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N.B.: When citing this work, cite the original article.

### Original Publication:

Henrik Green, Per Rosenberg, Peter Söderkvist, György Horvath and Curt Peterson,  $\beta$ -tubulin mutations in ovarian cancer using single strand conformation analysis – risk of false positive results from paraffin embedded tissues, 2006, Cancer Letters, (236), 1, 148-154.

<http://dx.doi.org/10.1016/j.canlet.2005.05.025>

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Postprint available at: Linköping University Electronic Press

<http://urn.kb.se/resolve?urn=urn:nbn:se:liu:diva-14242>

$\beta$ -Tubulin mutations in ovarian cancer using single strand conformation analysis  
– risk of false positive results from paraffin embedded tissues

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## **Abstract**

Mutations in the  $\beta$ -tubulin gene have been proposed as a resistance mechanism to paclitaxel. We therefore investigated the presence of mutations in the  $\beta$ -tubulin M40 gene in 40 ovarian tumours (16 paraffin-embedded and 24 freshly frozen) selected for good or poor response to chemotherapy with paclitaxel or non-tubulin-affecting regimens. The presence of mutations was investigated using single strand conformation analysis followed by sequencing of the products with altered mobility. No sequence variants in the exons of the  $\beta$ -tubulin M40 gene were detected. Non-reproducible shifts were identified, in eight out of 16 paraffin embedded samples. This may explain some of the previously published discrepancies. In conclusion, sequence variants in the  $\beta$ -tubulin M40 gene are rare and are unlikely to be a clinically relevant explanation of resistance to paclitaxel.

**Keywords:**  $\beta$ -tubulin; paclitaxel; ovarian cancer; mutation analysis; SSCA

## 1. Introduction

Ovarian cancer is a common malignancy in women and chemotherapy plays an important role in the treatment following the initial surgery. A major clinical advance was made in the early 1990s when paclitaxel (Taxol<sup>®</sup>) in combination with a platinum derivative was introduced in the treatment of ovarian carcinoma [1]. Paclitaxel has a unique mechanism of action in that it binds to  $\beta$ -tubulin in tubulin heterodimers [2]. These heterodimers, consisting of one  $\alpha$ -tubulin and one  $\beta$ -tubulin subunit, self-associate into polymers and cylindrical tubes that constitute the microtubule. Microtubules undergo rapid transitions between growth and shrinkage due to association and dissociation of tubulin dimers [3]. Microtubules containing paclitaxel bound tubulin are unusually stable and thus the drug suppresses the depolymerisation of microtubules [2]. The change in tubulin dynamics leads to interference with the formation of the mitotic spindle and the cells arrest at mitosis. Eventually, bcl-2 becomes hyperphosphorylated and the cells undergo apoptosis [1].

The clinical success of cancer chemotherapy is limited by the development of drug resistance. Several potential mechanisms have been proposed for paclitaxel resistance, including alterations in the cellular target tubulin such as changes in tubulin expression and mutations in the tubulin genes [3]. However, conclusive results have been difficult to obtain mainly due to the presence of multiple tubulin isoforms that are encoded by a large gene family consisting of both functional and non-functional genes with a high degree of nucleotide similarity. In humans, six different  $\beta$ -tubulin isoforms have been identified and are classified as follows (Roman numerals represent the protein class and Arabic numerals the gene): class I, *M40*; class II,  $\beta9$ ; class III,  $\beta4$ ; class IVa,  $5\beta$ ; class IVb,  $\beta2$ ; class VI,  $\beta1$ . The expression of these isoforms is tissue-dependent, but classes I and IVb are ubiquitously expressed and class I (gene *M40*) contributes to the major fraction of the  $\beta$ -tubulin isoforms [4]. Altered expression of the different  $\beta$ -tubulin isoforms, especially classes III and IVa, has been found in paclitaxel-resistant cell lines as compared to the parental cell line [3, 5]. It has also been suggested that paclitaxel-resistant cell lines contain “hyostable“ microtubules and that the tubulin equilibrium is shifted towards dimer formation [6]. This indicates that some of the paclitaxel-resistant cells contain less stable microtubule polymers, which has led to studies of the tubulin genes to identify mutations or polymorphisms that would explain the presence of tubulin with different microtubule dynamics. In resistant cell lines, point mutations have been found at several locations in the  $\beta$ -tubulin gene *M40* as well as in  $K\alpha1$ -tubulin [7-10].

Several research groups have studied the presence of tubulin mutations in human tumours. Monzo et al. (1999) identified  $\beta$ -tubulin mutations in 33% of patients with non-small-cell lung cancer [11]. Many groups have attempted to confirm this initial study; but conflicting results have been reported [12-15]. Most of these studies have used fluorescence-based DNA sequencing techniques, which may ignore small subpopulations of cells with altered DNA sequences. The analysis of the  $\beta$ -tubulin *M40* gene has also proved to be difficult due to nucleotide similarities with the other five known isoforms as well as the presence of several pseudogenes. The accuracy of the original gene sequence for  $\beta$ -tubulin *M40* (J00314) has also been questioned as it is thought to contain several discrepancies compared to the correct gene as well as the mRNA sequence [16-18]. We therefore designed a study to evaluate the presence of  $\beta$ -tubulin *M40* mutations in DNA isolated from ovarian tumours using single strand conformation analysis (SSCA) as mutation analysis, followed by sequencing of the shifted products, and to correlate the results with treatment effects.

## 2. Materials and methods

### 2.1. Tumour and Patient Characteristics

We selected 40 chemo-naïve epithelial ovarian tumours obtained at surgery from four patient groups. In the first group (n=10) the patients had a complete response (both clinically and chemically) during chemotherapy with paclitaxel in combination with carboplatin and were tumour-free for at least 18 months after chemotherapy. The second group (n=14) had been treated with the same chemotherapeutic agents, but the tumours progressed during treatment or the patients had a relapse within nine months. The patients were treated with paclitaxel for at least four cycles (median 8), except for one patient who received one cycle before chemotherapy was discontinued. The other two groups (n=12 and n=4) had the same clinical outcome, although they had been treated with non-tubulin-affecting chemotherapy (in most cases carboplatin, epirubicin and cyclophosphamide). Thirty-one of the 40 patients underwent non-radical surgery. The other nine had radical surgery (n=4) or the results of surgery were unknown (n=5). Six of these nine patients later suffered a relapse of ovarian cancer.

Sixteen tumours were collected from paraffin embedded tissues stored at the Division of Molecular and Immunological Pathology, Linköping University Hospital, and 24 tumours were freshly frozen and from a bio-bank at the Department of Oncology, Sahlgrenska Academy at Göteborg University. The study was approved by the local ethics committee.

### 2.2. DNA-Isolation

After tumour collection, genomic DNA was isolated from the paraffin embedded tissues using chloroform/phenol extraction. The paraffin was removed by extraction with xylene (Sigma, Stockholm, Sweden), ethanol 100% and ethanol 70% (Kemetyl, Stockholm, Sweden). DNA was extracted from the remaining tissue by adding 200µl buffer (50 mM Tris-HCL, 1 mM EDTA, 0.5% Tween-20, 0.2 mg/mL proteinase K) (Sigma) and incubated at 65°C over night. To remove heavy metal ions 100 µl of a chelex-100 slurry (Biorad, Stockholm, Sweden) was added (1:1 w/v in distilled water) and incubated at 100°C for 10 min. The genomic DNA in the supernatant was then extracted with phenol/chloroform/isoamyl alcohol (25:24:1) (Sigma) and with chloroform/isoamyl alcohol (24:1), precipitated with sodium acetate (0.3M)/ethanol, washed with ethanol, dried and

reconstituted in TE buffer (10 mM Tris-HCl, pH 7.5 and 1 mM EDTA). DNA was isolated from the freshly frozen tumours using QIAamp<sup>®</sup> DNA mini-kits (VWR, Stockholm, Sweden) according to the manufacturer's protocol. The amount of DNA extracted was quantified using absorbance spectroscopy (260 and 280 nm) and diluted to 10 ng/ $\mu$ l for working solutions (stored at -20°C).

### 2.3. PCR

The sequences of the PCR primers (Table 1) for amplification of exons 1 to 4 were designed using primer 0.5 free software to amplify specific regions of the  $\beta$ -tubulin *M40* gene (GenBank accession number AC006165). Primers were checked for specificity using the NCBI BLAST server (<http://www.ncbi.nlm.nih.gov/blast/>) and special attention was paid to the gene sequences of the other isoforms and the pseudogenes. For exons 1 to 3, the primers were placed in the 5'-UTR and in the introns. For exon 4, two rounds of amplification, one initial and one nested, generated PCR products (denoted 4.1-4.5) for further analysis. All primers were purchased from Invitrogen (Paisley, Scotland, UK).

The PCR reactions were carried out on a Mastercycler gradient (Eppendorf) in a total volume of 20  $\mu$ l with AmpliTaq Gold (Applied Biosystems, NJ, USA) (2  $\mu$ M PCR primers, 0.8 mM dNTPs, 1.5 mM MgCl<sub>2</sub> and 25 ng of human genomic DNA as template). The reactions were optimised for an annealing temperature and MgCl<sub>2</sub> concentration to yield single PCR products. For the initial PCR, the following temperature cycle was used: 1 cycle at 95°C for 10 min; 35 cycles at 95°C for 30 s, 61°C for 30 s and 72°C for 1 min; followed by 1 cycle at 72°C for 7 min. For the nested amplification, the product was diluted 10-fold in deionised water and 1  $\mu$ l was added to the second PCR reaction. The amplification of the final products as well as the reactions for exons 1-3 were carried out with the following temperature cycles: 1 cycle at 95°C for 10 min; 35 cycles at 95°C for 30 s, 61°C for 30 s and 72°C for 30 s; followed by 1 cycle at 72°C for 7 min.

### 2.4. Single Strand Conformation Analysis - SSCA

PCR products (0.5  $\mu$ l) were labelled with an addition of 0.5  $\mu$ Ci [ $\alpha$ -<sup>32</sup>P]dATP (Amersham Pharmacia Biotech, Piscatawa, NJ) to a new PCR reaction for 8-15 cycles and subsequently mixed with denaturising loading buffer (47% formamide, 0.1% SDS and 10 mM EDTA). After denaturation by heating to 90°C for at least 2 min, the samples were applied to

a non-denaturing polyacrylamide gel (6% acryl amide-Bis 19:1, 10% glycerol in TBE buffer) and run for 18 h at 5W for 200-235 bp fragments and at 8W for 270-300 bp fragments. The gel was then transferred to 3 mm filter paper, vacuum-dried and subjected to autoradiography at -70°C.

### *2.5. Sequencing of Shifted Products*

The PCR products displaying a mobility shift in the SSCA were excised, dissolved in H<sub>2</sub>O and sequenced using the Thermo Sequenase radiolabelled terminator cycle sequencing kit according to the manufacturer's (USB Corporation, Cleveland, OH) instructions. The products were purified using GFX<sup>TM</sup> PCR DNA and the Gel band purification kit (Amersham Pharmacia Biotech) and labelled with <sup>33</sup>P-dideoxy nucleotides during a PCR reaction with the original primers. The radiolabelled products were separated on a 6% denaturing polyacrylamide gel at 70 W constant power. The gel was dried after electrophoresis and subjected to autoradiography. All sequence variants and mobility shifts were confirmed in an independent PCR with original DNA.

The specificity of the PCR reactions was confirmed by sequencing the PCR products using both forward and reverse primers and the sequences were consistent with AC006165 (GenBank).

### 3. Results

DNA was successfully isolated from all tumour samples and produced single PCR-products as evaluated by agarose gel electrophoresis.

First we tried to amplify the gene described in the GenBank sequence J00314; however, we were never able to amplify PCR products corresponding to that sequence. We then redesigned the primers using the sequence AC006165, and the PCR reactions using these primers (Table 1) successfully amplified that sequence. We then screened the tumour material for mutations in  $\beta$ -tubulin *M40* using SSCA (Fig. 1).

When DNA from the freshly frozen tumours was analysed, one of the 24 showed a mobility shift corresponding to exon 3. This shift was reproduced in a second independent PCR and shifted fragment was estimated to correspond to about 2% of the total PCR product after studying the autoradiography results after the SSCA. However, the sequence of the shifted product corresponded to the sequence described in AC006165 with an overlapping sequence for about 17-22 bases near the forward primer placed in intron 2 (overlapped sequence -27 - -5 ACACCTCTTAACCTTTATTCTCT, overlapping sequence TAWSGTGTTGCARGGGTGCAA). The overlapping sequence did not correspond to any known gene sequence found in GenBank and did not overlap any of the bases in the exon. This shift was then considered to be a PCR artefact or, if present in the tumour DNA, it would not affect the amino acid sequence of the protein.

Eight of the 16 paraffin embedded tumours displayed a mobility shift in one or more exons in the first SSCA (Fig. 1, lanes 13 and 14). The sequence of the shifted products corresponded to non- $\beta$ -tubulin sequences, single or multiple nucleotide shifts in the  $\beta$ -tubulin gene some of which corresponded to pseudogenes and some being novel mutations. However, none of the shifts could be reproduced on subsequent SSCA. Finally, all samples displayed the same pattern on the SSCA gel as a non-mutated sequence.

#### 4. Discussion

After performing mutation analyses using SSCA on 200-300 bp fragments corresponding to all the exons including the exon/intron borders of the  $\beta$ -tubulin *M40* gene (GenBank AC006165), we conclude that no mutations or polymorphisms could be detected in our material. When using DNA material from paraffin embedded tumours we identified numerous false positive mutations, i.e. the mutations were not reproducible in independent PCRs with original DNA.

Designing primers for amplification of the  $\beta$ -tubulin gene has been shown to be difficult [15-18], which may explain some of the results in the first report by Monzo et al. (1999) [11]. This has been complicated by the presence of several gene sequences in the databases for the  $\beta$ -tubulin gene. Three sequences have been used in the literature: J00314 [12, 13], AC006165 [15] and AF000512 [19], of which the last two are identical, at least for the part corresponding to the  $\beta$ -tubulin gene, and are now considered to be the correct sequence. In our study we were not able to design primers that amplified the sequence in J00314 and we therefore redesigned the sequence to fit AC006165, which proved successful.

Getting the right sequence is also complicated by the presence of, to date, five other isoforms and especially the pseudogenes (K00842, K00840, K00841, AF252825, J00315, J00316, M24191, J00317, V00598, M28484 and AC109329 (bp117000-118400)). Most of the pseudogenes do not contain introns but are highly homologous in the coding part, which results in co-amplification of pseudogenes when using exonic primers [15]. Tsurutani et al. (2002) circumvented this problem by using both DNA and cDNA as templates, which may be useful if the pseudogenes do not produce any mRNA. However, at least one of the pseudogene sequences has been found in cDNA. In our study we used nested PCR to achieve higher specificity and produce PCR products of suitable sizes for SSCA.

For the paraffin embedded tissue samples, eight of the 16 tumours gave false positive results and some of these shifts corresponded to co-amplification of the pseudogenes. In four samples the same base substitution, codon 251 CGC>TGC, was identified. This sequence variant is present in a newly identified pseudogene (AC109329), as well as the pseudogene 14 $\beta$  (K00840), and turned out to be polymorphic (investigated using RFLP, data not shown). However, if one of the four samples had given the same base substitution in two subsequent PCRs, instead of in four parallel PCRs, the variant would have incorrectly been considered a mutation. In addition to co-amplification of pseudogenes, some of the false positive results

may be explained by the use of tissue that had been formalin fixed and paraffin embedded, which is known to induce sequence alterations [20], as well as DNA fragmentation. None of the identified mutations were confirmed in an independent PCR, SSCP and DNA sequencing and therefore were not considered to be true mutations.

The interpretation of previous studies on non-small-cell lung cancer (NSCLC) [11] is complicated since they are performed with non-specific primers [15, 19]. As shown in this study, the use of paraffin embedded tissue might also explain the high frequency of mutations in that material.

The present study on ovarian cancer confirms the results reported by Kelley et al. (2001), Kohonen-Corish et al. (2002) and Lamendola et al. (2003) indicating that no mutations were found in the  $\beta$ -tubulin gene [14, 15, 21]. Kelley et al. (2001) examined 20 NSCLC primary tumour samples, Kohonen-Corish et al. (2002) studied 29 patients with resected lung tumours and Lamendola (2003) examined 29 paired ovarian tumour samples without finding any mutations [14, 15, 21]. Kohonen-Corish et al. (2002) and Lamendola et al. (2003) only examined part of the  $\beta$ -tubulin gene, but in the present study all exons of  $\beta$ -tubulin *M40* were examined without finding any mutations or polymorphisms, and thus it does not seem to constitute a mutational target in ovarian cancer.

On the other hand, several studies have reported silent mutations in the  $\beta$ -tubulin gene, especially at codons 180 (GTC>GTT), 195 (AAT>AAC) and 217 (CTG>CTA). Hasegawa et al. (2002) found that 35% (22/62) of the breast cancer patients investigated had the CTG to CTA transition at codon 217, as well as an additional somatic mutation in one patient [19]. Similarly, Sale et al. (2002) reported a high frequency (17%) of the transition at codon 217 [13]. Tsurutani et al. (2002) found the codon 180 and 195 variants in 3 (for each transition) out of 17 NSCLCs investigated, but they did not find any variation at codon 217 in these patients [12]. Several of these variants are present in the pseudogenes, which complicates the interpretation. The discrepancies found in these studies and in ours may be due to ethnic differences between Caucasians and Asian populations. The materials presented by Hasegawa et al. (2002) and Tsurutani et al. (2002) were from a Japanese population and Sale et al. (2002) found most of the variants in Asian, African and Oceanic populations [12, 13, 19]. Our study as well as the other ones in which no variants in the  $\beta$ -tubulin gene have been reported, is based on Caucasian populations.

In conclusion, we did not find any sequence variants in the exons of the  $\beta$ -tubulin gene *M40* in genomic DNA from chemo-naïve ovarian tumours. Thus, it is not likely that mutations

in the tubulin gene constitute a resistance mechanism to paclitaxel in human tumours and therefore cannot be used to predict the clinical response to antitubulin drugs. We cannot rule out that these variations occur later in the carcinogenesis or in other isoforms. Our results from the mutational analysis of paraffin embedded tissues also indicate that, in addition to the use of exonic primers, the pre-treatment of the material may influence the results. The rarity of true somatic mutations as well as missense polymorphisms in clinical samples suggests that other mechanisms such as changes in  $\beta$ -tubulin isoform expression and changes in metabolism or drug efflux may be more important factors in antitubulin drug resistance.

## Acknowledgements

This study was supported by grants from the Swedish Cancer Society, Gunnar Nilsson's Cancer Foundation and County Council in Östergötland. The authors wish to thank Isaac Austin for proofreading the text.

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## Table

**Table 1:** PCR primers for each exon and corresponding PCR product size.

	<b>Forward primer, 5'-3'</b>	<b>Reverse primer, 5'-3'</b>	<b>Product, bp</b>
Exon 1	CTTGCCCCATACATACCTTG	AGGATAGCGGGTGCAAAT	199
Exon 2	GGGACTTGACCTGTTGT	GTGGGAGACAGGGAAG	196
Exon 3	AACCTTCCCTTCTGCCAGAT	CCTTGCACCCAAATAAGTTGA	224
Exon 4			
Nested 1	AGTTGAAAGATGGAAACATCATG	ATGTTCTTGGCATCGAAGAC	690
4.1	CCTGTTAATTGAGCTTTTCTCC	AGGCACCACACTGAAGGTAT	272
4.2	CCGAGAAGAATACCCTGAT	TCAGATCCCCGTAGGTT	212
4.3	TGCTTCCGCACTCTGAA	GTTCCGGCACTGTGAGAG	235
Nested 2	CTCCATTTCTTTATGCCTGG	GCCATTATCTACATGTGTTTTCA	913
4.4	CACCAGCCGTGGAA	CTGTGCTATTGCCAATG	299
4.5	CTCAAGATGGCAGTCA	AAGGGAAC TGAGAAGC	292

## Figure Legend

**Fig. 1.** SSCA of eight different PCR products corresponding to exons 1-4 of the  $\beta$ -tubulin gene *M40*. Lanes 13a and 13b correspond to exon 4.4 amplified from the same sample using two independent PCR reactions. The shifts found in lane 13a, corresponding to the arrows I, were excised and sequenced. The sequence in these fragments consisted of a single point mutation at codon 283 (GCT-GCC), giving rise to a silent mutation. However, the shift could not be reproduced using an independent PCR, shown in lane 13b. During the same SSCA, a shift was found in lane 14a corresponding to arrow II, but this shift could not be reproduced using an independent PCR at the following SSCA, shown in lane 14b.

**Fig. 1.**

