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Breast cancer metastasis suppressor-1 promoter methylation in cell-free DNA provides prognostic information in non-small cell lung cancer

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Background: Breast-cancer metastasis suppressor 1 (*BRMS1*) gene encodes for a predominantly nuclear protein that differentially regulates the expression of multiple genes, leading to suppression of metastasis without blocking orthotropic tumour growth. The aim of the present study was to evaluate for the first time the prognostic significance of *BRMS1* promoter methylation in cell-free DNA (cfDNA) circulating in plasma of non-small cell lung cancer (NSCLC) patients. Towards this goal, we examined the methylation status of *BRMS1* promoter in NSCLC tissues, matched adjacent non-cancerous tissues and corresponding cfDNA as well as in an independent cohort of patients with advanced NSCLC and healthy individuals.

Methods: Methylation of *BRMS1* promoter was examined in 57 NSCLC tumours and adjacent non-cancerous tissues, in cfDNA isolated from 48 corresponding plasma samples, in cfDNA isolated from plasma of 74 patients with advanced NSCLC and 24 healthy individuals.

Results: The *BRMS1* promoter was highly methylated both in operable NSCLC primary tissues (59.6%) and in corresponding cfDNA (47.9%) but not in cfDNA from healthy individuals (0%), while it was also highly methylated in cfDNA from advanced NSCLC patients (63.5%). In operable NSCLC, Kaplan–Meier estimates were significantly different in favour of patients with non-methylated *BRMS1* promoter in cfDNA, concerning both disease-free interval (DFI) ($P = 0.048$) and overall survival (OS) ($P = 0.007$). In advanced NSCLC, OS was significantly different in favour of patients with non-methylated *BRMS1* promoter in their cfDNA ($P = 0.003$). Multivariate analysis confirmed that *BRMS1* promoter methylation has a statistical significant influence both on operable NSCLC patients' DFI time and OS and on advanced NSCLC patients' PFS and OS.

Conclusions: Methylation of *BRMS1* promoter in cfDNA isolated from plasma of NSCLC patients provides important prognostic information and merits to be further evaluated as a circulating tumour biomarker.

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Lung cancer is the leading cause of cancer death worldwide, with over 1 million deaths each year (Parkin *et al*, 2005; Anglim *et al*, 2008). Patient's survival depends significantly on early detection, and for patients with operable stage IA, the 5-year survival can be as high as 55–80% (Wang *et al*, 2010). Early detection and precise diagnosis are critical for patients to receive proper therapeutic treatment as early as possible and thus could improve survival rates in non-small cell lung cancer (NSCLC) patients. Despite recent advancements in lung cancer therapies, the prognosis for patients with advanced NSCLC remains poor, so innovative, non-invasive, sensitive and reliable biomarkers still need to be discovered and exploited. Tumour biomarkers can have an important role in cancer screening, diagnosis, prognosis and therapeutic monitoring. Discovery and validation of novel biomarkers for early characterisation of carcinomas is one of the main aims of contemporary cancer research (Diamandis *et al*, 2013; Pavlou *et al*, 2013).

Methylation of DNA is one of the most frequently occurring epigenetic events taking place in mammalian genome and alterations in DNA methylation are very common in cancer cells. In particular, hypermethylation has been reported as an early event in carcinogenesis and progression to malignancy, frequently leading to gene silencing through methylation of CpG-rich regions near the transcriptional start sites of genes that regulate important cell functions (Laird, 2003). Methylation of specific genes appears to be an early event that has a fundamental role in the development and progression of cancer (Heyn and Esteller, 2012). Epigenetic changes such as individual gene promoter methylation are now under intensive evaluation as lung cancer biomarkers and present a strong potential to advance our understanding of its aetiology as well as provide novel early detection biomarkers (Zöchbauer-Müller *et al*, 2001; Anglim *et al*, 2008; Brock *et al*, 2008; Heyn and Esteller, 2012).

The genetic profile of solid tumours is currently obtained in an invasive way from surgical or biopsy specimens; moreover, information acquired from a single biopsy might fail to reflect tumour heterogeneity and reflects a limited snap-shot of a tumour that is continuously evolving and can acquire resistance to systemic treatment as a result of clonal evolution and selection. A 'liquid biopsy', or blood sample, can provide the genetic landscape of all cancerous lesions (primary and metastases) as well as offering the opportunity to systematically track genomic evolution (Crowley *et al*, 2013). Additionally, blood-based diagnostics can classify tumours into distinct molecular subtypes and monitor disease relapse and response to treatment (Hanash *et al*, 2011).

Circulating cell-free DNA (cfDNA) is an emerging non-invasive blood-based biomarker utilised to assess tumour progression and to evaluate prognosis, diagnosis and response to treatment (Marzese *et al*, 2013) and monitoring of the efficacy of anticancer therapies (Schwarzenbach *et al*, 2011). It was very recently shown that sequencing of cancer exomes in serial plasma samples can track genomic evolution of metastatic cancers in response to therapy (Murtaza *et al*, 2013).

Cancer cell-specific methylated DNA has been found in the blood of cancer patients, indicating that cfDNA is a tumour-associated DNA marker that can be used as a minimally invasive diagnostic test. Esteller *et al* have shown already in 1999 that detection of aberrant promoter hypermethylation of tumour suppressor genes could be detected in serum DNA from NSCLC patients (Esteller *et al*, 1999). Since then, many studies have described methylation of tumour suppressor genes in serum or plasma samples and in the corresponding primary tumours (Usadel *et al*, 2002; Hoque *et al*, 2006; Hsu *et al*, 2007). In the majority of these studies, the frequencies of methylation in plasma were lower in respect to those of the primary tumours (Hoque *et al*, 2006), while notably, in the majority of cases, DNA methylation was not detected in plasma or serum of healthy donors (Usadel *et al*, 2002; Hoque *et al*, 2006). Especially in lung cancer, DNA methylation of various genes has been detected in cfDNA circulating in plasma or serum, in sputum and in bronchoalveolar lavage samples (Palmisano *et al*, 2000; Fujiwara *et al*, 2005; Hsu *et al*, 2007).

Breast cancer metastasis suppressor 1 (*BRMS1*) is a predominantly nuclear protein that differentially regulates expression of multiple genes leading to suppression of metastasis without blocking orthotropic growth (Vaidya and Welch, 2007).This gene is significantly downregulated in some breast tumours, especially in metastatic disease, by epigenetic silencing (Metge *et al*, 2008). We have recently shown that *BRMS1* promoter was methylated in DNA extracted from circulating tumour cells (CTCs) isolated from peripheral blood of breast cancer patients (Chimonidou *et al*, 2011, 2013). We have also recently shown that *BRMS1* promoter methylation was not detected in non-cancerous breast tissues or benign fibroadenomas, while in breast cancer primary tumours it was significantly associated with reduced disease-free survival (Chimonidou *et al*, 2013). Although the role of BRMS1 in NSCLC has being recently studied in primary tumour tissues (Smith *et al*, 2009; Nagji *et al*, 2010; Yang *et al*, 2011), there is no information concerning the prognostic significance of *BRMS1* gene promoter methylation in cfDNA circulating in plasma.

The aim of the present study was to evaluate for the first time the prognostic significance of *BRMS1* promoter methylation in cfDNA circulating in plasma of NSCLC patients. Towards this goal, we examined the methylation status of *BRMS1* promoter-associated CpG island in NSCLC tissues, matched adjacent non-cancerous tissues and cfDNA as well as in healthy individuals.

PATIENTS AND METHODS

The outline of the workflow of our study is shown in Figure 1.

Clinical samples. The study material consisted of three different sets of clinical samples: (a) *Training set:* this set consisted of 57 NSCLC fresh-frozen tissues and corresponding adjacent non-neoplastic tissues and 48 corresponding plasma samples. There were 46 men and 11 women (median age: 61 years), all diagnosed with operable (stage I–III) NSCLC; 27 patients were diagnosed with adenocarcinoma (AD), 25 had squamous cell carcinoma (SQ) and 5 were diagnosed with undifferentiated NSCLC; in this group, the majority of patients (91.5%) were smokers and suffered from mild-to-moderate chronic obstructive pulmonary disease according to pulmonary function tests that were included as a part of the standardised preoperative evaluation of the patients. All patients were treatment naïve when the samples were collected, but after surgery all patients received standard chemotherapy protocols for adjuvant NSCLC, such as gemcitabine plus taxanes (90%) or platinum-based chemotherapy (10%). The majority of patients changed stage after the disease relapse to IIIB, (b) *Independent validation cohort:* this set consisted of 74 cfDNA samples isolated from plasma of advanced (stage IV) NSCLC patients. In this group, blood was obtained at diagnosis and before the initiation of any systemic treatment. Fifty patients had a non-squamous histology and 53 had distant metastases whereas 21 had inoperable stage IIIB disease. Twenty-three patients were treated with single agent chemotherapy in the context of geriatric chemotherapy protocols of the Hellenic Oncology Research Group (HORG), namely docetaxel or gemcitabine whereas the remaining 51 patients received chemotherapy combinations associating a taxane with a platinum compound. Among the evaluable for response patients, 18 achieved an objective response (CR: $n=1$; PR: $n=17$) and 11 stable disease. At the time of the present analysis, all but one patient were dead because of disease progression and (c) *Control population:* this set consisted of 24 cfDNA samples isolated from plasma of healthy donors. The tumour type and stages were

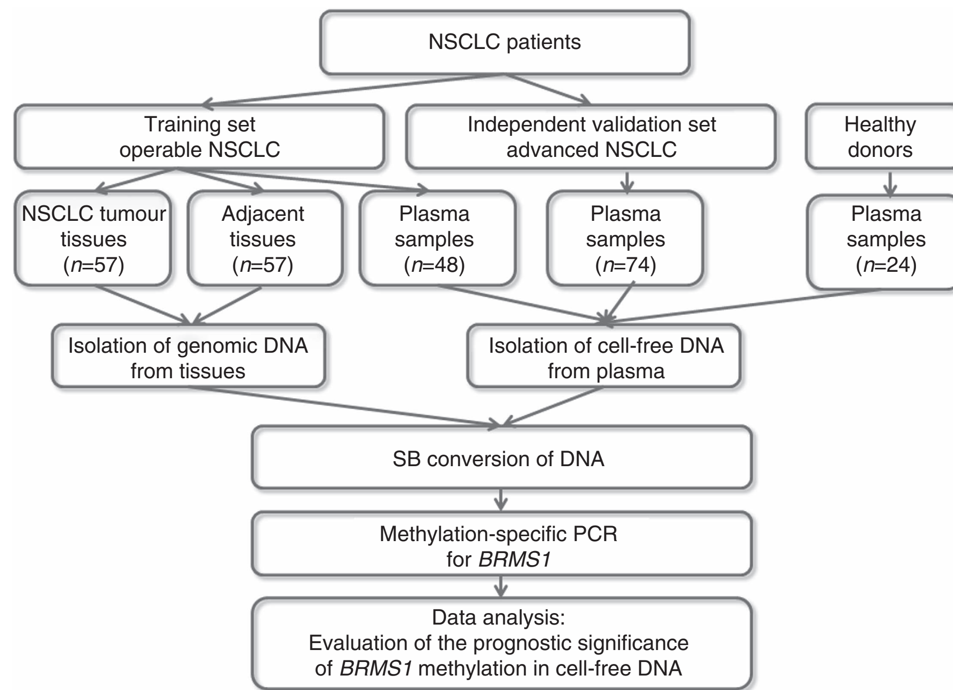

Figure 1. Workflow of the study.

analysed histologically and tissue sections containing >80% of tumour cells were used for DNA extraction and methylation-specific PCR (MSP) analysis. All patients gave their informed consent to participate in the study, which has been approved by the Ethical and Scientific Committees of our Institutions. At the time of surgery, all tissue samples were immediately flash frozen in liquid nitrogen and stored at −80 °C until use. Immediately after venipuncture, peripheral blood in EDTA was centrifuged at 2000 **g** for 10 min at room temperature and 1 ml aliquots of plasma samples were stored at −80 °C until use.

Isolation of genomic DNA from tumour tissues. Genomic DNA (gDNA) from NSCLC tissues and corresponding adjacent tissues was isolated using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Isolation of cfDNA circulating in plasma. Cell-free DNA was isolated from plasma samples using the High Pure Viral Nucleic Acid Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. In all, 200 μl of plasma was mixed with 200 μl of working solution and 50 μl proteinase K (18 mg ml^{-1}) and incubated for 10 min at 72 °C. DNA isolation was then processed as described in the manufacturer's protocol.

Sodium bisulfite conversion. The concentration of DNA was determined in the Nanodrop ND-100 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). In all, 1 μg of extracted DNA was modified with sodium bisulfite (SB), to convert all non-methylated cytosines to uracil, while methylated cytosines were not converted. Bisulfite conversion was carried out in 1 μg of denaturated DNA using the EZ DNA Methylation Gold Kit (ZYMO Research Co., Orange, CA, USA), according to the manufacturer's instructions and as previously described (Parkin *et al*, 2005; Pavlou *et al*, 2013). The converted DNA was stored at –70 °C until use. In each SB conversion reaction, dH_2O and DNA isolated from the MCF-7 cell line were included as a negative and a positive control, respectively.

Methylation-specific PCR. The methylation status of *BRMS1* gene in tissue samples was detected by conventional MSP by using specific primer pairs for both the methylated and unmethylated promoter sequences as previously described (Chimonidou *et al*, 2013). Each MSP reaction was performed in a total volume of 25 μl. In all, 1 μl of SB-converted DNA was added into a 24 μl reaction mixture that contained 0.1 μl of Taq DNA polymerase (5 U μl^{-1}, hot start GoTaq Polymerase; Promega, Madison, WI, USA), 5 μl of the supplied 10 × PCR buffer, 2.0 μl of $MgCl_2$ (50 mmol l^{-1}), 0.5 μl of dNTP's (10 mmol l^{-1}; Fermentas, Carlsbad, CA, USA) and 1 μl of the corresponding forward and reverse primers (10 μmol l^{-1}); dH_2O was added to a final volume of 25 μl. Sodium bisulfite-treated DNA was amplified in two separate MSP reactions, one with a set of primers specific for the methylated and one for the unmethylated *BRMS1* promoter sequences. Methylation-specific PCR products for methylated and unmethylated *BRMS1* promoter were fractionated on 2% agarose gels containing 40 mM Tris-acetate/1.0 mM EDTA (pH = 8) and visualised by ethidium bromide staining.

Real-time MSP. The methylation status of *BRMS1* gene in cfDNA samples was detected by a newly designed and more sensitive real-time MSP assay based on the same set of MSP-specific primer pairs as previously described (Chimonidou *et al*, 2013) and a newly designed hydrolysis (Taqman) LNA probe that is hybridising to a methylation-independent region (Table 1). Each reaction was performed in a total volume of 10 μl in the LightCycler 2.0 real time PCR instrument (Roche, Mannheim, Germany). One microlitre of SB-converted DNA was added into a 9-μl reaction mixture that contained 0.1 μl of Taq DNA polymerase (5 U μl^{-1}, DNA polymerase; Promega), 2 μl of the supplied PCR buffer (5 ×), 1.0 μl of $MgCl_2$ (25 mmol l^{-1}), 0.2 μl of dNTPs (10 mmol l^{-1}; Fermentas) and 0.2 μl of the forward and reverse primers (10 μmol l^{-1}), 0.15 μl BSA (10 μg μl^{-1}), 1 μl hydrolysis LNA probe (3 μmol l^{-1}); finally, dH_2O was added to a final volume of 10 μl. Similar thermocycling conditions were used: 1 cycle at 95 °C for 2 min, followed by 45 cycles at 95 °C for 10 s and 60 °C for 1 min. Sodium bisulfite-converted DNA from the DNA methylation standard (100%) was included in every run as a positive control.

In both cases (MSP and real-time MSP), human placental gDNA (Sigma-Aldrich, St Louis, MO, USA) methylated *in vitro* with SssI methylase (NEB, Ipswich, MA, USA) was used, after SB

Table 1. Oligonucleotide sequences for the real-time MSP for *BRMS1* promoter methylation

BRMS1	Primer name	Oligonucleotide sequence 5′-3′
Methylated	*BRMS1* M F1 *BRMS1* M R1	GTAGATGTTTTA**CG**TTATT**CG**GTG**C** CCTCCTACC**CG**TACAATC**CG**A
Unmethylated	*BRMS1 U F* *BRMS1 U R*	AGATGTTTTATGTTATTTGGTGTGT ATTAATCTTACTCCTCCTACCCATA
LNA probe	*BRMS1 probe*	5′-6FAM-ACAAATAAAA + [a]C + A + A + CT + A + C + AAC-BBQ

Abbreviations: *BRMS1* = breast-cancer metastasis suppressor 1; MSP = methylation-specific PCR.
[a] + indicates the position for LNA modification.

conversion, as fully methylated (100%) MSP-positive control; the same unmethylated placental gDNA, was used, after SB conversion, as a negative MSP control. The specificity and sensitivity of the MSP assay for *BRMS1* promoter methylation has been previously verified (Chimonidou *et al*, 2011, 2013).

Statistical analysis. Correlations between methylation status and clinico-pathological features of the patients were assessed by using the Chi-square test. Disease-free interval (DFI), progression-free survival (PFS) and overall survival (OS) curves were calculated by using the Kaplan–Meier method and comparisons were performed using the log-rank test. *P*-values <0.05 were considered as statistically significant. Statistical analysis was performed by using the SPSS Windows version 17.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Concordance between *BRMS1* promoter methylation in operable NSCLC tissues and corresponding cfDNA. We first compared our results on *BRMS1* promoter methylation in 48 operable NSCLC fresh tissues and corresponding cfDNA circulating in plasma of these patients. According to our findings, in 14 out of 48 (29.2%) NSCLC patients *BRMS1* promoter was found methylated both in tumour tissues and in cfDNA samples. In 16 out of 48 (33.3%) NSCLC patients, *BRMS1* promoter was not methylated both in tumour tissues and in cfDNA samples. The concordance between *BRMS1* promoter methylation in NSCLC fresh-frozen tissues and cfDNA was 30 out of 48 (62.5%). In 9 out of 48 (18.8%) cases, *BRMS1* promoter was not methylated in cfDNA, while it was methylated in corresponding primary tumours. In another 9 out of 48 (18.8%) cases, *BRMS1* promoter was methylated in cfDNA, while the corresponding tumours were found to be negative for methylation.

Evaluation of the prognostic significance of *BRMS1* promoter methylation in operable NSCLC fresh-frozen tissues. The methylation status of *BRMS1* promoter was first assessed in 57 pairs of fresh-frozen NSCLC tissues and their adjacent non-cancerous tissues, using the *BRMS1* conventional MSP assay. The *BRMS1* promoter was found to be methylated in 34 out of 57 (59.6%) tumour tissues and in 31 out of 57 (54.3%) of the corresponding adjacent non-cancerous tissues. The patient's characteristics according to the methylation status of *BRMS1* in NSCLC are presented in Table 2. Chi-square analysis did not reveal any statistically significant correlation between *BRMS1* promoter methylation and the clinico-pathological features of these patients.

During the follow-up period (73 months), 5 out of 57 patients without disease relapse died from other reasons and were thus not included in the survival analysis. In the remaining 52 patients, 36 out of 52 (69.2%) relapsed and 31 out of 52 (59.6%) died from the disease, during a median follow-up period of 45 months (range 1–73 months). Methylation of *BRMS1* was detected in 24 out of 36 (66.7%) of patients who relapsed, and in 20 out of 31 (64.5%) of patients who died. The incidence of relapses was similar between patients with methylated (24 out of 32, 75.0%) and non-methylated *BRMS1* promoter (12 out of 20, 60.0%) ($P=0.202$) while the incidence of deaths was also similar between patients with methylated (20 out of 32, 62.5%) and non-methylated *BRMS1* promoter (11 out of 20, 55.0%) ($P=0.402$) (Table 3).

Kaplan–Meier estimates of the cumulative DFI and OS for NSCLC patients with methylated and non-methylated *BRMS1* promoter in tumour tissues were not significantly different ($P=0.106$ and $P=0.376$, respectively; log-rank test, data not shown).

Evaluation of the prognostic significance of *BRMS1* promoter methylation in corresponding cfDNA. We further evaluated the prognostic significance of *BRMS1* methylation in corresponding cfDNA circulating in plasma of 48 out of the initial 57 enrolled patients. In addition, plasma samples from 24 control healthy individuals were also analysed.

The *BRMS1* promoter was methylated in 23 out of 48 (47.9%) plasma samples of operable NSCLC patients but not in any of the control plasma samples (0%). During follow-up, 4 out of these 48 patients died from reasons other than cancer and were thus not included in the survival analysis. In the remaining group of 44 patients, after a median follow-up period of 45 months (range 1–73 months), 32 out of 44 (72.7%) patients relapsed and 27 out of 44 (61.4%) died from the disease. Methylation of *BRMS1* promoter was detected in 19 out of 32 (59.4%) of patients who relapsed, and in 18 out of 27 (66.7%) of patients who died.

Table 3 indicates that the incidence of relapses was statistically different between patients with methylated (19 out of 22, 86.3%) and patients with non-methylated *BRMS1* promoter (13 out of 22, 59.1%) ($P=0.044$). The incidence of deaths was also statistically different between patients with methylated (18 out of 22, 82.0%) and patients with non-methylated *BRMS1* promoter (9 out of 22, 40.9%) ($P=0.006$).

Subsequently, the prognostic significance of *BRMS1* promoter methylation in these cfDNA samples was analysed. Kaplan–Meier estimates of the cumulative DFI and OS for NSCLC patients with methylated and non-methylated *BRMS1* promoter methylation in cfDNA were significantly different in favour of patients with non-methylated *BRMS1* promoter ($P=0.048$ and $P=0.007$, log-rank test, Figure 2A and B, respectively).

Multivariate analysis confirmed that only *BRMS1* promoter methylation has a statistical significant influence on patients' DFI (Table 4). Patients who present *BRMS1* promoter methylation have a significant lower DFI than those who do not (HR: 2.158, 95% CI: 1.030–4.517, $P=0.041$). Multivariate analysis revealed that only *BRMS1* promoter methylation has a statistical significant influence on patients' OS time. Patients who present *BRMS1* promoter methylation have a significantly lower OS time than those who do not (HR: 3.008, 95% CI: 1.295–6.989, $P=0.010$).

Table 2. Association of *BRMS1* promoter methylation in NSCLC tissues ($n=57$) and cfDNA ($n=48$) with patients' clinico-pathological features

	BRMS1 promoter methylation					
Patients	***n***			***n***		
Clinico-pathological feature	**57**	**Tissues (%)**	**P^a**	**44**	**cfDNA**	**P^a**
Tumour size (cm)			0.594			0.500
T1 ($\leqslant$3 cm)	13	8 (61.5)		11	6 (54.5)	
T2 (>3 cm)	35	22 (62.9)		31	15 (48.3)	
Unknown	9			2		
Lymph node			0.549			0.448
Positive	21	13 (61.9)		19	10 (52.6)	
Negative	28	18 (64.3)		24	11 (45.8)	
Unknown	8			1		
Stage			0.185			0.192
IA, IB	18	13 (72.2)		14	5 (35.7)	
IIA, IIB, IIIA, IIIB	31	17 (54.8)		29	16 (55.2)	
Unknown	8			1		
Histology			0.361			0.421
Adenocarcinoma	23	15 (65.2)		20	13 (65.0)	
Squamous cell carcinoma	25	14 (56.0)		23	11 (47.8)	
Unknown	9			1		
Sex			0.580			0.640
Male	41	25 (61.0)		34	17 (50.0)	
Female	11	7 (63.6)		10	5 (50.0)	
Unknown	5					

Abbreviations: *BRMS1* = breast-cancer metastasis suppressor 1; cfDNA = cell-free DNA; NSCLC = non-small cell lung cancer.
[a] χ^2 analysis.

Table 3. Incidence of disease relapses and disease-related deaths according to *BRMS1* promoter methylation in NSCLC tissues and corresponding cell-free DNA

NSCLC	*BRMS1* methylation	Relapses (%)	P	Deaths (%)	P
Tumour tissues ($n=52$)	M[a] ($n=32$)	24 (75%)	0.202	20 (62.5%)	0.402
	U[b] ($n=20$)	12 (60.0%)		11 (55.0%)	
Cell-free DNA ($n=44$)	M[a] ($n=22$)	19 (86.3%)	**0.044**	18 (81.8%)	**0.006**
	U[b] ($n=22$)	13 (59.1%)		9 (40.9%)	

Abbreviations: *BRMS1* = breast-cancer metastasis suppressor 1; NSCLC = non-small cell lung cancer. Statistically significant findings are shown in bold.
[a] Methylated.
[b] Unmethylated.

Evaluation of the prognostic significance of *BRMS1* promoter methylation in cfDNA in an independent validation cohort of advanced NSCLC patients. To further verify the prognostic significance of *BRMS1* promoter methylation in cfDNA circulating in plasma, we conducted a validation step in an independent cohort of 74 patients with advanced (stage IV) NSCLC.

The methylation status of *BRMS1* promoter in cfDNA of these patients revealed that it was methylated in 47 out of 74 (63.5%) cases. After a median follow-up period of 43 months (range 1–84 months), 73 out of 74 (98.6%) patients relapsed and 72 out of 74 (97.3%) died due to disease progression. Methylation of *BRMS1* was detected in plasma of all patients who relapsed and died.

Kaplan–Meier estimates of the cumulative PFS for patients with methylated and non-methylated *BRMS1* promoter had marginal significance ($P=0.059$, log-rank test) whereas that of the cumulative OS was significantly different among the two groups ($P=0.003$, log-rank test) (Figure 2C).

Multivariate analysis confirmed that only *BRMS1* promoter methylation has a statistical significant influence on patients' PFS time (Table 5). Patients who present *BRMS1* promoter methylation have a significant lower PFS time than those who do not (HR: 1.951, 95% CI: 1.175–3.238, $P=0.010$). Moreover, multivariate analysis revealed that *BRMS1* promoter methylation has a statistical significant influence on patients' OS time, while performance status has a marked but not significant trend (HR: 1.861, 95% CI: 0.989–3.500, $P=0.054$). Patients who present with *BRMS1* promoter methylation have a significant lower OS than those who do not (HR: 2.057, 95% CI: 1.247–3.386, $P=0.005$) (Table 5).

DISCUSSION

There is now an urgent need for blood-based, non-invasive molecular tests to assist in the detection, diagnosis and prognosis of cancers in a non-invasive and cost-effective manner especially at an early stage, when curative interventions are still possible.

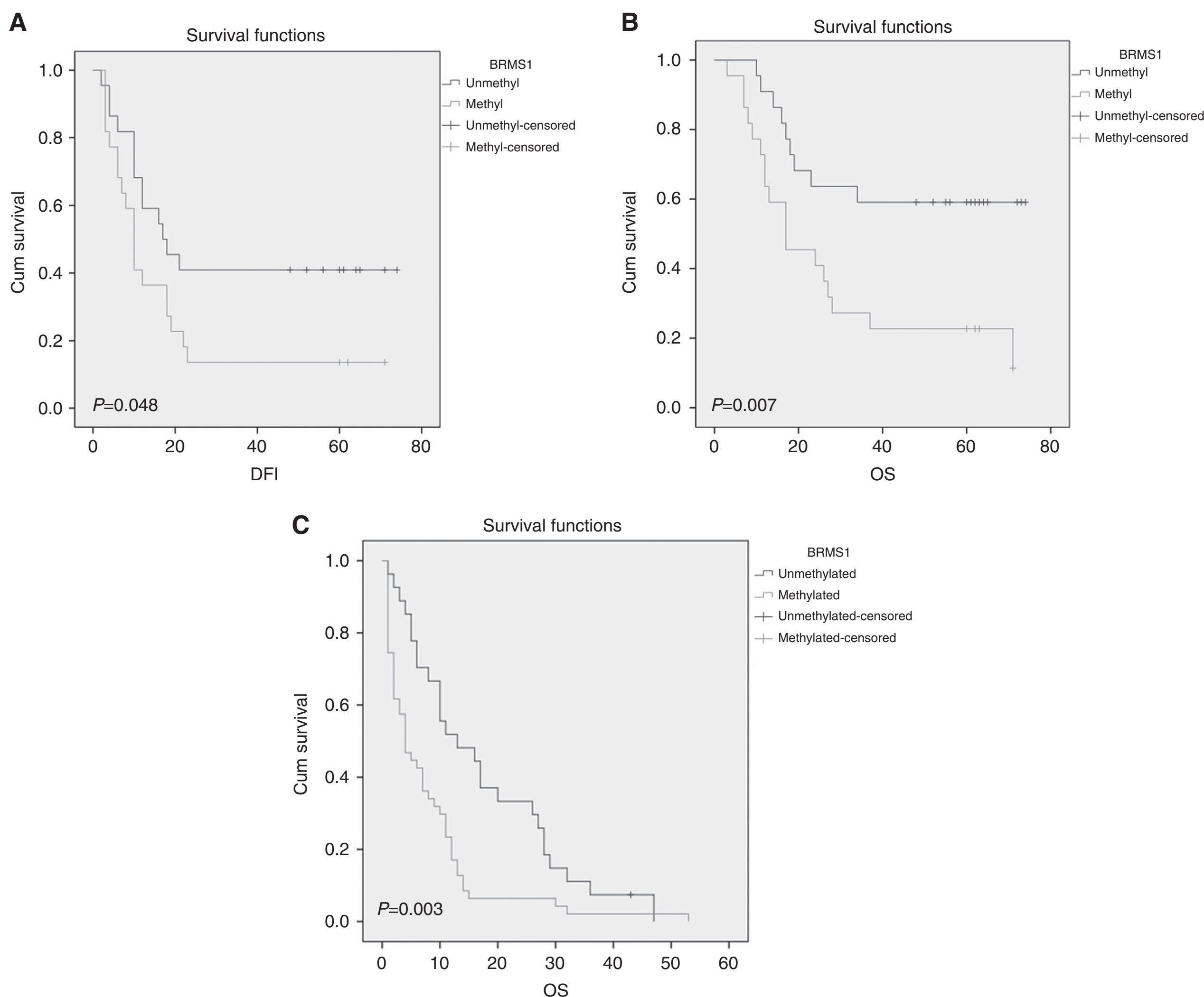

Figure 2. Prognostic significance of *BRMS1* promoter methylation in cell-free DNA circulating in plasma: (**A**) training set ($n=48$): Kaplan–Meier estimates for DFI ($P=0.048$), (**B**) training set ($n=48$): Kaplan–Meier estimates for OS ($P=0.007$) and (**C**) independent cohort ($n=74$): Kaplan–Meier estimates for OS ($P=0.003$).

Cell-free DNA is released from cancer cells into plasma, and is representing a non-invasive liquid biopsy approach that can now give important information as a blood-based tumour biomarker. Especially information on the methylation status of tumour suppressor genes in cfDNA is a very promising approach, since it can offer a useful tool for lung cancer diagnostics, evaluation of cancer treatment efficiency and post-treatment monitoring.

In this study, we evaluated for the first time the prognostic significance of *BRMS1* promoter methylation in cfDNA circulating in plasma of NSCLC patients. Our results clearly indicate that *BRMS1* promoter methylation is highly methylated in NSCLC tissues, and that detection of *BRMS1* promoter methylation in cfDNA isolated from plasma is highly specific and provides important prognostic information. The observed association between methylated *BRMS1* and reduced OS may be relevant to the known role of BRMS1 as a tumour suppressor. Methylation of *BRMS1* promoter, and subsequent silencing of this gene, may be indicative of a more aggressive tumour phenotype and, thus, the association with a poorer outcome. In the contrary, non-methylated *BRMS1* may be indicative of a slower progressing tumour as the gene may partially maintain its tumour suppressing capacity.

It was recently reported that *BRMS1* expression is diminished in NSCLC compared with non-cancerous lung tissues and it was also lower in squamous cell carcinoma compared with AD (Smith *et al*, 2009). Given these observations, the same group hypothesised that *BRMS1* transcription is decreased in NSCLC through increased *BRMS1* promoter methylation, and it was confirmed that *BRMS1* promoter-associated CpG island was hypermethylated in both NSCLC cells and human NSCLC specimens (Nagji *et al*, 2010). For that specific study cohort, *BRMS1* promoter methylation was significantly more robust in squamous cell carcinoma compared with AD histologies. Another recent study confirmed the above results. Yang *et al* (2011) have recently reported that *BRMS1* promoter-associated CpG island is aberrantly methylated in NSCLC and that patients with a high level of *BRMS1* mRNA expression had significantly better OS than those with low expression and that promoter methylation of *BRMS1* was a significantly unfavourable prognostic factor.

The mechanism through which BRMS1 suppresses metastasis is not clearly understood. A recent study revealed that ubiquitous BRMS1 expression suppresses pulmonary metastasis and promotes apoptosis of tumour cells located in the lung but not in the mammary glands, suggesting that cell-location specific over expression of *BRMS1* is important of BRMS1-mediated metastasis suppression (Cook *et al*, 2012).

Other studies focus on the strong correlation between loss of BRMS1 protein expression and reduced disease-free survival in

Table 4. *BRMS1* promoter methylation in cell-free DNA circulating in plasma of patients with operable NSCLC cancer ($n = 44$): univariate and multivariate analysis for DFI and OS

	Univariate analysis[a]			Multivariate analysis[a]		
	Hazard ratio	**95% CI**	***P***	**Hazard ratio**	**95% CI**	***P***
Disease-free interval (DFI)						
Age (⩾ 65 *vs* <65)	1.302	0.635–2.670	0.472			
Age (⩾70 *vs* <70)	1.526	0.700–3.326	0.288			
Smoking (yes *vs* no)	1.330	0.543–3.258	0.520			
Histology (squamous *vs* non-squamous)	1.088	0.525–2.252	0.821			
Stage (III *vs* I/II)	1.298	0.528–3.188	0.570			
Adj. Chemo (no *vs* yes)	1.495	0.730–3.062	0.272			
BRMS1 promoter methylation (yes *vs* no)	2.191	1.054–4.555	0.036	2.158	1.030–4.517	0.041
Overall survival						
Age (⩾ 65 *vs* <65)	1.578	0.722–3.452	0.253			
Age (⩾70 *vs* <70)	2.297	1.005–5.249	0.049			
Smoking (yes *vs* no)	1.592	0.545–4.650	0.395			
Histology (squamous *vs* non-squamous)	0.985	0.446–2.174	0.969			
Stage (III *vs* I/II)	1.392	0.519–3.731	0.511			
Adj. Chemo (no *vs* yes)	1.908	0.865–4.206	0.109			
BRMS1 promoter methylation (yes *vs* no)	3.114	1.350–7.183	0.008	3.008	1.295–6.989	0.010

Abbreviations: *BRMS1* = breast-cancer metastasis suppressor 1; CI = confidence interval; NSCLC = non-small cell lung cancer; OS = overall survival.
[a]Cox regression analysis.

Table 5. *BRMS1* promoter methylation in cell-free DNA circulating in plasma of patients with advanced NSCLC cancer ($n = 74$): univariate and multivariate analysis for PFS and OS

	Univariate analysis[a]			Multivariate analysis[a]		
	Hazard ratio	**95% CI**	***P***	**Hazard ratio**	**95% CI**	***P***
Progression-free survival (PFS)						
Sex (male *vs* female)	1.141	0.584–2.233	0.699			
Age (⩾ 65 *vs* <65)	1.122	0.706–1.784	0.626			
Age (<70 *vs* ⩾70)	1.052	0.632–1.751	0.845			
Performance status (2 *vs* 0-1)	1.548	0.826–2.904	0.173			
Histology (non-squamous *vs* squamous)	1.391	0.834–2.320	0.206			
Regimen (non-plat. *vs* plat.)	1.328	0.776–2.273	0.302			
BRMS1 promoter methylation (yes *vs* no)	1.945	1.174–3.223	0.010	1.951	1.175–3.238	0.010
Overall survival						
Sex (male *vs* female)	1.532	0.754–3.112	0.238			
Age (⩾ 65 *vs* <65)	1.012	0.632–1.624	0.959			
Age (<70 *vs* ⩾70)	1.060	0.625–1.800	0.828			
Performance status (2 *vs* 0-1)	**1.924**	**1.027–3.606**	**0.041**	1.861	0.989–3.500	0.054
Histology (non-squamous *vs* squamous)	1.282	0.780–2.107	0.327			
Regimen (non-plat. *vs* plat.)	1.037	0.605–1.776	0.896			
BRMS1 promoter methylation (yes *vs* no)	2.061	1.255–3.384	0.004	2.057	1.247–3.386	0.005

Abbreviations: *BRMS1* = breast-cancer metastasis suppressor 1; CI = confidence interval; NSCLC = non-small cell lung cancer; OS = overall survival. Statistically significant findings are shown in bold.
[a]Cox regression analysis.

subsets of breast cancer patients (Hang *et al*, 2006; Hicks *et al*, 2006). Furthermore, the loss of BRMS1 is associated with a decreased survival in patients with NSCLC (Nagji *et al*, 2010; Yang *et al*, 2011). In addition, aberrant methylation of *BRMS1* is responsible for its loss of expression in breast cancer and in NSCLC. The BRMS1 low expression is also correlated with poor patient survival in nasopharyngeal carcinoma (Cui *et al*, 2012). These results suggest that downregulation caused by *BRMS1* promoter methylation has an important role in tumorigenesis in many different types of cancer.

Our results clearly indicate that aberrant methylation of the *BRMS1* gene promoter is a common event in operable NSCLC tissues. Our primers are designed to recognise *BRMS1* promoter methylation in specific CpG sites, since we have already shown that

methylation of these regions is of clinical importance in breast cancer (Chimonidou *et al*, 2013). However, the prognostic significance of *BRMS1* promoter methylation in operable NSCLC tissues was not evident in our patients' cohort. On the contrary, we report here for the first time that detection of aberrant methylation of the *BRMS1* gene promoter in cfDNA circulating in plasma of these NSCLC patients provides prognostic information, both for DFI and for OS. Our results were further confirmed in an independent validation cohort of advanced NSCLC patients. In both groups of patients, training and independent cohort, the frequency observed for *BRMS1* methylation was high. This may suggest that loss of *BRMS1* expression is an early event in NSCLC tumorigenesis and remains in high levels in advanced stages of the disease. In operable NSCLC, Kaplan–Meier analysis has shown a strong correlation of *BRMS1* promoter methylation in plasma and poor DFI and OS, and these results were further verified by multivariate analysis. In advanced NSCLC, Kaplan–Meier analysis has shown a strong correlation between *BRMS1* promoter methylation in plasma and poor OS, while multivariate analysis has shown that *BRMS1* promoter methylation has a statistical significant influence on both patients' PFS and OS.

In conclusion, our data indicate for the first time that detection of *BRMS1* promoter methylation in cfDNA circulating in plasma provides important prognostic information for NSCLC patients. We believe that *BRMS1* promoter methylation in cfDNA should be further evaluated and validated as a non-invasive circulating tumour biomarker in a larger cohort of patients.

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