Physiol. Res. 64 (Suppl. 4): S539-S547, 2015

Contribution of *ABCB1* and *CYP2D6* Genotypes to the Outcome of Tamoxifen Adjuvant Treatment in Premenopausal Women With Breast Cancer

S. ARGALÁCSOVÁ[1,2], O. SLANAŘ[2,3], P. VÍTEK[4], P. TESAŘOVÁ[1], H. BAKHOUCHE[2], M. DRAŽĎÁKOVÁ[3], O. BARTOŠOVÁ[2], T. ZIMA[3], L. PERTUŽELKA[1]

[1]Department of Oncology, First Faculty of Medicine, Charles University and General Teaching Hospital, Prague, Czech Republic, [2]Institute of Pharmacology, First Faculty of Medicine, Charles University and General Teaching Hospital, Prague, Czech Republic, [3]Institute of Clinical Biochemistry and Laboratory Diagnostics, First Faculty of Medicine, Charles University and General Teaching Hospital, Prague, Czech Republic, [4]Institute of Radiotherapy, First Faculty of Medicine, Charles University and Hospital Na Bulovce, Prague, Czech Republic

Received September 17, 2015
Accepted October 12, 2015

Summary

Recent pre-clinical evidence suggests that the active metabolite of tamoxifen, endoxifen, is a substrate for efflux pump P-glycoprotein. The aim of our study was to evaluate, if the polymoprhisms within *ABCB1* gene alter tamoxifen adjuvant treatment efficacy in premenopausal women. Totally 71 premenopausal women with estrogen receptor positive breast cancer indicated for tamoxifen adjuvant treatment were followed retrospectively for median period of 56 months. The gentic polymorphisms of *CYP2D6* and *ABCB1* were analyzed and potential covariates as tumor grading, staging, age at the diagnosis, comedication, quantitative positivity of ER or PR were also evaluated. Cox proportional-hazards regression model indicated that patients carrying at least one variant allele in *ABCB1* rs1045642 had significantly longer time to event survival compared to wild type subjects. Non-significant trend was noted for better treatment outcome of patients carrying at least one variant allele in the SNP rs2032582, while for the *CYP2D6* polymorphism poor metabolizer phenotype resulted in worse outcome in comparison to extensive metabolizers subjects with HR of 4.04 (95 % CI 0.31-52.19). Similarly, patients using CYP2D6 inhibitors had non-significantly shorter time-to-event as compared to never users resulting in hazard ratio of 2.06 (95 % CI 0.40-10.63). *ABCB1* polymorphisms may affect outcome of tamoxifen adjuvant treatment in premenopausal breast cancer patiens. This factor should be taken into account in addition to the *CYP2D6* polymorphism or phenotypic inhibition of CYP2D6 activity.

Key words

P-glycoprotein • SNP • Pharmacogenomics • Tamoxifen • CYP2D6

Corresponding author

O. Slanař, Institute of Pharmacology, First Faculty of Medicine and General Teaching Hospital, Albertov 4, 128 00 Prague 2, Czech Republic. Fax: +420224968104. E-mail: oslan@lf1.cuni.cz

Introduction

Breast cancer is the most frequent cancer affecting women worldwide. Its incidence and prevalence has been steadily rising and search for predictive biomarkers of prognosis and therapeutic success is one of the major challenges for oncologists today.

The large interindividual variability in the treatment response is driven by both genetic diversity of patients as well as factors associated with tumor (Higgins *et al.* 2009). One of the therapeutic modalities of choice and one of the first targeted therapies for patients with hormone-positive breast cancer is hormone therapy. Since the early 1980s, adjuvant therapy with tamoxifen has been the gold standard for reducing risk of recurrence in women with estrogen receptor positive breast cancer.

Long-term data have demonstrated that the use of tamoxifen reduced recurrence and mortality by more than 30 %. However, meta-analyses of comparative studies on the adjuvant hormonal therapy in postmenopausal patients with hormone-dependent tumor indicate higher efficacy of aromatase inhibitors (AI) over tamoxifen treatment (Forbes *et al.* 2008, Howell *et al.* 2005). Despite the conclusions drawn from these studies tamoxifen remains the important treatment option in patients with hormone-dependent breast cancer. It remains a first line option for premenopausal women and in the prophylaxis of carcinoma *in situ*. Also in postmenopausal women tamoxifen is still frequently used.

The results of the ATAC study show difference in recurrence rates between tamoxifen and IA groups less than 5 % (Forbes *et al.* 2008, Schroth *et al.* 2009). Therefore, if there were any biomarkers for prediction of treatment response to tamoxifen, some subgroups of postmenopausal women could benefit from tamoxifen to the same extent or even more than from IA application. CYP2D6 activity has been proposed as one of the pontential biomarkers, since it is the liver drug-metabolizing enzyme responsible for *in vivo* conversion of tamoxifen. The antiestrogenic activity of tamoxifen is dominantly mediated through the active metabolites 4-hydroxytamoxifen, and 4-hydroxy-N-desmethyl-tamoxifen (endoxifen) that possess approximately 100 times higher afinity to estrogen receptores than the parent compound itself (Brauch *et al.* 2008). It has been shown that extensive metabolizers of CYP2D6 have higher endoxifen plasma levels compared to patients with genetic CYP2D6 deficiency and similar decrease in endoxifen exposition has been observed in patients receiving paoxetine, a strong CYP2D6 inhibitor, as a comedication to tamoxifen compared to patients, who did not use any CYP2D6 inhibitor (Stearns *et al.* 2003).

Based on pharmacokinetic data it could be expected that less enzymatic activity reduces the bioactivation of tamoxifen and its efficacy may be reduced.

Pharmacogenetic studies estimated that a considerable proportion of caucasian patients carried a functionally deficient C*YP2D6* allele, while the *CYP2D6*4* variant (1846G>A) was the most prevalent one (Bradford 2002, Buzkova *et al.* 2008, Sachse *et al.* 1997). Approximately 5-10 % of Caucasians are predicted as CYP2D6 poor metabolizers (PM) with complete enzyme dificiency and approximately 40 % of the population represent heterozygous extensive metabolizers or intermediate metabolizers (IM), who possess partially decreased CYP2D6 activity in comparison with extensive metabolizers (homozygous wilde type allele carriers, EM) (Buzkova *et al.* 2008, Kirchheiner 2008, Zanger *et al.* 2004).

Previous publications provided evidence that endoxifen is substrate for an active efflux transport pump P-glycoprotein (Iusuf *et al.* 2011, Teft *et al.* 2011). Plasma endoxifen levels did not significantly differ between wild-type and Mdr1-deficient mice. However, brain concentrations of endoxifen were nearly 20-fold higher in Mdr1-deficient mice compared to wild-type animals. Because P-glycoprotein is highly expressed at the blood-brain barrier and in some breast cancer tumors, variation in expression and function of this transporter may alter the therapeutic outcome of tamoxifen treatment. Many polymorphisms have been identified in the *ABCB1* gene coding for P-glycoprotein (multidrug-resistance gene-MDR-1) some of which have functional consequence for the expression of the gene in the target tissoues and/or transport capacity of the substrates (Hoffmeyer *et al.* 2000, Sakaeda *et al.* 2003). Therefore, we conducted this study to evaluate, if the genetic polymorphisms rs1045642 and rs2032582 within the *ABCB1* gene that may affect the expression of the P-glycoprotein efflux pump affect the therapeutic efficacy of tamoxifen treatment in premenopausal women, while CYP2D6 status was taken into account as well.

Methods

Premenopausal patients with breast cancer indicated to 5 year adjuvant tamoxifen treatment with or without adjuvant chemotherapy between 1985-2011 complying with the inclusion criteria have been selected for this study. The study was conducted in the single centre at the Department of Oncology, General Teaching Hospital in Prague and the responsible Ethics committee approval was obtained before the study start up. All enrolled patients signed informed consent.

The main inclusion criteria included histologically confirmed breast cancer, estrogen receptor positivity of at least 10 %, primarily local or loco-regional stage of the disease (I-III).

The exposure to comedications known as CYP2D6 inhibitors that could confound the CYP2D6 activity was recorded. The comedication effect on

CYP2D6 has been reviewed based on interaction table at http://medicine.iupui.edu/clinpharm/ddis/table.aspx

We summarized each patient's medical history by searching the hospital records. Tumor stage was classified according to Union for International Cancer Control guidelines and summarized as stage I, II, or III. TNM classification was considered at the time of diagnosis. Adverse effects were recorded and classified according to organ system that has been affected. Patients receiving tamoxifen for less than 6 months due to adverse effects have been exluded from this study. This study was retrospective; the primary analysis was conducted on time-to-event end points as time to recurrence-disease free survival (DFS) or time to progression (TTP).

Genotyping

Samples of peripheral venous blood for DNA isolation were collected in tubes containing K_2EDTA and immediately frozen and stored at –20 °C until further processing. DNA was subsequently isolated using QIAamp DNA Blood Mini Kit (Quiagen Ltd.). Detection of the polymorphisms in C*YP2D6* gene was done by AmpliChip (Roche) microarray based method. The following alleles have been detected *CYP2D6*3, *4, *5, *6, *7, *8, *9, *10, *11, *14, *15, *17, *19, *20, *25, *26, *29, *30, *31, *35, *36, *40, *41* and duplication/multiplication of the gene. After exclusion of the presence of any of the above mentioned alleles, wild type *CYP2D6*1* genotype has been assigned.

The genotypes of *ABCB1* (rs2032582 and rs1045642) were determined using a modified analysis by Cascorbi *et al.* (2001). DNA amplification was done in a termocycler My-Cycler (BioRad, USA). Reaction mixture consisted from 60 ng of DNA template, 8 nM primers (sequence was identical to the primers used by Cascorbi *et al.* (2001), PCR Buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTP and 0.5 U of Tag DNA polymerase. Water was added to a final volume of 25 µl. PCR amplification consisted of initial denaturation for 2 min at 94 °C followed by 35 cycles denaturation at 94 °C for 30 s, annealing at 60 °C for 45 s and extension at 72 °C for 1 min. Terminal elongation ran at 72 °C for 7 min. Restriction by enzymes BseNI, BshNI, and Bsp1431 produced DNA fragments that were separated on 3.5 % agarose gel and visualized after ethidium bromide staining on the ultraviolet transilluminator.

Statistical analysis

The time to recurrence/progression was calculated from the date of diagnosis. All recurrences/ progressions were taken into account. Data of event-free patients were censored as of the date when the last follow-up information was obtained. Allelic distribution was evaluated comparing with Hardy-Weinberg equilibrium.

The statistical significance of differences was tested using the Kruskal-Wallis test. A Pearson´s χ^2 test was used for categorical data. The primary analysis of time-to-event end points was performed with the use of Mantel-Cox test.

Analysis was performed on subgroups defined by the factors that had been determined: age, staging and grading of the disease, degree of ER positivity and degree of PR positivity. The probability of event-free survival was estimated with the Kaplan-Meier technique. For the analysis of prognostic factors, a Cox proportional-hazards model was used. All relevant tests were performed at the 5 % level of significance.

Results

Seventy-one patients have been enrolled into the study. Median age of the patients at the time of diagnosis was 44 years (range 26-52 years) and median follow-up was 56 months (range 8-198 months). The basic clinical characteristics of the study population are summarized in the Table 1. The genotypes of *CYP2D6*, and *ABCB1* polymorphisms rs2032582 and rs1045642 are summarized in the Table 2. The distribution of the variant alleles in our study population did not substantially differ from normal distribution. We noted 12 (16.9 %) of patients, who received inhibitors of CYP2D6 as comedication to tamoxifen treatment.

There were statistically non-significant differences between subgroups of patients according to tumor size at the time of diagnosis, although the tendency towards less frequent progression among T1 stage patients was observed with 12.1 %, 31.5 %, and 20.0 % of progressing patients within the subgroups of T1, T2, and T3, respectively.

Further analyses revealed that neither the quantification of estrogen/progesterone receptor positivity nor age of the patients nor tumor grading at time of diagnosis represented a prognostic factor for treatment outcome.

Table 1. Clinical characteristics of the study population (n=71).

Characteristics	n (%)
Stage – tumor size (T)	
T1 ≤ 2 cm)	43 (60.6 %)
T2	19 (26.8 %)
T3	3 (4.2 %)
T4	1 (1.4 %)
Tx	2 (2.8 %)
Tis	3 (4.2 %)
N status	
N0	45 (63.4 %)
N1	21 (29.6 %)
N2-3	2 (2.8 %)
Nx	3 (4.2 %)
Histology	
IDC	52 (73.2 %)
ILC	10 (14.1 %)
Mixed (ILC+IDC)	4 (5.6 %)
Other	5 (7.4 %)
Grade	
G1	15 (21.1 %)
G2	24 (33.8 %)
G3	11 (15.5 %)
Gx	21 (29.6 %)
PR status	
PR positive (≥ 10 %)	62 (87.3 %)
PR negative	7 (9.9 %)
PR unknown	2 (2.8 %)
Her-status	
Negative	46 (64.8 %)
FISH positive	6 (8.5 %)
Unknown	19 (26.7 %)
Chemotherapy	
Adjuvant	27 (38.0 %)
Neoadjuvant	9 (12.7 %)
Both – adjuvant plus neoadjuvant	4 (5.6 %)
No chemotherapy	31 (43.7 %)
Radiotherapy	
Yes	48 (67.6 %)
No	20 (28.2 %)
Unknown	3 (4.2 %)
Local therapy	
Mastectomy	29 (40.8 %)
Segmentectomy/Tumorectomy	42 (59.2 %)

Table 2. Distribution of the CYP2D6 predicted phenotypes and *ABCB1* polymorphisms in the study population.

	n (%)
CYP2D6 predicted phenotype	
UM	1 (1.4 %)
EM	34 (47.9 %)
hetEM	29 (40.8 %)
PM	7 (9.9 %)
rs1045642	
CC	17 (23.9 %)
CT	41 (57.8 %)
TT	13 (18.3 %)
rs2032582	
GG	24 (33.8 %)
GT	34 (47.9 %)
TT	11 (15.5 %)
GA	1 (1.4 %)
TA	1 (1.4 %)
AA	0 (0 %)

With a median follow-up of 56 months in the whole group the median time to event was 156 months. Survival analysis shows that although there was no significant difference of the time-to-event end points among CYP2D6 genotype groups a tendency towards shorter recurrence/progression free survival in PM group in comparison to EM group was noted. The hazard ratio in PM group was 4.04 (95 % CI 0.31-52.19) in comparison to EM subjects. Similarly, patients using CYP2D6 inhibitors had non-significantly shorter time-to-event as compared with never users resulting in hazard ratio of 2.06 (95 % CI 0.40-10.63). Tendency towards more frequent progression/relapse has been observed in patients with CYP2D6 deficiency as suggested by cumulative progression/relaps rates over the follow up period of 11.8 %, 20.7 %, and 28.6 % in the EM, heterozygous EM, and PM groups, respectively. The Figure 1 shows Cox proportional-hazards regression model for the whole study population. The Kaplan-Meier analyses showing survival curves for subgroups as determined by CYP2D6, and *ABCB1* polymorphisms rs1045642, rs2032582 and comedication status are shown in the Figure 2. Out of these possible predictors for the time-to-event endpoint, only *ABCB1* polymorphism rs1045642 reached statistical significance. The treatment outcome was significantly better in either homozygous or

heterozygous carriers of the variant allele compared to the wild-type (P=0.012).

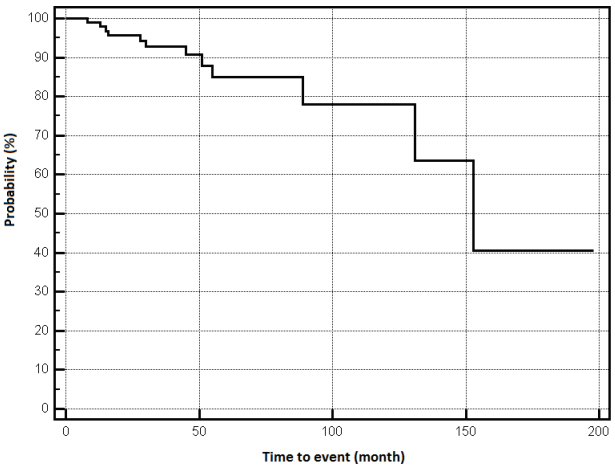

Fig. 1. Time to event survival probability in the study population.

Discussion

The genotype distribution in the study population is not only compliant with the theoretical normal distribution, but also very similar to distribution of *CYP2D6* and *ABCB1* variants in the Czech healthy population as documented by comparison with a „historical control groups“ formed in our previous studies (Buzkova *et al.* 2008, Pechandova *et al.* 2006). Such a comparison suggests that there was no obvious selection preference of a specific CYP2D6 or ABCB1 phenotype/genotype, as could for e.g. happen in case of intolerance of some treatment. Further, the *CYP2D6* or *ABCB1* polymorphisms do not belong to known susceptibility factors for breast cancer (Rodriguez-Antona *et al.* 2006), therefore the distribution of the polymorphic alleles is expected to be similar to that in the healthy population in the same region as noted in our study.

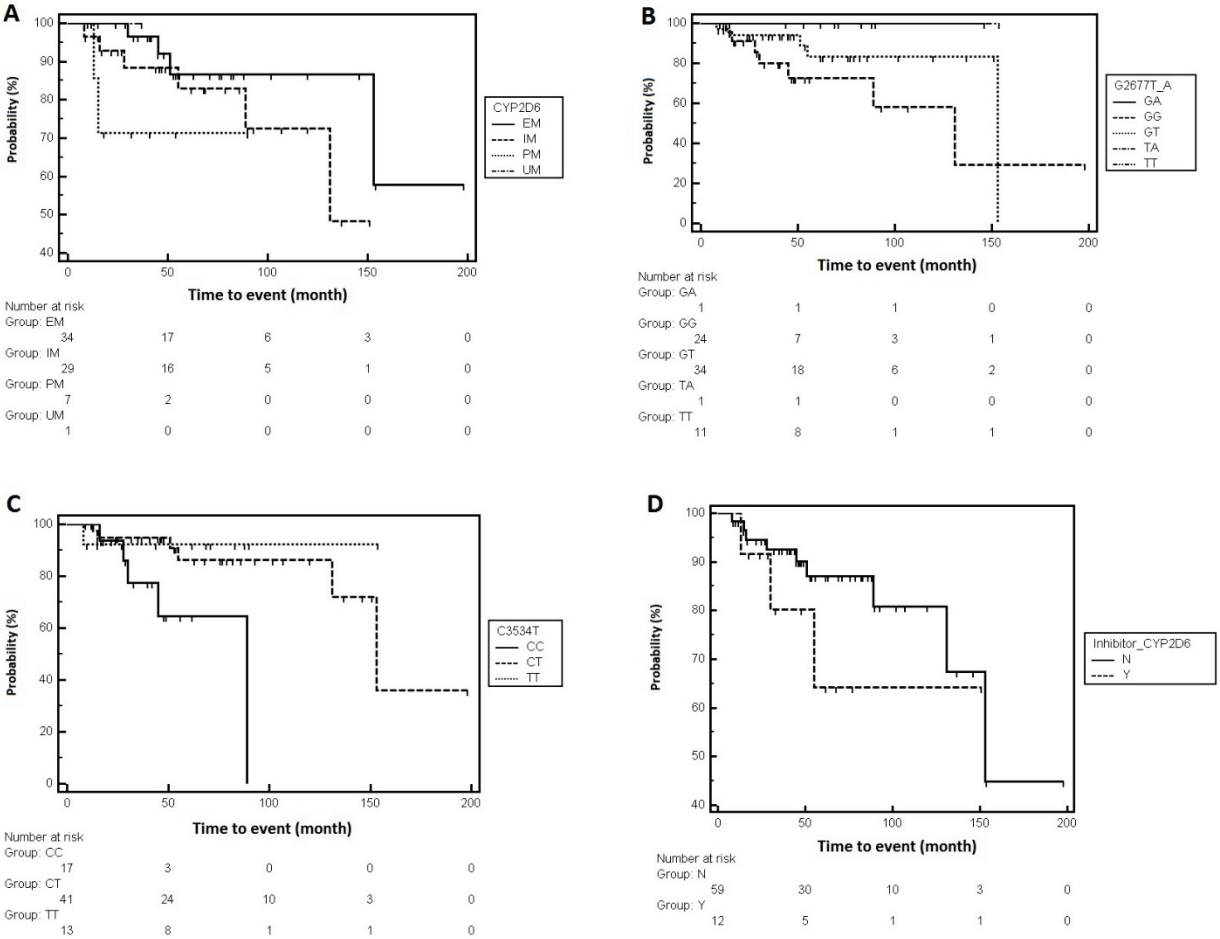

Fig. 2. Kaplan-Meier analysis of the time to event stratified by (**A**) CYP2D6 predicted phenotype status, (**B**) *ABCB1* polymorphism rs2032582 *(G2677T/A*), (**C**) *ABCB1* polymorphism rs1045642 *(C3435T)*, and (**D**) presence of CYP2D6 inhibitory comedication.

This study was conducted in premenopausal patients, who represent currently the most appropriate primary target population for tamoxifen treatment according to current therapeutic guidelines (Burstein *et al.* 2014). Since this subpopulation represents approximately one third of the breast cancer patients, this inclusion criterion contributed to the lack of statistical significance of the survival analyses noted for all the genetic factors except *ABCB1* rs1045642. However, the results of our study suggest that diminished activity of CYP2D6 may represent an important risk factor for worst treatment outcome of adjuvant tamoxifen therapy. Firstly, when taking into account only the extreme genotypes of PM and EM groups the hazard ratio of 4.04 of PM subjects found in our study is considerably high although displaying substantial variability. Secondly, the event free survival time is shorter also in CYP2D6 deficiency combining both genotypic CYP2D6 deficiency and possible drug-drug interactions in EM subjects although the hazard ratio value is less (2.06). Such a hazard ratio is potentially still of clinical relevance for individual patients. Thirdly, PM group displayed not only shorter time to event, but also a proportion of patients with detected progression/relaps during the folow up tended to be higher, though again not reaching level of statistical significance. In this aspect our study endorses the previous findings suggesting CYP2D6 polymorphism as a possibly important risk factor for the prognosis of adjuvant treatment.

Indeed, some published studies demonstrate a higher risk of recurrence of the disease and shortening of DFS in patients carrying functionally variant alleles of CYP2D6 (Bonanni *et al.* 2006, Goetz *et al.* 2005, 2007, Newman *et al.* 2008, Schroth *et al.* 2007, 2009, Trojan *et al.* 2013, Yazdi *et al.* 2015).

Meta-analysis of ten previous clinical reports (n=5183) reported significantly increased risk of breast cancer recurrence in patients carrying variant CYP2D6 genotypes (Jung *et al.* 2014). Significant effect of CYP2D6 on treatment outcome in pre-menopausal subpopulation was published recently (Saladores *et al.* 2015).

By analogy, a decrease of tamoxifen efficacy could be expected in patients using comedications with CYP2D6 inhibitors (e.g. antidepressants) (Kelly *et al.* 2010).

However, there are clinical trials with contradictory results not confirming the CYP2D6 dependency of tamoxifen effects either as a result of inherent deficiency or comedications (Abraham *et al.* 2010, Nowell *et al.* 2005, Wegman *et al.* 2005, 2007). Therefore, due to the inconclusive evidence standardized testing patients has not been implemented into the clinical praxis (Dezentje *et al.* 2009) while the drug regulatory agencies in the EU adopted a label change for tamoxifen providing information on the discrepant results and the clinical need for factors predicting efficacy of the treatment that remain largely unmet.

Our study is different from most previously published as only premenopausal women have been enrolled. It is well documented that the premenopausal women have worse prognosis in general, either as consequence of less awareness of this disease in the younger population or due to a different pathophysiological background of the disease in pre-, and post-menopausal population for e.g. more intense estrogen load in premenopausal women. Thus some discussion still continues if the disease in the two menopausal status groups shall not be regarded as substantially different. In that aspect our study contributes by providing the data in subgroup representing minority of the general patient population. However, since the recent therapeutic guidelines change, the premenopausal population is the primary target population for tamoxifen adjuvant therapy.

This is to our best knowledge the first study describing possible impact of A*BCB1* polymorphisms (rs2032582, rs1045642) on the treatment outcome of tamoxifen therapy in premenopausal breast cancer patients. Significantly shorter time to event has been observed for the wild-type homozygous patients in the rs1045642 polymorphism as compared with the heterozygous or homozygous carriers for the variant allele. The variant allele in this polymorphism has been reported to result in decreased P-glycoprotein expression leading to reduced efflux activity of the active transporter. Therefore our observation may reflect increased intracellular exposure to the tamoxifen active moiety in the target tissue leading to better treatment outcome in patients carrying the variant allele. However, our study was not designed in a way to clarify the mechanisms behind the findings. Although the differences between the genotype subgroups for the rs2032582 polymorphisms have not reached statistical significance, the survival analysis shows similar tendency as was noted in the rs1045642 polymorphism, i.e. trend for decreased time to event time in the wild type homozygous group as compared with the variant allele carriers.

ABCB1 polymorphisms may affect outcome of tamoxifen adjuvant treatment in premenopausal breast cancer patients. This pharmacogenetic factor should be taken into account in addition to the CYP2D6 polymorphism or phenotypic inhibition of CYP2D6 activity.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

This project was supported by a grant IGA MZ CR No N10563-3 and a Charles University project PRVOUK P25/LF1/2.

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