Development of high-resolution melting analysis for *ABCB1* promoter methylation: Clinical consequences in breast and ovarian carcinoma

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Abstract. Multidrug resistance to anticancer drugs, which is often associated with enhanced expression of the ATP-binding cassette (ABC) transporter P-glycoprotein (encoded by the *ABCB1* gene) may limit the effects of cancer therapy. Epigenetic regulation of *ABCB1* expression may thus have a clinical impact. A detailed assessment of ABCB1 promoter methylation is of importance for predicting therapy outcome and prognosis. Thus, validated methods for the analysis of *ABCB1* promoter methylation are urgently required. In the present study, high-resolution melting (HRM) analysis of the CpG island regions covering the distal promoter of the *ABCB1* gene was developed and compared with pyrosequencing. In addition, the clinical effects of the methylation status of the *ABCB1* promoter were analyzed in patients with breast and ovarian carcinoma prior and subsequent to chemotherapy treatment. HRM analysis of *ABCB1* methylation correlated with the results of pyrosequencing (P=0.001) demonstrating its analytical validity and utility. Hypermethylation of the analyzed *ABCB1* promoter region was significantly correlated with low levels of the *ABCB1* transcript in tumors from a subset of patients with breast and ovarian carcinoma prior to chemotherapy but not following treatment. Finally, high *ABCB1* transcript levels were observed in tumors of patients with short progression-free survival prior to chemotherapy. Our data suggest the existence of functional epigenetic changes in the *ABCB1* gene with prognostic value in tumor tissues of patients with breast and ovarian carcinoma. The clinical importance of such changes should be further evaluated.

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Introduction

Multidrug resistance (MDR) to cytostatics, whether intrinsic or acquired, remains a barrier to successful therapy for solid tumors. The MDR phenotype often correlates with high expression of P-glycoprotein, which is the most commonly studied ATP-binding cassette (ABC) transporter (1). The protein is encoded by the *ABCB1* gene [Online Mendelian Inheritance in Man (OMIM) entry: 171050], which is located on chromosome 7q21.1, and functions as a cellular efflux pump for numerous xenobiotics, including anticancer drugs (2-4). ABCB1 is mostly expressed in excreting organs (such as the liver and kidney) and physiological barriers, including the blood-brain, -testis and -placental barriers (5,6), as well as tumor tissues such as breast and ovarian carcinomas (7-11). Correlations between *ABCB1* expression and overall or disease-free survival and response to chemotherapy have been reported in patients with breast carcinoma (12-14). A previous study on ovarian carcinoma has

shown that *ABCB1* gene/protein expression is associated with MDR (15).

Epigenetic mechanisms (i.e. mainly DNA and histone modifications) result in the regulation of genes without changes in their coding sequence (16). Epigenetic changes can be inherited (such as imprinting) and relatively stable (such as chromosome X inactivation), but more often reflect rapidly changing cell needs (17). Epigenetic changes can be induced by DNA damage (18,19). It is therefore not surprising that errors in DNA methylation are linked to a variety of effects, including imprinting defect syndromes and cancer (17).

The contribution of DNA methylation to cancer prognosis and progression has been extensively studied in recent years. Methylation of the *ABCB1* promoter occurs early during breast tumorigenesis, and it has been detected in ductal carcinoma *in situ* and invasive breast tumors (20-22). A significant association between *ABCB1* promoter methylation and protein expression was observed in invasive ductal carcinomas and paired serum samples (23). Other authors have reported an association between *ABCB1* promoter methylation and treatment response and survival of patients with breast carcinoma (21,24).

Taken together, previously published studies suggest potential epigenetic effects of promoter methylation on *ABCB1* expression and prognosis of patients with breast carcinoma, but the data are inconsistent. Different studies include different regions of the *ABCB1* gene and use non-validated technologies. Furthermore, the clinical impact of *ABCB1* promoter methylation on ovarian carcinoma prognosis or survival is currently unknown. In particular, ovarian carcinoma patients are predominantly treated with a combination of platinum derivatives and taxanes. Taxanes are substrates of ABC membrane transporters including P-glycoprotein and epigenetic regulation of the *ABCB1* promoter affecting its function may play an important role in therapeutic efficiency and development of chemoresistance to taxanes in ovarian carcinomas.

DNA methylation of the *ABCB1* promoter region can be detected and quantified by various technologies, including pyrosequencing (20-22) and methylation-specific polymerase chain reaction (PCR) mainly encompassing the binding SP-1 site in the 3'-region of the *ABCB1* gene (23-25).

The aim of the present study was to develop a novel, rapid and simple method for *ABCB1* promoter methylation analysis overlapping a transcript site using gene-specific, high-resolution melting (HRM) analysis. The present study also evaluated the functional consequences of *ABCB1* promoter methylation by the analysis of correlation between the methylation status with the expression levels of *ABCB1* gene. Finally, associations of *ABCB1* promoter methylation with the prognosis of patients with breast and ovarian carcinoma were assessed. Such associations may have a clinical impact on prognosis and individualized patient therapy, thus, offering potential socio-economic benefits.

Patients and methods

Patients. This retrospective study utilized the following historical pre-treatment and post-treatment cohorts of patients with breast and ovarian carcinoma (Fig. 1).

Breast tumor samples. Tumor tissues of 83 patients with breast carcinoma collected prior to chemotherapy (pre-treatment set). The patients were diagnosed at Motol University Hospital (Prague, Czech Republic; n=71) and the General University Hospital (Prague, Czech Republic; n=12) between February 2000 and December 2006. The collection and handling of tissue samples was described in detail elsewhere (8,26). The post-treatment set of patients with breast carcinoma was used for validation of the HRM method, and the samples were collected from hospitals in Oslo, Norway as described previously (22,27). The post-treatment set of patients was treated with 5-fluorouracil and mitomycin (n=34) or doxorubicin (n=78). The patients were enrolled in an Institutional Review Board approved protocol evaluating the drug response in a neoadjuvant setting (28-30). Paired adjacent control tissues without morphological signs of carcinoma were available for 6 patients.

Ovarian tumor samples. A total of 61 samples of patients with epithelial ovarian carcinoma (EOC) diagnosed at Motol University Hospital (Prague, Czech Republic) during the 2009-2013 period were used in the present study. In total, 11 samples were collected upon neoadjuvant treatment (post-treatment set) based on a combination of paclitaxel and platinum derivatives, while the remaining samples (n=50) were collected at the time of surgery prior to chemotherapy treatment. A total of 11 samples of ovarian tissues without morphological signs of carcinoma were used as controls, and were obtained from patients who underwent surgery for reasons other than ovarian malignancy at Motol University Hospital. The collection and handling of these tissue samples has been described in detail elsewhere (8,26,31,32).

All patients had given informed consent, and the project was approved by the Ethics Commission of the National Institute of Public Health in Prague (ethic codes: IGA no. 9799-4 of 30 January 2008, 14055-3 of 2 July 2012, and 14056-3 of 2 July 2012), and the Institutional Review Board of Norwegian Radiumhospital (Oslo, Norway) in the frame of the Norwegian Cancer Society (D-03067) and the Norwegian Research Council (163027/V409) projects. The methods were carried out in accordance with guidelines approved by the above Ethics Commissions.

ABCB1 methylation analysis by pyrosequencing. The overall study design is described in Fig. 1. DNA was extracted from freshly frozen breast tumor tissue samples by the standard phenol/chloroform extraction method. The extracted DNA was bisulfite modified using the EpiTect® Bisulfite kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's protocol. Three CpG island regions (as shown in Fig. 2) overlapping the ABCB1 promoter and transcription start site were identified using MethPrimer software (33). Quantitative DNA methylation analysis was performed by pyrosequencing of bisulfite-treated DNA from pre-treatment (n=66) and post-treatment (n=105) breast carcinoma specimens, as previously described (22,27,34).

ABCB1 methylation analysis by HRM. In the present study, HRM analysis covering the entire region of the *ABCB1* promoter was estimated using pyrosequencing and was

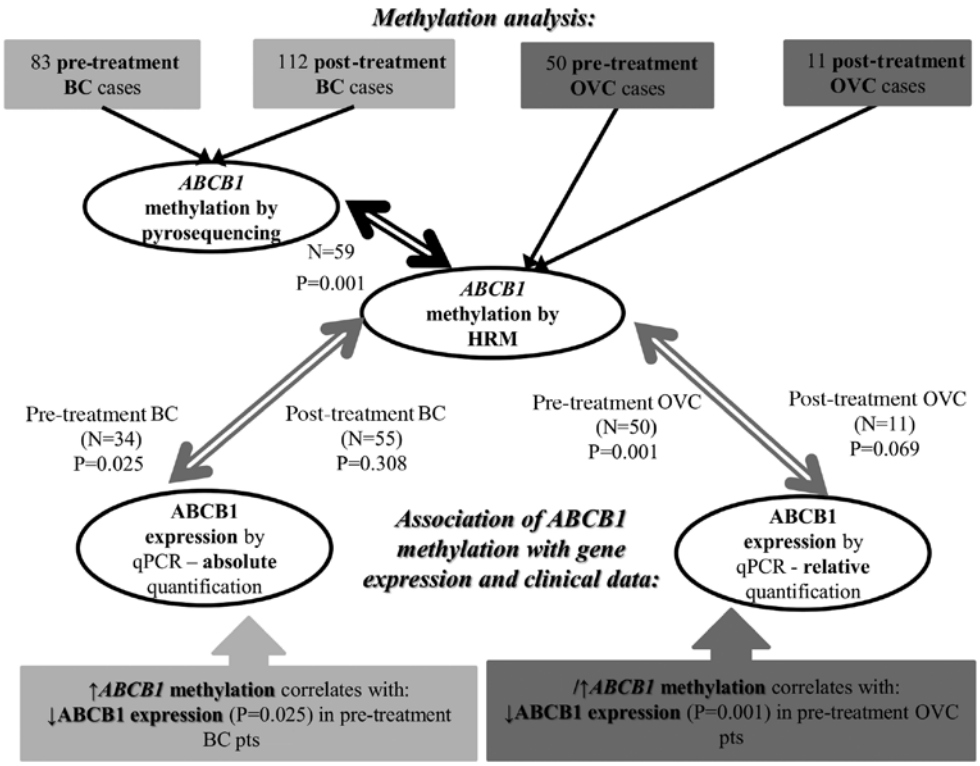

Figure 1. Flow chart of the present study. The chart describes the sample types, methods used and results of high-resolution melting estimation, as well as the major clinical findings for the sets of patients with breast and ovarian carcinoma evaluated in the present study. *ABCB1*, ATP binding cassette subfamily B member 1; BC, breast cancer; OVC, ovarian cancer; HRM, high-resolution melting; pts, patients.

established in genomic DNA samples (100-500 ng) from pre-treatment breast tumor tissues (n=59). A total of 10 ng bisulfite-converted DNA sample was used for each HRM methylation analysis. PCR amplification and subsequent HRM analysis was performed on a RG6000 system (Corbett Life Science; Qiagen GmbH) using the EpiTect HRM kit (Qiagen GmbH) according to the recommendations of the manufacturer. The reverse-transcription-quantitative PCR (RT-qPCR) cycling conditions and the sequences of the primers for HRM analysis of all the examined ABCB1 regions are summarized in Table I. A standard curve including bisulfite-converted human control DNA (Qiagen GmbH) as the fully methylated control and dilutions (0, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100%) with unmethylated control DNA (Qiagen GmbH) was included in each replicate. The collected HRM data were analyzed using Rotor-Gene software version 6.0 (Corbett Life Science; Qiagen GmbH). Subsequently, the optimal conditions for HRM analysis were used for *ABCB1* promoter methylation status in ovarian tumor tissue samples (n=61), as described in Table I.

Quantification of ABCB1 gene expression. Total RNA was isolated from frozen tissue using TRIzol reagent (Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the procedure provided by the manufacturer. The quality and quantity of the extracted RNA were assessed by spectrophotometry and agarose gel electrophoresis (28S/18S ribosomal ratio). Complementary DNA (cDNA) was synthesized using 0.5 μg total RNA and random hexamer primers with the RevertAid First Strand cDNA Synthesis kit (Fermentas; Thermo Fisher Scientific, Inc). Contamination with genomic DNA was assessed by PCR amplification of ubiquitin C (UBC) fragment capable of discriminating between products amplified from cDNA (190 bp) and from genomic DNA (1009 bp) as previously described (35).

Subsequently, absolute quantification of *ABCB1* transcript levels in breast carcinoma samples (n=34 pre-treatment and n=55 post-treatment) was performed by RT-qPCR as previously described (8). Human peptidylprolyl isomerase A (PPIA) was used as a reference gene for normalization of the *ABCB1* transcript levels. Standards for the construction of the calibration curve were prepared using Gateway Cloning Technology (Thermo Fisher Scientific, Inc.) by cloning *ABCB1* and *PPIA* gene fragments into the pDONR201 vector (Thermo Fisher Scientific, Inc.) and propagation of vectors in *Escherichia coli* DH5α Maximum Efficiency Cells (Thermo Fisher Scientific, Inc.) as previously described (8). In ovarian carcinoma samples (n=61), our recently published method of relative quantification of *ABCB1* expression with normalization to 3 reference genes, namely peptidylprolyl isomerase A (PPIA), ubiquitin C (UBC) and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein ζ (YWHAZ, 32) was used. Amplification efficiencies for each reference and target gene were calculated applying the formula Efficiency=$10^{-1/slope}$-1. The qPCR study design adhered to the Minimum Information for Publication of Quantitative Real-time PCR Experiments Guidelines (36).

Statistical analyses. Due to the deviation of the data from the normal distribution, gene methylation and expression levels were analyzed with non-parametric statistical tests. The correlation between transcript and methylation levels was assessed using Spearman's rank correlation. Mann-Whitney test,

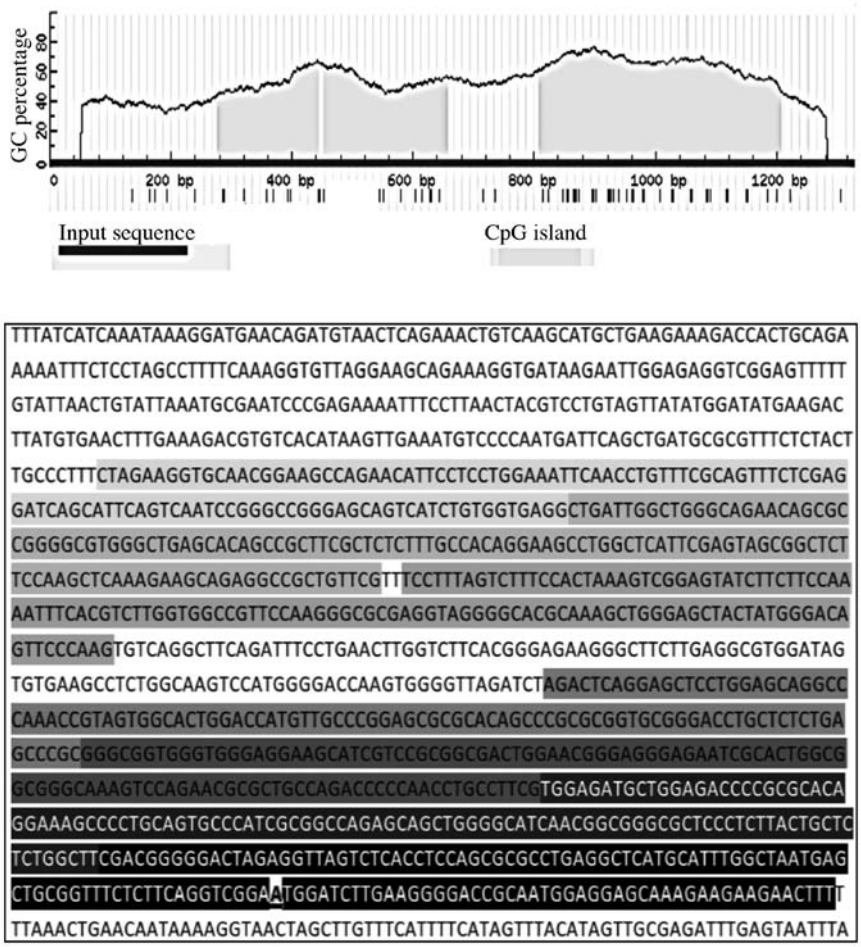

Figure 2. Schematic representation of the 3 CpG islands examined in the *ABCB1* promoter region (source sequence, NC_000007.13), as defined using MethPrimer software. Methylation of the same region of all 3 CpG islands was estimated using pyrosequencing and the newly developed HRM analysis. For optimal HRM analysis, the examined region of CpGs was divided into 7 regions as shown by the grayscale. The start site of the coding region of the *ABCB1* gene is represented by position 113,140 and underlined as **A**. HRM, high-resolution melting; *ABCB1*, ATP binding cassette subfamily B member 1.

Kruskal-Wallis test and Spearman's rank correlation were used for analysis of associations of transcript and methylation levels with clinical data. In general, the evaluated clinical and pathological variables in breast carcinomas were as follows: Stage (I/II vs. III/IV), grade (1 or 2 vs. 3), and histological type (invasive ductal vs. other invasive carcinoma). Pathological lymph node categorization (pN) was performed as follows; pN0 indicates that regional lymph node metastasis was not found, pN1-3 indicates that micrometastases or metastases in axillary lymph nodes were identified in 1-3 nodes (pN1), 4-9 nodes (pN2) or ≥10 nodes (pN3); pNx indicates that regional lymph nodes cannot be assessed. Pathological tumor size (pT) was described as follows: pT1, the tumor is ≥2 centimeters (cm) large; pT2, the tumor size is >2 but ≤5 cm; pT3, the tumor is ≥5 cm large; pT4, a tumor of any size with direct extension to chest wall and/or to the skin; pTx, a tumor size cannot be assessed (37). Expression of estrogen and progesterone receptors and p53 protein was estimated as positive vs. negative. Expression of p53 protein, as a potential marker of aggressive type of breast cancer, was available in a limited number of samples because unlike ER and PR, main prognostic factors for breast cancer, p53 is not routinely assessed in clinical practice. In ovarian carcinomas, the evaluated clinical data were: Stage (I/II vs. III/IV), grade (1 or 2 vs. 3), tumor size (pT1 vs pT2-4), histological type (high grade serous vs. others) and expression of the proliferation marker Ki67 (expressed as a percentage). Survival functions were plotted by Kaplan-Meier curves, and the statistical significance was evaluated by the log-rank test. Progression-free survival (PFS), which was defined as the time elapsed between surgical treatment and disease progression or mortality from any cause, was used for survival analysis. Two-sided $P<0.05$ was considered to indicate a statistically significant difference. The Bonferroni's test was used for adjustment of P-values due to multiple comparisons. All statistical analyses were performed using SPSS v16.0 (SPSS Inc., Chicago, IL, USA). Due to the heterogeneity between the pre-treatment and post-treatment sets, the data were analyzed separately.

Results

Study population characteristics. Breast carcinoma samples from pre-treatment and post-treatment patients (n=83 and 112, respectively) were collected in the present study. The clinical data from all patients with breast carcinomas are described in Table II. Both sets of patients significantly differed in stage distribution and estrogen and progesterone receptors expression ($P<0.001$; according to the results of χ^2 test). As an additional

Table I. Primer sequences, amplicon sizes and conditions for real-time PCR and the following HRM analysis of the examined *ABCB1* promoter.

Target *ABCB1*	Primers (5'-3')	Amplicon size (bp)	Annealing[a]
Region 1	Forward: TTAGAGAGGTGTAATGGAAGTTAGAATATTTT Reverse: CACTATTCCTACCCAACCAATCAA	140	30 sec/59˚C
Region 2	Forward: GTTGATTGGTTGGGTAGGAAT Reverse: CAAACAACAACCTCTACTTCTTTAAA	132	30 sec/55˚C
Region 3	Forward: TTTTTTAGGTTTTTTTATTAAAGT Reverse: CTTAAAAACTATCCCATAATAACTC	124	30 sec/50˚C
Region 4	Forward: AGATTTAGGAGTTTTTGGAGTAG Reverse: CTCAAAAAACAAATCCCC	101	30 sec/50˚C
Region 5	Forward: TTGTGGAGATGTTGGAGATT Reverse: ACACAAAATCTCCAACATCTCCA	132	30 sec/61˚C
Region 6	Forward: TTGTGGAGATGTTGGAGATTT Reverse: CCATCAAAACCAAAAAACAAT	116	60 sec/58˚C
Region 7	Forward: TGATGGGGGATTAGAGGTTAGTT Reverse: AAAATTCTTCTTCTTTACTCCTCCATTA	136	30 sec/57˚C

[a]PCR conditions for all regions were as follows: Initial denaturation for 10 min at 95˚C, 45 cycles at 94˚C for 15 sec, annealing temperature specified for each region in Table I and 72˚C for 30 sec with final extension at 72˚C for 10 min, followed by HRM analysis in the range of 65-95˚C at a rate 0.1˚C/cycle. ABCB1, ATP binding cassette subfamily B member 1.

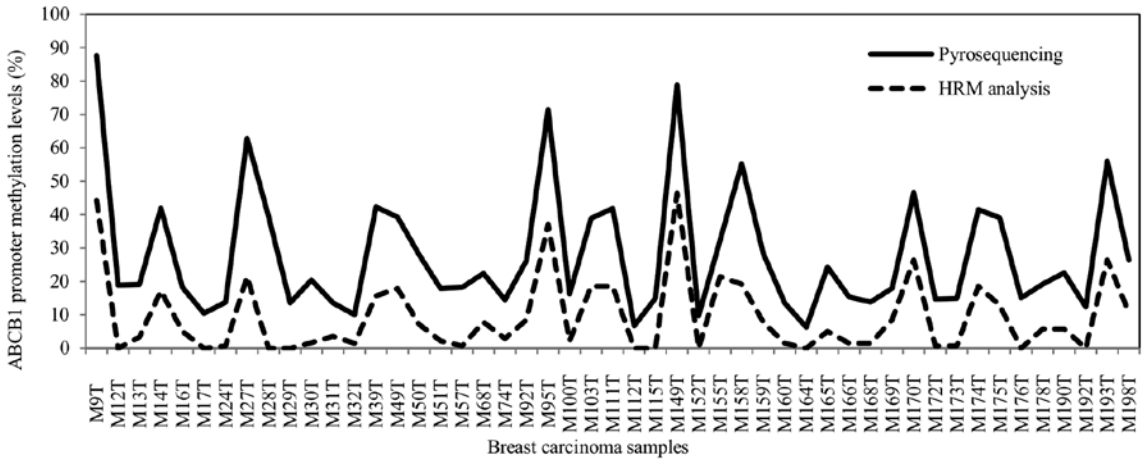

Figure 3. Comparison of *ABCB1* promoter methylation analysis by high-resolution melting analysis and pyrosequencing in individual breast carcinoma samples. HRM, high-resolution melting; *ABCB1*, ATP binding cassette subfamily B member 1.

type of solid tumor, samples from patients with ovarian carcinoma were collected in the present study (n=61). Of these, 50 samples were collected during surgery prior to any treatment while 11 samples were collected following neoadjuvant chemotherapy. All the post-treatment samples were tumors of high-grade serous carcinoma subtype with advanced stages (III or IV) and grade 3. The clinical data of the examined patients with ovarian carcinoma are described in Table III.

Development of HRM analysis for estimation of ABCB1 promoter methylation levels. For rapid, affordable and simple screening of the *ABCB1* promoter methylation status, a HRM methylation analysis was developed. First, the *ABCB1* methylation levels were estimated in a subset of the aforementioned pre-treatment breast carcinoma samples (n=59) (Fig. 1). DNA methylation levels were then compared with the DNA methylation levels of the *ABCB1* promoter that were assessed previously in tumor samples from 66 pre-treatment and 105 post-treatment patients with breast carcinoma by pyrosequencing (22,27,34). The methylation levels estimated using HRM analysis were closely associated with the pyrosequencing methylation levels in the breast carcinoma samples as estimated by Spearman's rho correlation (P=0.001, ϱ=0.699, n=59; significant after the Bonferroni's test) (Fig. 3).

The ABCB1 sequence covered by pyrosequencing and HRM analysis was the same and overlapped the transcriptional start site of the *ABCB1* gene. Neverthless, for accurate and optimal PCR amplicon lengths in HRM analysis, this

Table II. Clinical characteristics of the patients with breast carcinoma involved in the present study.

Characteristics	Pre-treatment set (n=83) n (%)[a]	Post-treatment set (n=112) n (%)[a]
Stage[b]		
I[c]	31 (37.3)	0 (0)
II	35 (42.1)	0 (0)
III	7 (8.4)	87 (77.7)
IV	2 (2.4)	20 (17.9)
N/A	8 (9.6)	5 (4.5)
Lymph node metastasis		
Present (pN1-3)	44 (53.0)	71 (63.4)
Absent (pN0)	32 (38.6)	36 (32.1)
pNx	7 (8.4)	5 (4.5)
Tumor size[b]		
pT1	45 (54.2)	0 (0)
pT2	30 (36.1)	5 (4.5)
pT3	1 (1.2)	63 (56.3)
pT4	3 (3.6)	39 (34.8)
pTx	4 (4.8)	5 (4.5)
Histological type		
Invasive ductal carcinoma	67 (80.7)	93 (83.0)
Other types of carcinoma[c]	16 (19.3)	19 (17.0)
N/A	0 (0)	0 (0)
Expression of ER[b]		
Positive	48 (57.8)	89 (79.5)
Negative	31 (37.3)	23 (20.5)
N/A	4 (4.8)	0 (0)
Expression of PR[b]		
Positive	46 (55.4)	76 (67.9)
Negative	33 (39.8)	36 (32.1)
N/A	4 (4.8)	0 (0)
Expression of p53		
Positive	21 (25.3)	35 (31.3)
Negative	49 (59.0)	67 (59.8)
N/A	13 (15.5)	10 (8.9)

[a]n (%)=number of patients with percentage in parentheses; [b]Significantly different between both sets (P<0.01, Pearson Chi-square test); [c]Including one patient with ductal carcinoma *in situ*. N/A, not available; ER, estrogen receptor; PR, progesterone receptor.

estimated sequence of the *ABCB1* gene was divided into 7 regions (Fig. 2). Typical results of methylation analysis using HRM are presented in Fig. 4A (normalized graph of distribution of differentially methylated samples along calibration curves ranging from 0 to 100% methylation). Fig. 4B shows the distribution of *ABCB1* methylation using a baseline of 50% methylation. Subsequently, this newly developed HRM method was used for *ABCB1* methylation analysis and evaluation of the clinical consequences of *ABCB1* methylation status in ovarian carcinoma samples (n=61).

Variability of ABCB1 promoter methylation. Extensive variability was observed in the *ABCB1* promoter methylation status ranging from 0 to 80%, with mean methylation levels of 19 and 14% in breast and ovarian carcinoma samples, respectively. Comparison of pre-treatment and post-treatment levels of *ABCB1* methylation revealed significantly higher methylation of *ABCB1* prior to chemotherapy treatment in ovarian carcinoma samples as estimated by the Mann-Whitney test (n=61; P=0.001; significant after the Bonferroni's test; Fig. 5A), in which paclitaxel and platinum derivatives were normally used. By contrast, the difference in *ABCB1* methylation between pre-treatment and post-treatment breast carcinoma samples was not statistically significant, as shown in Fig. 5B (Mann-Whitney test, n=171; P=0.424).

When comparison between tumor and control tissue samples were performed, no significant changes in *ABCB1* methylation were observed for patients with breast carcinoma. In ovarian carcinoma samples, the comparison of all (pre-treatment and post-treatment) ovarian tumor tissues with control

Table III. Clinical characteristics of the patients with ovarian carcinoma involved in the present study.

Characteristics	Pre-treatment set (n=50) n (%)[a]	Post-treatment set (n=11) n (%)[a]
Stage		
I	2 (4.0)	0 (0)
II	2 (4.0)	0 (0)
III	37 (74.0)	10 (90.9)
IV	4 (8.0)	1 (9.1)
N/A	5 (10.0)	0 (0)
EOC type		
High grade serous	40 (80.0)	11 (100.0)
Other type	5 (10.0)	0 (0)
N/A	5 (10.0)	0 (0)
Histological grade		
1	1 (2.0)	0 (0)
2	9 (18.0)	0 (0)
3	35 (70.0)	11 (100)
N/A	5 (10.0)	0 (0)
Ki-67 protein expression		
Median ± SD (%)	39.0±24.0	31.8±17.4
N/A	15 (30.0)	0 (0)

[a]n (%)=number of patients with percentage in parentheses is shown. EOC, epithelial ovarian cancer; N/A, not applicable.

ovarian tissues (n=11) revealed significant hypermethylation in 85.2% of tumor samples as estimated by non-parametric the Kruskal-Wallis test (n=61; P<0.001, Table IV). This hypermethylation was confirmed by the multiple comparison post-hoc Bonferroni's test; (n=50; P=0.002) in pretreatment ovarian tumor samples when compared with control ovarian tissues.

Functional analysis of ABCB1 methylation levels. To assess a potential functional meaning of the observed promoter methylation status, *ABCB1* transcript levels were determined by RT-qPCR, and the effect of methylation on *ABCB1* transcript levels was analyzed. In breast carcinoma samples, correlations of *ABCB1* methylation and transcript levels were evaluated separately in samples from pre- and post-treatment tumors (n=34 and 55, respectively), in which both total RNA and DNA from tumor tissues were available. In the pre-treatment set, *ABCB1* DNA methylation levels negatively correlated with *ABCB1* transcript levels, as estimated by Spearman's rho correlation (P=0.025, ϱ=0.397; P=0.05 after the Bonferroni's test), while the results were not statistically significant in the post-treatment set (P=0.308). Similar findings were observed in ovarian carcinoma samples, in which high *ABCB1* DNA methylation levels significantly correlated with lower transcript levels (Spearman's rho correlation test; P=0.001, ϱ=0.470; P=0.002 after the Bonferroni's test) in the pre-treatment set of ovarian carcinoma samples, but not in the post-treatment set (P=0.069).

Clinical associations of ABCB1 promoter methylation and expression with prognosis of patients. Comparison of *ABCB1* promoter methylation levels and clinical features revealed various significant associations. In the post-treatment breast carcinoma samples, significantly higher *ABCB1* intratumoral methylation levels were observed in patients with negative p53 expression (Mann-Whitney test; n=70, P=0.039; Table V). In the pre-treatment ovarian carcinoma samples, higher *ABCB1* methylation levels were observed in tumors at stage I in comparison with a cohort of advanced stages II-IV cases (Mann-Whitney test; n=45, P=0.028; Table V).

Subsequently, analyses of *ABCB1* transcript levels and clinical features were performed. Intratumoral *ABCB1* transcript levels in the post-treatment breast or ovarian carcinoma samples did not exhibit any association. In the pre-treatment carcinoma samples from breast patients with grade-1 or 2 tumors, significantly higher *ABCB1* transcript levels were observed compared with patients exhibiting grade-3 tumors (Mann-Whitney test; n=70, P=0.011). However, the trend test did not show a statistically significant difference (P>0.05), and thus, this association was not further discussed. Conversely, significantly higher levels of *ABCB1* in high-grade (grade 3) tumors compared with low-grade (grades 1 or 2) tumors were observed in the pre-treatment set of ovarian carcinoma samples (Mann-Whitney test; n=45, P=0.021; Table VI).

In the survival analysis, *ABCB1* promoter methylation or *ABCB1* transcript expression was not significantly associated with PFS of patients with breast or ovarian carcinoma in the post-treatment set. In the pre-treatment sets, low expression levels of *ABCB1* were associated with longer PFS estimated by the Kaplan-Meier method in patients with breast carcinoma. Differencies between groups were compared using the log-rank test (n=68; P=0.001; significant after the Bonferroni's test; P=0.004; Fig. 6A). The same trend, although non-significant, was also found in ovarian carcinoma (n=50; P=0.05; P=0.2 after the Bonferroni's test; Fig. 6B).

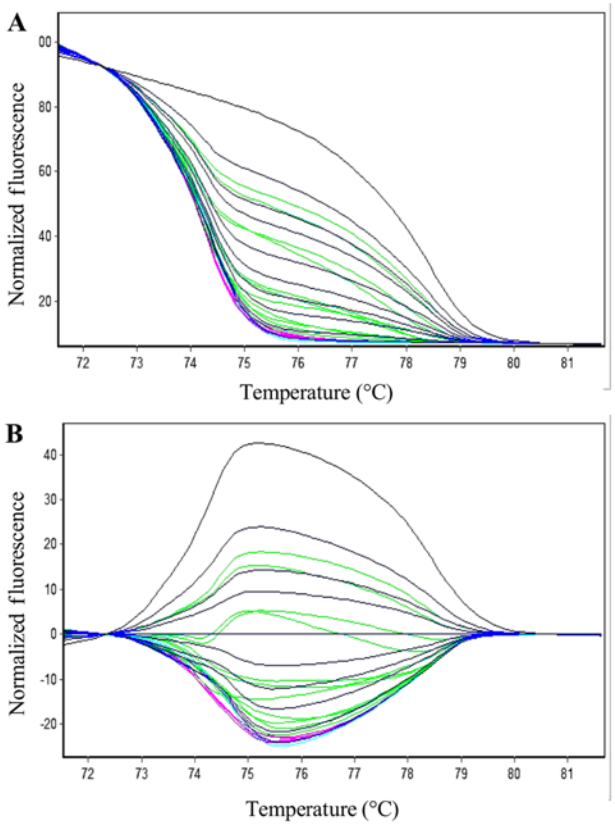

Figure 4. (A) Normalized and (B) difference graphs of distribution of *ABCB1* promoter methylation. ABCB1 promoter methylation of DNA samples from breast and ovarian carcinomas was estimated in the range of calibration curve of DNA-methylated standards (0-100%, blue curves); DNA samples from tumor tissues are shown in green, while DNA samples with methylation levels ≤5% are shown in purple. *ABCB1*, ATP binding cassette subfamily B member 1.

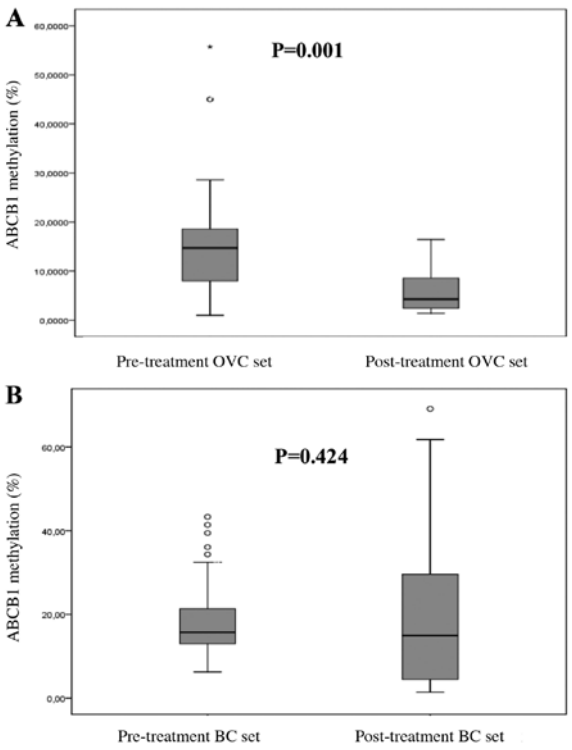

Figure 5. Boxplot charts comparing *ABCB1* promoter methylation levels between the pre-treatment and post-treatment unpaired sets of (A) ovarian (OVC) and (B) breast carcinoma (BC) samples. Due to the deviation of the methylation data from the normal distribution, the non-parametric Mann-Whitney test was applied to identify significant differences (P<0.05). *ABCB1*, ATP binding cassette subfamily B member 1.

Discussion

In the present study, HRM methylation analysis of the most frequently studied ABC drug-efflux transporter (namely *ABCB1*, which is associated with MDR in cancer cells), was developed. This method was compared with *ABCB1* promoter methylation status obtained by pyrosequencing and a strong correlation of both methods was found.

DNA methylation and histone modifications are important reversible mechanisms of epigenetic gene expression regulation and serve a role in cancer development. Numerous recent studies have suggested a direct role for epigenetic inactivation of genes in determining tumor chemosensitivity (38). Methylation-specific PCR technology, which has previously been used for *ABCB1* gene analysis mainly encompassing the SP-1 site in the 3'-region of *ABCB1*, was applied (23-25). In the present study, the three CpG islands identified by MethPrimer software in a promoter region encompassing the start site were analyzed. This type of analysis allows an exact estimation of a realistic methylation pattern of the complete promoter region in contrast to only using small regions of the *ABCB1* gene separately. Furthermore, HRM analysis allows a simple, rapid and reasonably economic (and therefore, suitable for routine clinical practice) method for detection of the percentage of methylated CpG islands of genes with potential clinical importance.

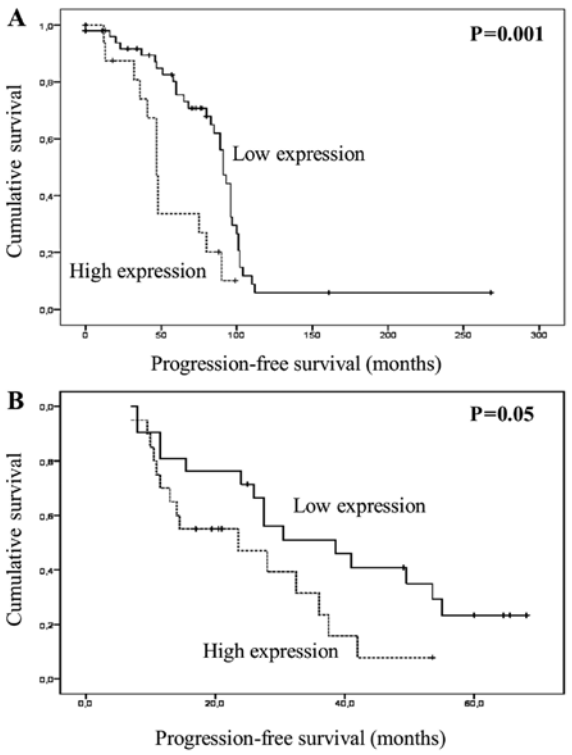

Figure 6. Statistically significant results of survival analysis. Associations between *ABCB1* gene expression level and progression-free survival in the pre-treatment group of patients with (A) breast and (B) ovarian carcinoma. Survival analysis was performed by the Kaplan-Meier method and the log-rank test was applied to identify significant associations. *ABCB1*, ATP binding cassette subfamily B member 1.

Table IV. Significant changes in methylation status of the *ABCB1* promoter in the set of pre-treatment and post-treatment ovarian carcinomas in comparison with control ovarian tissue samples.

Gene	*ABCB1* methylation status	Ovarian control tissue samples (n=11) n (%)[a]	Ovarian carcinoma tissue samples (n=61) n (%)[a]
ABCB1	Hypomethylated	0 (0)	0 (0)
	Normal-like	11 (100)	9 (14.8)
	Hypermethylated	0 (0)	52 (85.2)[b]

[a]n (%), number of patients with percentage in parentheses; [b]P<0.001, significantly different methylation levels between tumors and controls by the Kruskal-Wallis test. ABCB1, ATP binding cassette subfamily B member 1.

Table V. Evaluation of associations between *ABCB1* promoter methylation levels in breast and ovarian carcinoma tissues and clinicopathological data of patients.

Patient sets	Associated clinical characteristics (n)[a]	ABCB1 methylation (%)	P-value
Post-treatment breast carcinoma set	*p53 expression*		
	Positive (21)	21.2±2.0	0.039[b]
	Negative (49)	15.9±2.8	
Pre-treatment ovarian carcinoma set	Stage		
	I (2)	31.1±13.9	0.028[b]
	Advanced stages II-IV (43)	18.6±2.1	

ABCB1 methylation is expressed as mean ± standard error. [a]Number of patients involved in the analysis; [b]P-values by the Mann-Whitney test. ABCB1, ATP binding cassette subfamily B member 1.

Table VI. Evaluation of associations between ABCB1 transcript levels in breast and ovarian carcinoma tissues and clinicopathological data of the patients.

Patient set	Associated clinical characteristics (n)[a]	ABCB1 expression normalized to control genes[b]	P-value
Pre-treatment ovarian carcinoma set	Grade		
	Low grade 1/2 (10)	1.74±0.03	0.021[c]
	High grade 3 (35)	1.59±0.03	

[a]Number of patients involved in the analysis; [b]ABCB1 transcript expression is expressed as mean ± standard error. For analyses of associations of clinicopathological characteristics with transcript levels in tumors, a ratio of Ct for ABCB1 to arithmetic mean of Ct for all reference genes (ABCB1/REF) was calculated for each sample. Therefore, the lower ABCB1/REF ratio the higher the ABCB1 transcript level; [c]P-values by the Mann-Whitney test.

The present study reports the first set of results of *ABCB1* promoter methylation assessments in ovarian carcinoma. In breast carcinoma, *ABCB1* promoter methylation was estimated for the first time in 2010 (20). The patients with breast carcinoma in that study displayed widespread aberrant CpG island methylation (mean, 39.3% in 28 invasive breast tumors and 40.7% in 27 ductal carcinoma *in situ*). Furthermore, comparison of tumor tissues with normal breast tissues revealed significant *ABCB1* hypermethylation in breast tumors in comparison with normal breast tissue (20). Dejeux *et al* (21) analyzed the methylation patterns in the promoter regions of 14 genes including *ABCB1* in 75 well-described pre-treatment samples from patients with locally advanced breast carcinoma in comparison with 6 normal breast tissues. Absence of methylation was observed in all normal breast tissue samples, while 3 amplification products of the *ABCB1* gene were observed to be methylated in 70, 64 and 81% of tumor samples. Klajic *et al* (22) estimated *ABCB1* DNA methylation by pyrosequencing in a series of 238 breast carcinoma tissue samples (ranging from ductal carcinoma *in situ* to invasive tumors of stages IV). *ABCB1 was* the most frequently hypermethylated gene in all invasive and ductal carcinoma *in situ* samples (mean methylation, 16.2%) in comparison with normal breast tissue samples (mean methylation, 2.6%). Collectively, it

was demonstrated by previous studies and confirmed in the present study that breast carcinoma exhibits frequent *ABCB1* promoter hypermethylation. The present study extends the knowledge on this topic by the additional observation of such hypermethylation in ovarian carcinomas.

The negative significant correlation between *ABCB1* methylation and gene expression levels observed in samples prior to chemotherapy treatment in the two types of tumors evaluated in the present study demonstrates a clear functional effect of *ABCB1* methylation status. It confirms the results of Sharma *et al* (24), who reported *ABCB1* hypomethylation in tumor samples (n=41) with high P-glycoprotein levels (as estimated using immunohistochemistry) of patients with breast carcinoma prior to chemotherapy or radiation treatment. The lack of correlation between methylation status and expression levels in samples following neoadjuvant therapy that was observed in the present study is in the agreement with the results of Dejeux *et al* (21), who reported a lack of such correlation in post-treatment sets of samples. In ovarian carcinoma, the correlation of *ABCB1* promoter methylation with the transcript levels of this gene that was observed in the present study is the novelty of the present report.

Regarding clinical consequences, the present study observed associations between *ABCB1* expression and patient survival. High *ABCB1* expression levels significantly predicted poor progressive-free survival (PFS) of patients with breast and ovarian carcinoma prior to treatment. These observations indirectly suggest that silencing of *ABCB1* expression may affect patient prognosis. Although a few significant associations have been suggested between *ABCB1* methylation levels and prognostic factors of patients with breast or ovarian carcinomas, none of these associations were confirmed by the post-hoc tests.

Nevertheless, the present observation that *ABCB1* methylation does not appear to have a direct prognostic role was unexpected, suggesting that the connection between *ABCB1* and drug resistance may be a complex phenomenon. By contrast, it has been previously shown that patients with breast carcinoma and hypermethylated *ABCB1* promoter had significantly longer median overall survival (OS) compared with patients exhibiting a hypomethylated *ABCB1* promoter (21). Similarly, *ABCB1* promoter hypermethylation in circulating DNA was significantly associated with longer OS (23). By contrast, Klajic *et al* (22) did not observe a significant correlation between *ABCB1* methylation and survival. Taken together, the correlation of *ABCB1* methylation with gene expression reported in the present study may be of interest as a potential chemoresistance biomarker although its biological relevance for drug transport remains to be evaluated *in vitro* and confirmed *in vivo*.

A modest sample size and a relatively low number of samples corresponding to patients with ovarian carcinoma upon receiving treatment may be considered as major limitations of the present study. Our study primarily focused on the establishment and validation of a novel method for assessment of epigenetic regulation of an important gene implicated in chemoresistance. Thus, all the present clinical findings should be interpreted with caution and replicated by independent validation studies. Protein levels of *ABCB1* gene product called P-glycoprotein (P-gp) are usually estimated in different tumor cells and they are associated with multidrug resistance of tumor cells. In our previous studies, we successfully identified P-gp by western blot assay, but only in highly taxane-resistant SK-BR-3/PacR, MCF-7/PacR and NCI/ADR-RES carcinoma cell lines (39,40). We tried to estimate P-gp protein levels in breast and ovarian tumor tissues with high and low level of methylation in the frame of the present study. However, we did not succeed to unambiguously detect the levels of P-gp in any of these tumor tissue samples by immunoblotting analysis and thus it was not possible to connect differences reflecting methylation status of the *ABCB1* gene with its protein product levels. Finally, but equally important, methylation belongs to epigenetic factors controlling the rate of transcription and may or may not contribute also to the translation rate. This is probably a gene- and protein-specific process and also presumably individually different due to variation in RNA processing and protein translation and degradation machineries. Nevertheless, we perceive this fact as a limitation of the study and we are planing to collect more samples and estimate P-gp levels by immunohistochemistry in the near future.

In conclusion, the present study reports the successful development of a cost-effective HRM methylation analysis method of the *ABCB1* promoter region. Hypermethylation of the analyzed *ABCB1* promoter region significantly correlated with downregulation of its transcript levels in tumors from pre-treatment subsets of patients with breast and ovarian carcinoma. The observed association of low *ABCB1* transcript levels with longer survival suggesting good prognosis in the pre-treatment subsets of patients with breast and ovarian carcinoma opens the potential use of *ABCB1* as a prognostic biomarker. Its clinical utility should be further evaluated in larger independent cohorts of samples.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

RV, VNK and PaS conceived and planned the study. RV, VB, KE and GIGA performed the experiments. JK, RV and JT analyzed the data and carried out statistical analyses. LR, RK, PeS and MM prepared and characterized the tumor samples and collected the clinical data. RV, VNK and PaS wrote the manuscript. All authors read and approved the final manuscript, and agree to be accountable for all aspects of the

research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The present study was approved by the Ethics Commission of the National Institute of Public Health in Prague (ethic codes: IGA no. 9799-4 of 30 January 2008, 14055-3 of 2 July 2012, and 14056-3 of 2 July 2012) and Institutional Review Board of Norwegian Radiumhospital (Oslo, Norway) in the frame of the Norwegian Cancer Society (D-03067) and the Norwegian Research Council (163027/V409) projects. The methods were carried out in accordance with guidelines approved by the above Ethics Commissions. The study was conducted in accordance with the ethical standards of the Declaration of Helsinki of 1975 and its most recent version. All subjects were informed about the study topic and provided their written consent to participate in the study.

Patient consent to publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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