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Original Article

Influence of *CYP3A4* genotypes in the outcome of serous ovarian cancer patients treated with first-line chemotherapy: implication of a CYP3A4 activity profile

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Abstract: CYP3A4 is a key enzyme involved in the metabolism of numerous compounds, such as paclitaxel, and its activity shows an extensive inter-individual variation which can influence treatment response. The study's purpose was to investigate the potential predictive role of a CYP3A4 profile (*CYP3A4*1B*, rs2740574 and *CYP3A4*22*, rs35599367) in serous ovarian cancer patients treated with first-line chemotherapy (paclitaxel and cisplatin or carboplatin), after cytoreductive surgery. *CYP3A4*1B* and *CYP3A4*22* genotypes were determined by Nested PCR-RFLP and Taqman® Allelic Discrimination, respectively. We observed that the mean survival rates were statistically different according the patients *CYP3A4* genotypes. The group of patients carrying the *CYP3A4*1B* G allele present a decreased mean survival rate when compared with AA genotype patients (103.93 and 134.44 months, respectively, $p = 0.010$). This result is consistent after multivariate Cox regression analysis (HR, 2.15; 95% CI, 1.03-4.52; $p = 0.043$). The combination of *CYP3A4*1B* and *CYP3A4*22* polymorphisms result in the definition of a CYP3A4 activity profile: the group of patients with a higher CYP3A4 activity profile had significantly diminished survival when compared with patients with a lower CYP3A4 activity profile (101.06 and 134.44 months, respectively, $p = 0.012$). Multivariate Cox regression analysis revealed a diminished overall survival time for patients with CYP3A4 high activity profile (HR, 2.29; 95% CI, 1.05-5.02; $p = 0.038$). The definition of a CYP3A4 activity profile resulted in the increase of prediction ability, using Harrels's concordance indexes (C-index from 0.617 to 0.626). To conclude, our results demonstrate an association between *CYP3A4*1B* and a diminished overall survival of patients with serous ovarian cancer. The definition of a CYP3A4 activity profile proved to be benefic and the CYP3A4 high activity profile was associated with a lower overall survival. We consider that the definition of a CYP3A4 activity profile might be useful as molecular marker for predicting the clinical outcome of serous ovarian cancer patients.

Keywords: Serous ovarian cancer, CYP3A4, polymorphism, pharmacogenetic, paclitaxel, predictive value

Introduction

Ovarian cancer (OC) is the third most common gynecological cancer among women worldwide, with an estimate 225 000 new cases and 140 000 deaths due to this disease each year [1]. In Europe, ovarian cancer is the fifth most incident cancer in women but the main cause of death among gynecological cancers [2].

Standard treatment for OC patients is based on cytoreductive surgery, followed by first-line chemotherapy with platinum (cisplatin or carboplatin) and a taxane agent (paclitaxel or docetaxel). However, despite this aggressive approach, the 5-year survival rates remains only around 45% [3]. The high fatality rate results in part from the frequent diagnosis of OC at an advanced stage, with 75% of all cases being diagnosed in stage III and IV [4, 5]. Furthermore, although nearly 80% of patients initially respond to treatment, long-term survival remains poor because of eventual tumor recurrence and emergence of drug-resistant disease [6].

The development of chemotherapy resistance is poorly understood and many investigations

are made to comprehend the biological mechanisms responsible for acquisition of resistance. Although many non-genetic factors may influence the effects of therapy, there are numerous studies that show that inter-individual differences in drug response and toxicities can be due to genetic polymorphisms in genes encoding drug-metabolizing enzymes, drug transporters and drug targets. Therefore, genetic polymorphisms in these genes can be useful as prognostic and predictive markers in cancer, namely in OC [7-12].

One of the most studied drug-metabolizing enzymes is CYP3A4 (Cytochrome P450, family 3, subfamily A, Polypeptide 4), a key enzyme involved in the metabolism of numerous exogenous and endogenous compounds, accounting for 30-60% of total liver cytochrome P-450 protein [13-15]. Compounds metabolized by CYP3A4 include chemotherapeutic agents like tamoxifen or taxanes, namely paclitaxel [16-18].

CYP3A4 activity or protein content shows 10 to 100 fold inter-individual variation, influencing drug response/toxicity and hence therapy outcome [15, 19-21]. The most known and studied *CYP3A4* polymorphism is *CYP3A4*1B* (rs-2740574), which consists in an A/G transition in the promoter region at position-392 (A-392G) [22]. Initially, *CYP3A4*1B* G allele was associated with an increased CYP3A4 activity due to reduced binding of transcriptional repressor [23]. Nevertheless, besides being intensively studied, the biological effect of *CYP3A4*1B* polymorphism is still controversial although remaining as one of the most important polymorphisms in *CYP3A4* gene [18, 23-32].

Recently, Wang and collaborators identified a functional SNP (Single Nucleotide Polymorphism) located in intron 6 of *CYP3A4* gene (*CYP3A4**22 C/T, rs35599367), which markedly affects expression of CYP3A4 and could be a promising biomarker for predicting response to CYP3A4-metabolized drugs [33]. This study showed that *CYP3A4*22* is significantly linked to an early defect in transcription and RNA processing and could affect the folding of single-stranded DNA or nascent RNA and hence RNA elongation, potentially affecting the binding of regulatory proteins. Consequently, CYP3A4*22 T allele was associated with the decreased CYP3A4 mRNA expression and enzymatic activity [33].

For its role in the metabolism of chemotherapeutic agents, including paclitaxel, inter-individual differences in expression of CYP3A4 enzyme can be conditional for different responses to the first-line treatment of OC, affecting the patients overall survival. The main purpose of this work was to investigate the potential predictive role of *CYP3A4*1B* polymorphism in OC patients treated with paclitaxel/platinum first-line chemotherapy. Additionally, we also intended to combine *CYP3A4*1B* and *CYP3A4*22* genotypes in order to evaluate the overall survival rates according to a CYP3A4 activity profile.

Materials and methods

Patients

We conducted a hospital-based study on 261 European female patients with histologically confirmed ovarian cancer admitted and treated, between 1996 and 2009, in the departments of gynecology and oncology of the Portuguese Institute of Oncology, Porto, Portugal. Within this group of patients were excluded those who did not have serous ovarian tumors and who did not underwent first-line chemotherapy, consisting in paclitaxel (175 mg/m^2) and cisplatin (75 mg/m^2) or carboplatin (AUC 5-7.5) at 21-day intervals for six cycles, after cytoreductive surgery. This combination chemotherapy was the standard treatment in our institute for these patients. The therapy was concluded when objective disease progression was observed or unacceptable toxicity appeared. Patients' clinical characteristics were obtained from their medical records (n = 120). The tumor stage was evaluated according to the staging system of the International Federation of Gynecology and Obstetrics (FIGO) and the assessment of the tumor response to chemotherapy was based on World Health Organization (WHO) criteria. Blood samples were obtained with the written informed consent of participants prior to their inclusion in the study, according to Helsinki Declaration principles. The study was approved by the ethics committee of Portuguese Institute of Oncology-Porto.

DNA extraction and genotyping

Blood samples were obtained with a standard technique and collected in ethylenediaminetetraacetic acid (EDTA)-containing tubes. Genomic DNA was extracted from the white blood cell

Table 1. Relation between *CYP3A4*1B* and *CYP3A4*22* genotypes (high or low activity genotype) and clinicopathological parameters in serous OC

Parameter	High activity genotype[a)]	Low activity genotype[b)]	p*
CYP3A4*1B genotype			
Age (years), mean ± SD	50.3 ± 11.2	50.8 ± 10.6	0.78**
FIGO stage			
I/II	2 (12.5%)	22 (21.2%)	0.42
III/IV	14 (87.5%)	82 (78.8%)	
Hormonal Status			
Pre-menopause	5 (31.3%)	36 (37.1%)	0.40
Post-menopause	11 (68.7%)	61 (62.9%)	
Residual Disease			
Optimal Surgery	4 (26.7%)	30 (32.6%)	0.85
Residual tumor ≤ 2 cm	3 (20%)	14 (15.2%)	
Residual tumor > 2 cm	8 (53.3%)	48 (52.2%)	
Relapse			
Yes	11 (73.3%)	61 (61.6%)	0.42
No	4 (26.7%)	38 (38.4%)	
Survival			
Alive with no evidence of cancer	7 (43.8%)	64 (63.4%)	0.14
Dead, or alive with evidence of cancer	9 (56.2%)	37 (36.6%)	
CYP3A4*22 genotype			
Age (years), mean ± SD	53.9 ± 12.3	54.6 ± 12.5	0.88**
FIGO stage			
I/II	17 (18.3%)	2 (33.3%)	0.36
III/IV	76 (81.7%)	4 (66.7%)	
Hormonal Status			
Pre-menopause	33 (37.5%)	3 (60%)	0.32
Post-menopause	55 (62.5%)	2 (40%)	
Residual Disease			
Optimal Surgery	28 (32%)	2 (40%)	0.60
Residual tumor ≤ 2 cm	15 (17%)	-	
Residual tumor > 2 cm	45 (51 %)	3 (60%)	
Relapse			
Yes	58 (63.7%)	4 (80%)	0.11
No	33 (36.3%)	1 (20%)	
Survival			
Alive with no evidence of cancer	52 (55.9%)	4 (66.7%)	0.60
Dead, or alive with evidence of cancer	41 (44.1%)	2 (33.3%)	

*χ^2 test with the exception of *t-student* analysis for the age comparison (**). [a)]For *CYP3A4*1B* and *CYP3A4*22*, G carrier genotype and CC genotype were considered as high activity genotypes, respectively. [b)]For *CYP3A4*1B* and *CYP3A4*22*, AA genotype and T carrier genotype were considered as low activity genotypes, respectively.

fraction of each study subject by using QIAamp DNA Blood Mini Kit (QIAGEN), according to the manufacturer's protocol.

*CYP3A4*1B* genotypes were determined by Nested PCR-RFLP (*Polymerase Chain Reactio n–Restriction Fragment Length Polymorphism)* method adapted from a previously established protocol by Zeigler-Johnson *et al*. [34]. The restriction fragments were then visualized by 3% (p/v) agarose gel electrophoresis, with ethidium bromide staining. Three types of band patterns were obtained: wild type homozygote (A/A), one band corresponding to 207 bp; het-

Table 2. Relation between CYP3A4 activity profile (high or low activity profile) and clinicopathological parameters in serous OC

Parameter	High activity profile[a)]	Low activity profile[b)]	p*
Age (years), mean ± SD	50.5 ± 11.7	50.9 ± 10.7	0.88**
FIGO stage			
I/II	-	22 (21.2%)	0.66
III/IV	13 (100%)	82 (78.8%)	
Hormonal Status			
Pre-menopause	5 (38.5%)	36 (37%)	0.93
Post-menopause	8 (61.5%)	61 (63%)	
Residual Disease			
Optimal Surgery	2 (16.7%)	28 (34.6%)	0.40
Residual tumor ≤ 2 cm	3 (25%)	12 (14.8%)	
Residual tumor > 2 cm	7 (58.3%)	41 (50.6%)	
Relapse			
Yes	9 (75%)	61 (61.6%)	0.32
No	3 (25%)	38 (38.4%)	
Survival			
Alive with no evidence of cancer	5 (38.5%)	64 (63.4%)	0.08
Dead, or alive with evidence of cancer	8 (61.5%)	37 (36.6%)	

*χ^2 test with the exception of *t-student* analysis for the age comparison (**). a)*CYP3A4*1B* G carrier or *CYP3A4*22* CC genotype were considered as high activity profile .b)*CYP3A4*1B* AA genotype or *CYP3A4*22* T carrier were considered as low activity profile.

erozygote (A/G), three bands corresponding to 18, 189 and 207 bp; polymorphic homozygote (G/G), two bands corresponding to 18 and 189 bp.

The *CYP3A4*22* genotyping was performed using Taqman® Allelic Discrimination (C__5-9013445_10). All allelic discrimination PCR reactions were carried out in 6 µL volumes using 2.5 µL of TaqMan® Universal PCR Master Mix (2X), 0.125 µL of 40x assay mix, 2.375 µL of sterile water and 1 µL of genomic DNA. Amplification of DNA was carried out using the following amplification conditions: 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. Data capture and analysis was carried through the ABI7300 Real Time PCR System (Applied Biosystems) and the Sequence Detection Systems software (Applied Biosystems version 1.2.3).

Quality control was assured by the use of non-template controls in all runs and blind replicate genotype assessment on 10% of the samples.

Statistical analysis

Analysis of data was performed using the Statistical Package for Social Sciences (SPSS) computer software for Windows™ (Version 17.0). Differences in proportions were evaluated by the χ^2 test. The probabilities of survival were calculated and the means life tables were computed using the product-limit estimate of Kaplan-Meier method. The curves were analyzed by the Breslow (generalized Wilcoxon) test, a statistical test for equality of survival distributions. A level of $p < 0.05$ was considered statistically significant. Survival duration was defined as the time between diagnosis and either death or the time of the last clinical evaluation of the patient. Cause of death was determined from the patient's records, death certificate, or by speaking with her general practitioner.

The associations between *CYP3A4* polymorphisms and survival were estimated by Cox regression analysis. Cox regression models were used to adjust for potential confounders with *CYP3A4* genotypes fitted as indicator variables. The concordance (*c*) index was used to compare the predictive ability of *CYP3A4* genotypes, with C > 0.5 being considered with good prediction ability.

In relation to CYP3A4 activity profile, based on published data, we defined that patients carrying *CYP3A4*1B* G allele or *CYP3A4*22* CC gen-

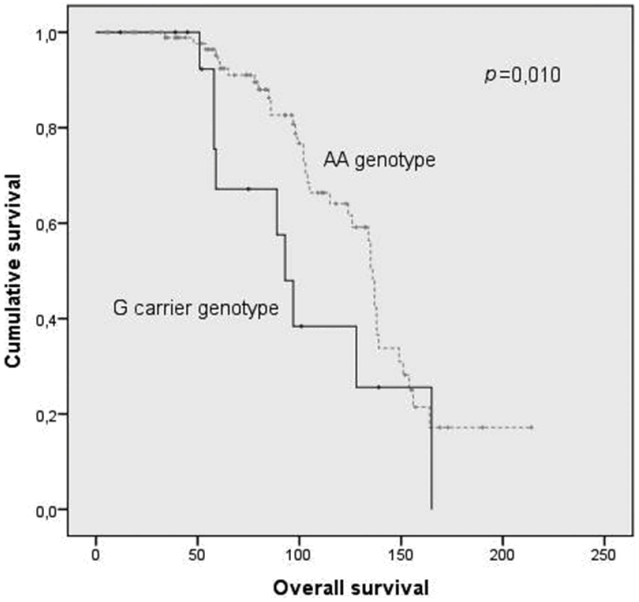

Figure 1. Overall survival by Kaplan-Meier and Breslow test of serous OC patients, according to *CYP3A4*1B* genotypes. The group of patients with G allele carrier genotypes had significantly diminished survival when compared with patients with AA genotype (HR, 2.15; 95% CI, 1.03-4.52; $p = 0.043$).

otype have a CYP3A4 high activity profile whereas patients with *CYP3A4*1B* AA genotype or carrying *CYP3A4*22* T allele have a CYP3A4 low activity profile [18, 23, 33, 35-38].

Results

The patients' clinic characteristics according to *CYP3A4*1B* and *CYP3A4*22* genotypes are shown in **Table 1**. There were no significant statistical differences between the group of patients with the high activity genotype (*CYP3A4*1B* G carriers or *CYP3A4*22* CC genotype) and the low activity genotype (*CYP3A4*1B* AA genotype or *CYP3A4*22* T carriers) regarding age at diagnosis, clinical stage, histological subtype, hormonal status, extent of residual disease, relapse or survival. Furthermore, we assessed the patients' clinic characteristics according to CYP3A4 activity profile (**Table 2**). Likewise, there were no significant statistical differences between the CYP3A4 activity profiles and the clinicopathological parameters assessed.

Concerning the overall survival rates obtained using Kaplan-Meier method and Breslow test, we observed that the mean survival rates were statistically different according to *CYP3A4*1B* patients' genotypes. The group of patients with G allele carrier genotypes had significantly diminished survival when compared with patients with AA genotype (103.93 and 134.44 months, respectively, $p = 0.010$) (**Figure 1**). Thus, individuals with AG/GG genotypes showed a lower overall survival, conferring a worse prognosis for G allele carrier patients. Using a multivariate Cox regression model, we found a diminished overall survival for G carrier patients, when compared with AA genotype, with age as covariate (hazard ratio (HR), 2.15; 95% confidence interval (95% CI), 1.03-4.52; $p = 0.043$).

Additionally, we analyzed the overall survival rates according CYP3A4 activity profile. The group of patients with a higher CYP3A4 activity profile had significantly diminished survival when compared with patients with a lower CYP3A4 activity profile (101.06 and 134.44 months, respectively, $p = 0.012$) (**Figure 2**). We observed that 5-year survival rate was 68% and 94% for patients with high activity and low activity profile, respectively. Once again, using a Cox regression model with age as covariate, we found that individuals with a higher activity profile had an inferior survival when compared to individuals with a lower activity profile (HR, 2.29; 95% CI, 1.05-5.02; $p = 0.038$). Therefore, individuals with *CYP3A4*1B* G carrier genotype or *CYP3A4*22* CC genotype (high activity profile) had a lower overall survival than individuals with *CYP3A4*1B* AA genotype or *CYP3A4*22* T carrier (low activity profile).

The increased predictive value of CYP3A4 high activity profile compared to *CYP3A4*1B* polymorphism, in relation to serous OC overall survival, was assessed with Harrell's concordance indexes, with an improvement in the prediction ability from 0.617 to 0.626.

Discussion

Ovarian cancer is one of the most common causes of cancer-related death among women and the major cause of death due to gynecological cancer [2]. This high mortality rate is mainly due to the late diagnosis of the disease, frequently detected at advanced stages, and to the low rate of therapy efficacy, with a great number of the patients developing resistance to therapy and a consequently recurrence of

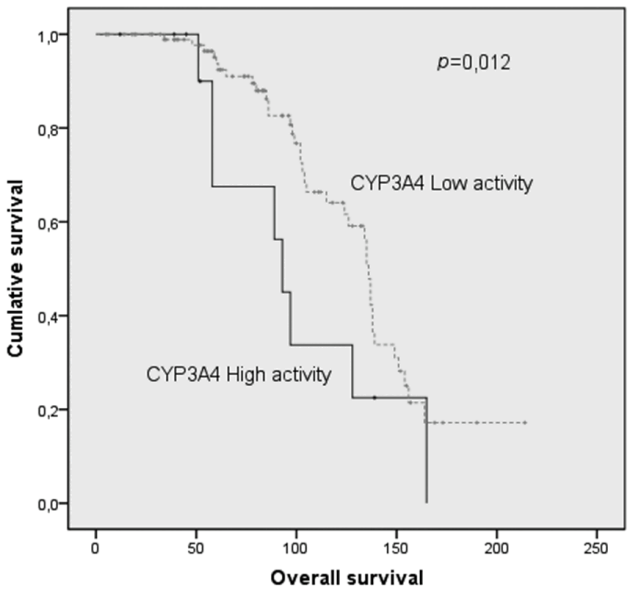

Figure 2. Overall survival by Kaplan-Meier and Breslow test of serous OC patients, according to CYP3A4 activity profile. The group of patients with a higher CYP3A4 activity profile had significantly diminished survival when compared with patients with a lower CYP3A4 activity profile (HR, 2.29; 95% CI, 1.05-5.02; $p = 0.038$).

the disease [5, 6, 39, 40]. Nowadays, it is accepted that inter-individual variations among patients, which are often associated to genetic polymorphisms in specific genes, can be responsible for different responses to the therapy as well as being useful as prognostic and predictive factors [7-9, 41-44].

The CYP3A4 enzyme, essential for phase I metabolism, can metabolize a panoply of endogenous and exogenous compounds, promoting their transformation in hydrophilic compounds easily eliminated by the organism [13, 14]. One of the most important oncologic drugs utilized in the clinical practice is paclitaxel and, like the other members of the taxanes family, it mediates their cytotoxic effect by the polymerization and stabilization of the microtubules, conducting to the arrest of cellular cycle in G2/M transition and to apoptosis [45]. Paclitaxel is partially metabolized by CYP3A4 enzyme and the resulting metabolites are less toxic and are present in a lower concentration in plasma when compared with the initial compound. In this way, the resulting metabolites are less potent than paclitaxel and it seems like they don't have a significant therapeutic effect [46, 47].

CYP3A4 shows great importance in a metabolic level and is greatly studied in the field of translational research with many efforts to identify genetic variation in CYP3A4 gene capable to alter enzyme activity. Despite its controversial biological significance, many studies continue to assess the predictive role of *CYP3A4*1B* in cancer research [27, 48-51]. However, to the best of our knowledge there are few studies that evaluate the role of this polymorphism as prognostic factor and none of them in relation to OC overall survival. Our results demonstrate an association between *CYP3A4*1B* and a diminished survival of patients with serous OC. Multivariate Cox regression analysis indicated a decreased overall survival for *CYP3A4*1B* G carrier, when compared with AA genotype, after adjustment for age (HR, 2.15; 95% CI, 1.03-4.52; $p = 0.043$). As suggested by previous published studies, we believe that the presence of the G allele of *CYP3A4*1B* polymorphism might increase the CYP3A4 activity [18, 23, 35-37]. This increased activity might be responsible for a great metabolism of paclitaxel to less active metabolites, which are less effective in the treatment of OC, conducting to the development of resistance phenotypes and, consequently, to a worse therapy efficacy. Consistent with enhanced expression of the minor allele, G allele carrying patients had a decrease of overall survival around 31 months ($p = 0.010$).

Regarding the contradictory results about the effect of *CYP3A4*1B* polymorphism, a newly discovered SNP in intron 6 may now introduces new knowledge and assists in the explanation of the inter-individual variation in CYP3A4 activity [33]. In this way, as suggested by the literature, we define a CYP3A4 activity profile using *CYP3A4*1B* and *CYP3A4*22* genotypes: individuals *CYP3A4*1B* G carriers or with *CYP3A4*22* CC genotype have a CYP3A4 high activity profile whereas individuals with *CYP3A4*1B* AA genotype or *CYP3A4*22* T carriers have a CYP3A4 low activity profile [18, 23, 33, 35-38]. Using these combinations, we observed a significant association between the high activity profile and a diminished overall survival of serous OC patients. Combining *CYP3A4* genotypes revealed a decreased of overall survival around 34 months to patients with a high activ-

ity profile (p = 0.012). Multivariate Cox regression analysis, with age as covariate, indicated a decreased overall survival for CYP3A4 high activity profile, when compared with low activity profile (HR, 2.29; 95% CI, 1.05-5.02; P = 0.038). This result, which is in agreement with the literature, indicated that as occurs with *CYP3A4*1B* G allele, patients with *CYP3A4*22* CC genotype have an enhanced enzymatic activity and, presumably, a higher capability to metabolize paclitaxel to less active metabolites [33, 38]. Additional data showed that the definition of CYP3A4 activity profiles increased the significance of the observed effects in the outcome of serous OC patient, with an improvement in the prediction ability for CYP3A4 high activity profile (C-index from 0.617 to 0.626).

Although serous tumors are frequently characterized as having an aggressive behavior but usually being good responders to standard chemotherapy, the increase of activity of CYP3A4 might conduct to the acquisition of a resistance phenotype by serous OC and consequently to a lower overall survival of these patients [6, 52].

In this study, we investigate one of the most important and studied metabolizing enzymes with a putative impact on the pharmacokinetics of paclitaxel. Under this assumption, we thought that genetic polymorphisms are capable to alter CYP3A4 activity and consequently influence survival of serous OC patients submitted to first-line chemotherapy. However, additional studies are required to confirm our findings and to provide more detailed analysis of the influence of these polymorphisms in ovarian cancer patients' survival. The influence in survival of serous OC patients might be due directly through the excessive clearance of paclitaxel or indirectly through the diminished concentration of active paclitaxel in the tumor microenvironment since *CYP3A4* is not only expressed in the digestive tract but also in the ovary [53]. Consequently, it would be a future aim to combine the studied *CYP3A4* polymorphisms with other genetic polymorphisms in metabolic enzymes, such in *CYP3A5*, *CYP2C8* or Glutathione S-Transferases (*GSTM1* and *GSTT1*), and in drug transporters, such in *ABCB1*, to evaluate their impact in first-line chemotherapy response, either alone or in combination. In this study we did not directly measure CYP3A4 expression or activity so further research is planned to quantify the impact of *CYP3A4*1B* and *CYP3A4*22* on CYP3A4 activity and paclitaxel biotransformation, as performed by Henningsson and colleagues [57].

Nowadays, new strategies have been developed in order to overtake the problematic of resistance to treatment and to improve clinical outcome in advanced-stage ovarian cancer. One of the strategies to enhance anti-tumor activity and prolong survival is the dose-dense weekly administration of paclitaxel [54-58]. In a study developed by Leiser and colleagues [59], their pharmacokinetics findings suggest that repeated doses of weekly paclitaxel might induce its own hepatic metabolism. In general, paclitaxel dose is determined by the patient's body surface area rather than by the patient's metabolic profile. Inter-individual differences in paclitaxel metabolism can implicate major alterations in blood concentration of paclitaxel and it metabolites with implication in toxicity and treatment efficacy [60-62].

Conclusion

To the best of our knowledge this is the first study that evaluate the presence of *CYP3A4*1B* polymorphism in the overall survival of OC patients and the first study to evaluate the influence of the new genetic *CYP3A4* intron 6 polymorphism in ovarian cancer patients. We consider that both polymorphisms might alter CYP3A4 activity and affect consequently dose requirements, response and toxicity to drugs with a narrow therapeutic window as chemotherapy agents. Therefore, the definition of a CYP3A4 activity profile could help to define a pharmacogenetic profile of serous OC, improving the clinical response of these patients to the standard first-line regimen. With the development of new therapeutic regimens for advanced ovarian cancer, the CYP3A4 activity evaluation can also be important in the prediction of weekly dose-dense paclitaxel regimen effectiveness.

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Disclosure of conflict of interest

The authors have declared that no competing interests exist.

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