.5 to 3.0 mmol/L $MgCl_2$, 200 μmol/L each deoxynucleotide triphosphate, 0.5 pmol/μL each primer, and 2 ng/μL genomic DNA. PCR was checked for yield and specificity by agarose gel electrophoresis. Intronic primer sets used for PCR amplification of the class I *β-tubulin* gene are shown in Table 2 and Fig. 1. These intronic-based primers as previously described (22) do not coamplify the previously reported class I β tubulin pseudogenes (J00315, J00316, J00317, K00840, K00841, K00841, M24191, V00598, and AF252825). Mutational analysis was conducted using one primer pair for each of exons 1, 2, and 3. Nested PCR for was done for exon 4, where the entire (1,809 bp) exon was first amplified using intronic primers, and then four segments (exons 4-1 to 4-4) were amplified separately using inner primer sets. Cycling conditions for all amplicons except exon 4 were 95°C 12 minutes, four cycles of (95°C for 30 seconds, 60°C for 30 seconds, 72°C for 1 minutes) followed by 19 cycles during which the annealing temperature was reduced by 0.5°C/cycle followed by 15 cycles with an annealing temperature of 50°C and completed by incubation at 72°C for 10 minutes with a final hold at 15°C. The whole of exon 4 was amplified at 95°C 12 minutes, 36 cycles of (95°C for 30 seconds, 64°C for 30 seconds, 72°C for 2 minutes). Products were then diluted with water and nested PCR of 4-1, 4-2, 4-3, and 4-4 carried out using the cycling conditions as described for exons 1, 2, and 3. Sequences were compared with wild-type reference gene (cDNA AF070600, genomic DNA 6p21.3 AP00512).

Mutation screening. Denaturing high-performance liquid chromatography of the PCR products was carried out using a Transgenomic WAVE Nucleic Acid Fragment Analysis system and DNASep column (Transgenomic, Elancourt, France). Heteroduplex formation was induced by denaturation of PCR products at 95°C for 5 minutes and cooling to 65°C. Previous studies carried out on these samples had indicated that there was sufficient contaminating normal DNA from infiltrating cells to make spiking of the samples to detect homozygous somatic mutations unnecessary. The composition of buffer A was 0.1 mol/L triethylammonium acetate and buffer B was 0.1 mol/L triethylammonium acetate and 25% acetonitrile. Analysis was carried out at a flow rate of 0.9 mL/min and a buffer B gradient increase of 2%/min for 4 minutes. Start and end concentrations of buffer B were determined empirically for each fragment. Heteroduplexes were identified by the appearance of two or more peaks on the chromatograms, which were examined independently by two observers. Column temperatures were as follows: exon 1, 56°C and 61°C; exon 2, 61°C and 65°C; exon 3, 57°C and 60°C; exon 4-1, 60°C and 64°C; exon 4-2, 62°C; exon 4-3, 63°C; exon 4-4, 62°C and 64°C.

DNA sequencing. All samples in which denaturing high-performance liquid chromatography indicated the presence of a potential mutation were subjected to direct DNA sequencing. The corresponding buffy coat DNA sample was examined alongside the tumor sample. Two further samples shown mutation-negative were also sequenced as controls. Unincorporated primers and deoxynucleotides were removed from PCR products using shrimp alkaline phosphatase and exonuclease I (Amersham Pharmacia, Amersham, United Kingdom). The same primers used in the PCR were used for sequencing of the PCR products. Cycling conditions for all exons were 25 cycles of (96°C for 10 seconds, 55°C for 5 seconds, 60°C for 4 minutes) with a final hold at 15°C. Sequencing reactions were carried out using ABI Prism BigDye Terminator Cycle Sequencing Kit version 2 (Applied Biosystems) with electrophoresis of the products on a 3100 Genetic Analyser (Applied Biosystems). Data analysis was carried out using Sequence Analysis 3.0 (Applied Biosystems) and by visual inspection of electropherograms. Sequencing data obtained from the clinical specimens were compared with the class I β-tubulin sequence as reported on the Genbank database (AP00512).

Cell culture and chemosensitivity assays. The established cell lines used in this study (RCC lines: HTB47, SN12K, TK-10, HTB46, and CRL1933) and the breast cancer cell line MCF-7 as a chemosensitive control were obtained from the American Type Culture Collection (Rockville, MD) or the cell repository National Cancer Institute-Frederick Cancer Research and Development Centre (Frederick, MD). Cell lines were routinely cultured in RPMI 1640 supplemented with 1% v/v L-glutamine and 10% FCS at 37°C in 5% CO_2. The IC_{50} (defined as the drug concentration that resulted in a 50% reduction in cell viability in drug-treated wells relative to control wells) of these cell lines for paclitaxel has been reported previously (8).

Protein extraction and Western blotting. Protein lysates from tumor and matched distal normal cortical kidney tissue from 17 of the patients were prepared in Laemmli buffer [62.5 mmol/L Tris-HCl (pH 6.8), 10% v/v glycerol, 2% w/v SDS, 5% v/v β-mercaptoethanol, and trace bromophenol blue]. These tissue blocks had been stored in parallel with those extracted for DNA but had been rinsed in isotonic sucrose before being snap frozen and stored. Additionally lysates were prepared from each of the cell lines above. Protein concentrations of all lysates were determined using the Bio-Rad protein assay (Bio-Rad, Hemel Hempstead, United Kingdom), based on a modified Bradford assay. In each case, the protein concentration of each sample relative to each other was also then checked by densitometric analysis of colloidal Coomassie blue stained SDS-PAGE gels (10% T, 4% C) using 2 μg protein per lane as previously described (28).

For Western blotting, samples were electrophoresed as described above and transferred to Hybond-C nitrocellulose paper (Amersham Biosciences) using Towbin's buffer [25 mmol/L Tris-HCl, 192 mmol/L glycine, 10% v/v methanol (pH 8.3)] and the Bio-Rad Trans-blot system (100 V, 1 hour). Blots were blocked in 10% nonfat dried milk w/v in TBST [20 mmol/L Tris-HCl, 140 mmol/L NaCl (pH 7.6), 0.1% v/v Tween 20] for 2 hours and incubated with primary antibody diluted in 1% nonfat dried milk w/v in TBST for 1 hour. Primary antibodies, anti–class I β-tubulin clone SAP.45 and anti–class III β-tubulin clone SDL.3D10 (both Sigma, Poole, United Kingdom) were used at previously optimized concentrations of 1 and 84 μg/mL, respectively. After washing with TBST (4 × 5 minutes) and incubation with horseradish peroxidase-conjugated mouse EnVision+ reagent

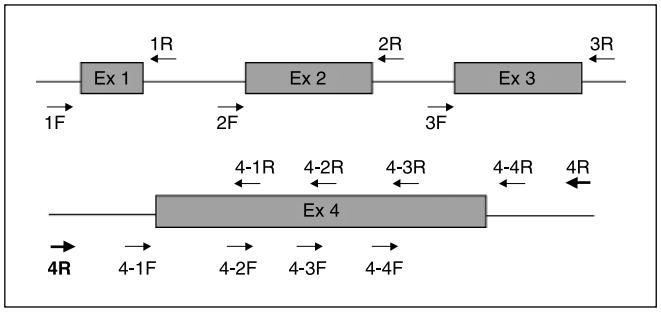

Fig. 1. Primer design for the class I *β-tubulin* gene (reference gene AF00512). Primer sequences are shown in Table 2. Exons 1 to 3 were amplified using single individual PCR reactions. Exon 4 was amplified using nested PCR, where the entire exon was amplified using the outer primers (4F and 4R), and four segments of exon 4 were amplified separately using the inner primer sets (22).

(DakoCytomation, Ely, United Kingdom) diluted 1:200 in 5% nonfat dried milk w/v in TBST for 1 hour (29), blots were developed using Supersignal West Dura Substrate (Pierce, Tattenhall, United Kingdom), exposed to Kodak Biomax MS film for multiple exposure times to ensure results were obtained within the linear range of the film and images scanned using a Personal Densitometer SI (Amersham Biosciences). Images were analyzed using ImageQuant. Results were not normalized to housekeeping proteins as we have shown previously that expression levels of the commonly used housekeeping proteins such as glyceraldehyde-3-phosphate dehydrogenase, α-actin, and pan β-tubulin vary considerably between normal and renal cancer tissue (28). Results of Western blotting could be directly compared between samples however with standardization of protein loads for each sample having been assured by both protein assay and parallel stained gels as described. Additionally, electrophoresis, Western blotting, and analysis were done on three separate occasions for each antigen and sample to ensure reproducibility.

Statistical analysis. Spearman rank correlation analyses were done using the Institute of Phonetic Sciences (IFA) software (Amsterdam, the Netherlands; http://fonsg3.let.uva.nl/Service/Statistics/).

Results

Class I β-tubulin mutation status in renal cancer. The clinicopathologic characteristics of the patients whose tissues were examined in this study are listed in Table 1, with the majority of patients classified as conventional RCC with an overall male-to-female ratio of 1.9:1.

Mutation screening followed by sequencing identified four different sequence changes in a total of 15 samples. Only one change was exonic, specifically in exon 4-2, and this was present in three (3.3%) of the tumor specimens (Table 3; Fig. 2). However, this substitution does not result in alteration of the corresponding amino acid at codon 217 (Leu/Leu; CTG/CTA) and was also present in the corresponding buffy coat DNA samples. Three additional substitutions were observed, two of which were in introns flanking exon 3: G > A 58 bases upstream of exon 3 and C > T 11 bases downstream of exon 3. The remaining change was a C > T substitution in the 3′ untranslated region. To distinguish between germ line variants and somatic mutations, we did mutational analysis of the buffy coat DNA of the corresponding patients, which revealed the same sequence variations, indicating that none of these changes were specifically tumor associated. Using the sequence analysis program ESEfinder Release 2.0 to examine possible effects of exonic splicing enhancers (http://exon.cshl.edu/ESE/) and NNSPLICE 0.9 (http://www.fruitfly.org/seq_tools/splice.html) to examine the predicted effects of the intronic sequence changes on alteration of cryptic splice sites, no effect of these nucleotide changes was predicted.

β-Tubulin isotype expression in renal cell carcinoma and normal kidney tissues. As the mutation analysis did not support a role for mutations in the class I *β-tubulin* gene in the resistance of renal cancer to TBAs, we subsequently investigated whether β-tubulin isoform expression was altered in RCC, which may be an alternative resistance mechanism. In this preliminary study, class I and III isoform protein levels of matched normal and RCC tissue were examined in a representative subgroup of samples from 17 patients covering various stages and grades. In 16 of 17 cases analyzed, class I β-tubulin was more highly expressed in tumor tissue displaying as much as a 5.6-fold difference (patient 120) in densitometric signal in the patient-matched tissue pairs (Fig. 3). Two bands were apparent with the anti–class III antibody, one of which runs at the expected molecular mass for tubulin at ~50 kDa (Fig. 3) and another at an apparent molecular weight of 70 kDa, the expression levels of which were similar to the 50-kDa species in each of the tissue lysates examined. It was noteworthy that this extra band was only seen in tissue samples and was not present in cell lines (see below). There was considerable interpatient variability in the expression levels of the class III isotype, being undetectable in two patients (patients 42 and 120) as well as some variability between patient-matched normal and tumor tissue, showing no consistent tumor-associated alteration.

β-Tubulin isotype expression in renal cell carcinoma cell lines differentially resistant to the tubulin-binding agents. Because an increased class I isotype expression in tumor versus matched normal kidney tissue emerged, to gain some insight into the possible role of these isotypes in resistance the expression levels of the class I and III were examined in a panel of RCC cell lines exhibiting a wide range in sensitivity to the TBAs (e.g., ~15-fold range for paclitaxel). Figure 4 shows their expression levels among the panel of established RCC cell lines and the chemosensitive breast line MCF-7 together with the previously determined IC_{50} values for the cell lines following a 1h exposure to four TBAs (8). Western blotting for both class I and III isoforms of β-tubulin revealed single bands at the expected molecular weights of ~50 kDa. Class I expression was similar among the cell lines, irrespective of sensitivity to paclitaxel. In contrast, class III expression was variable, being most highly expressed in the SN12K RCC cell line. No significant correlation with

Table 3. Nucleotide changes in the class I *β-tubulin* gene in renal cancer patients

Location	Effect	% Frequency
IVS 2-58G › A intron	None predicted	2.2 (*n* = 2; cRCC)
IVS 3+11C › T intron	None predicted	6.7 (*n* = 6; 4 cRCC, 2 pRCC)
651 G›A exon	Leu 217 Leu	3.3 (*n* = 3; cRCC)
*1335 + 57 C › T 3′UTR	None predicted	4.4 (*n* = 4; cRCC)

NOTE: Nucleotides are numbered from the ATG start codon.

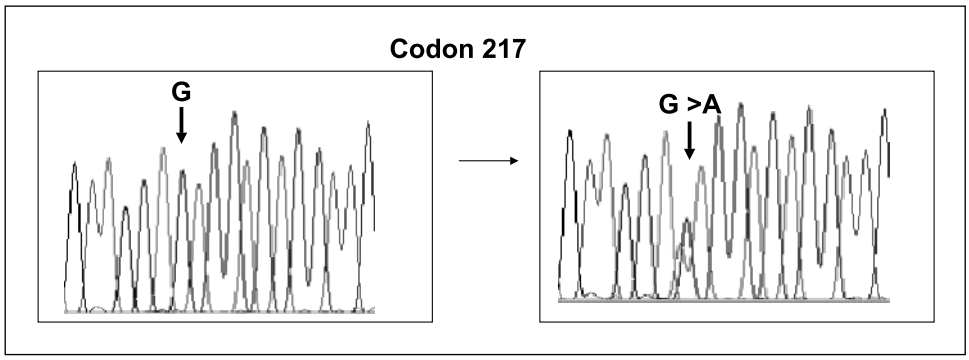

Fig. 2. Sequencing analysis of PCR products showing the G > A alteration in exon 4-2.

isotype expression and drug sensitivity could be shown except for the class III isotype and vinblastine (r = 0.89, P = 0.033).

Discussion

Most patients with RCC present with or develop metastases and clinical drug resistance is a major barrier to overcome before chemotherapy can become curative in this disease. Understanding the underlying mechanisms of drug resistance is key to developing new approaches. The microtubule represents one the most successful antitumor drug targets to date, yet RCC remains one of the most chemoresistant of tumors, showing minimal response even to these agents (5). A number of mechanisms have been implicated in drug resistance to these agents, and include pharmacologic, multicellular, and intracellular mechanisms. Recently, published cellular models of resistance to these agents have been associated with alterations in the cellular target itself, β-tubulin (9, 22). Two mechanisms in particular have been reported: altered β-tubulin isotype expression and point mutations in the class I *β-tubulin* gene. Publication of the β-tubulin structure has enabled the precise identification of the paclitaxel-binding site, thereby enabling the consequence of sequence variations to be determined (30). Acquired β-tubulin mutations in human cancer cell lines following selection for resistance to TBAs have been reported to confer resistance to the taxanes and epothilones by impairing their binding to microtubules (15, 31, 32). However, in a study using DNA from 25 unselected NSCLC cell lines differing in their inherent sensitivity to paclitaxel (19), two cell lines showed variant sequences, but neither caused a change in the encoded amino acid. Similarly in an analysis of exon 4 of the *β-tubulin* gene in 10 NSCLC and 10 SCLC cell lines (20) only silent mutations were seen in four of the SCLC and three of the NSCLC cell lines.

Although β-tubulin mutations have been shown to play a role in resistance in cultured cell lines selected for resistance to these agents (15–18, 31, 33), their role in clinical resistance remains controversial and the process of studying the functional gene in clinical samples is complicated. In a landmark study, a reported link between β-tubulin mutations and patient survival after paclitaxel-based chemotherapy was described (21) with 33% of 49 NSCLC patients having somatic β-tubulin mutations, none of whom responded to paclitaxel. Despite this tantalizing evidence for a role of β-tubulin mutations in resistance, the study design has since been questioned. A number of subsequent studies (19, 22, 23) have suggested that exonic primers used in this study were likely to coamplify the highly homologous pseudogenes, of which nine have been described to date. In a similar study analysing NSCLC samples but where sequencing was done with cDNA rather than genomic DNA to avoid amplification of pseudogenes, only silent mutations were observed (20). In a comprehensive study of paclitaxel-naive ovarian cancer samples, silent polymorphisms only were detected (23).

In the renal cancer samples examined here which represented a range of pathologic stages and grades and with all patients being untreated, only a single exonic nucleotide alteration in exon 4 which was present in 3% of the tumor specimens and did not result in substitution of the corresponding amino acid and was also present in the corresponding buffy coat DNA. This indicates that mutation of the class I β-tubulin target is not the cause of intrinsic resistance to the TBAs in RCC. Three additional substitutions were observed, two of which were in introns flanking exon 3, and one alteration in the 3′ untranslated region. The exonic alteration has been identified previously as a silent polymorphism in several cancer studies, including breast (22, 34), lung, ovarian (23), and gastric cancers (35), as well as in a comprehensive study of a control group of ethnically diverse individuals representing five continents (23). Although observed at higher frequencies (17-22%) than was observed in our study of renal cancer patients, this may simply reflect the variation in single nucleotide polymorphism frequencies in different populations. Single nucleotide polymorphisms in the 3′ untranslated region have also been described previously (refs. 22, 23; dbSNP:1054419).

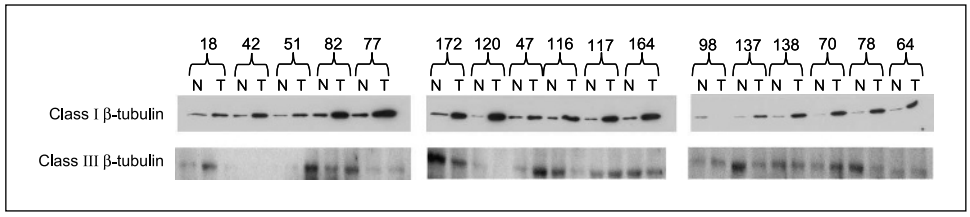

Fig. 3. Expression of tubulin isotypes I and III in paired normal and renal cancer tissues. Protein extracts were prepared from matched normal and tumor kidney tissue from each of seventeen patients with RCC and 2 μg of total protein were analyzed by Western blotting for the expression of the class I and III tubulin isotypes as indicated.

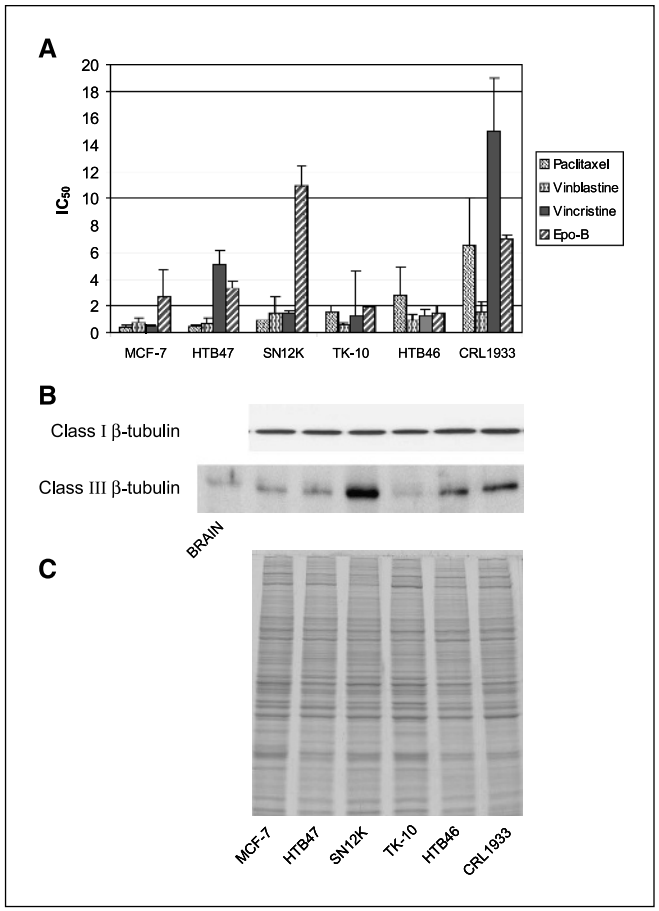

Fig. 4. Expression of tubulin isotypes I and III among cancer cell lines of differing intrinsic resistance to theTBAs. *A,* chemosensitivity profile of the RCC cell line panel (and MCF-7 breast cancer cell line) to a number ofTBAs [paclitaxel, vinblastine, vincristine, and epothilone-B (*Epo-B*)]. IC_{50} values were determined previously using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (8) following 1 hour of exposure to each of the drugs. IC_{50} (units in μmol/L except for epothilone-B where units are in nmol/L) is defined as the concentration of drug required to reduce cell viability to 50% of that of untreated controls. *B,* 2 μg of whole cell lysates were separated by 10% T SDS-PAGE and analyzed by Western blotting with anti – isotype I β-tubulin (~50 kDa) and anti – isotype III β-tubulin (~50 kDa) antibodies to the proteins as indicated. Lane 1, 2 μg of human brain lysate as a positive control for the neural-specific class III isotype. Lanes 2-7, the following cell lines: MCF-7, HTB47, SN12K,TK-10, HTB46, and CRL1933 that exhibit varying resistance to theTBAs. *C,* parallel Coomassie-stained gels of the separated cell lysates loaded in the same order as in (*B*) were used to confirm equal total protein loads as described in Materials and Methods.

Apart from the possible coamplification of pseudogenes another complexity in mutational analysis of the class I *β-tubulin* gene derives from the misuse of Genbank J00314 gene sequence as the wild-type sequence of the class I *β-tubulin* gene. The vast majority of previous mutation analyses have employed J00314 as a reference, which differs from the recently published wild-type sequence (cDNA AF070600, genomic DNA AP00512) by eight nucleotides (22, 36). Such findings are particularly important in a mutational analysis of β-tubulin, as mismatches between these reference genes have been reported as somatic mutations (21). Furthermore, although at least nine β-tubulin pseudogenes have been described, analysis has shown that several Genbank assignations have the same sequence; for example, accessions V00598, J00317, K00841, K00840, and M24191 are named HSTUB1, HUMTBB46P, HUMTBB21P, HUMTBB14P, and HUMTBB46QP, respectively. There is a further level of confusion in nomenclature. Several β-tubulin pseudogenes originally recorded in Genbank are now included in the ENSEMBL database both as new genes (e.g., Genbank accession no. K00840 corresponds to ENSESTG00000019284) and as sequences with known transcripts (e.g., Genbank accession no. J00316 corresponds to OTTHUMG00006003856; ref. 37). Such issues must be given careful consideration to avoid confusion in further studies.

In mammalian cells, each tubulin isotype has specific expression patterns. Classes I and IVb are constitutively and ubiquitously expressed; classes III and IVa are restricted to neural tissue; class II is predominantly in brain and at low levels in a broad range of tissues; and class VI is restricted to haematopoeitic tissues (6). We examined the levels of two isotypes in particular, classes I and III, because class I is the predominant isotype accounting for 88% to 99% of total β-tubulin expression (6) and has been implicated previously in taxane resistance in breast cancer (25). Of the remaining isotypes, class III has been frequently implicated in paclitaxel resistance (12, 38, 39). Analysis of protein expression was done in our study as several studies have shown poor correlation between gene expression and protein abundance (40). Furthermore, analysis at this level removes issues such as possible coamplification of homologous pseudogenes. Class III isotype expression was variable among RCC patient material and we observed no consistent tumor-associated alteration in its expression; however, a consistent increase in class I expression was observed in tumor versus matched normal kidney tissue. This observation is intriguing because increased class I expression has been shown associated with resistance to the paclitaxel and docetaxel in ovarian and breast cancers, respectively (12, 25). The significance of the absence of class III isotype in both the RCC and normal kidney tissue in two patients is unclear but is unlikely to represent degraded sample as Western blots of these lysates with several other antigens (e.g., pan β-tubulin, class I β-tubulin, and β-actin) have been successful. This may represent an alternative splice form of this class, which is not recognized by the particular monoclonal antibody used in this study.

To gain some insight into the possible effect of such increased class I expression in RCC on chemoresistance, we examined class I and III expression levels in a panel of established RCC cell lines with a broad range in intrinsic resistance to the TBAs (8). A significant correlation in expression of class III tubulin and resistance to vinblastine only was observed. Of the six isotypes, class III seems unique in that it has a destabilizing effect on the microtubule and previously has generally, been implicated in paclitaxel resistance, the notion being that it counters the stabilizing effect of paclitaxel on the microtubule (24). However, the findings have been somewhat mixed as in previous cell line-based studies in various cancer types, virtually all the β-tubulin isotypes (including I, II, III, IVa) have been implicated in resistance to the TBAs (9). This may partly reflect the use of cell lines in some cases which have been selected based on drug resistance following repeated *in vitro* exposure and which may in itself reflect an adaptation representing acquired resistance rather than intrinsic. Studies using antisense nucleotides used to block expression of class III isotype in NSCLC cells sensitized the cells to paclitaxel (11). Similarly, increases in class III and IVa tubulin have been described in human prostate lines in cells made resistant to estramustine (38). However, the same investigators observed that transfected class III failed to confer resistance in

these cells (39). Interestingly, in this particular study, counter-regulatory increases in class II and IVb tubulin in these cells was observed; possibly a compensatory mechanism to overcome the effects of the elevated class III levels, thus highlighting the difficulty in determining the contribution of individual isotypes in drug response studies and the importance of analysis of the total isotype profile. Other studies have shown that transfection of rodent I, II or IVa into Chinese hamster ovary cells and shown that these do not confer resistance to paclitaxel (41).

Studies of tubulin isotype expression in resistance using patient material are few but have been undertaken in ovarian and breast cancers (12, 25) and in both cases, correlations in isotype expression with resistance to the taxanes have been shown. These studies have examined mRNA expression rather than protein. Significant increases in classes I, III, and IVa isotypes were observed in paclitaxel resistant versus untreated primary ovarian tumors (12), although the number of samples analyzed was rather small. Similarly, in a study of 39 breast cancer patients, class I and III isotype mRNA levels were higher in nonresponders than in responders to docetaxel. Furthermore, when patients were grouped according to their class I and III isotype expression status; the class I high/class III high group showed low response rates to docetaxel (~15%), whereas the class I low/class III low group showed very high response rates to docetaxel of ~75%, suggesting that the levels of these isotypes may be useful in prediction of response to docetaxel (25). Following this, the increase in class I β-tubulin isotype that we have shown in RCC compared with normal kidney tissue is interesting but the lack of correlation between class I expression and resistance to paclitaxel in RCC cell lines *in vitro* may indicate that this plays little role in intrinsic resistance to TBAs. However, it is possible that expression levels of these isotypes may not be the sole determinants of resistance because β-tubulin undergoes a number of post-translational modifications, including phosphorylation, polyglutamation, and polyglycation (reviewed extensively ref. 6) and this area is the subject of further investigation.

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