Serum DNA as a tool for cancer patient management

Ramirez JL, Taron M, Balaña C, Sarries C, Mendez P, de Aguirre I, Nuñez L, Roig B, Queralt C, Botia M, Rosell R

Medical Oncology Service, Institut Català d´Oncologia, Hospital Germans Trias i Pujol, Badalona (Barcelona), Spain

Abstract

Purpose: Genetic analysis has shown that cell-free circulating DNA in plasma or serum of cancer patients shares similar genetic alterations to those described in the corresponding tumor. One of the most important alterations involved in carcinogenesis is aberrant promoter methylation. The interest in this field has grown due to the implementation of the methylation-specific PCR (MSP) assay. The main objective of this study is to analyze the methylation status of different genes in tumor and serum DNA obtained at the time of surgery in two different tumor models (glioblastoma [GBM] and non-small-cell lung cancer [NSCLC]) and their relationship to clinico-pathological characteristics and response to chemotherapy.

Material and methods: Using MSP assay, we assessed the methylation status of MGMT, RASSF1A, p16, DAPK, TMS-1 in tumor and serum DNA obtained at time of surgery or stereotactic biopsy from 28 GBM patients and from 51 NSCLC patients.

Results: In GBM patients, the prevalence of MGMT, p16, DAPK, and RASSF1A promoter methylation was 38.1%, 66.7%, 52.4%, 57.1%, respectively, in glioma tissue, and 39.3%, 53.6%, 34.3%, 50%, respectively, in serum. A high correlation between methylation in tumor and serum (Spearman test p=0.0001) was observed. In NSCLC patients, RASSF1A, DAPK and TMS-1 were methylated in 34%, 45% and 35% tumors, respectively, and in 34%, 40% and 34% serum, respectively. A good correlation was found between alterations found in tumor and serum (Spearman test p=0.0001).

Conclusions: The study of serum or plasma DNA has opened new roads for translational research and new strategies for molecular diagnosis. Due to the similarities of alterations found in serum DNA and primary tumor, we can use this tool to calculate the risk of local or distant recurrence and its relationship with survival and its value in patient follow-up to evaluate response to therapy.

Key words: serum DNA, tumor DNA, aberrant methylation, NSCLC, GBM.

Introduction

Free DNA can be detected in different body fluids, e.g.: urine, synovial fluid, pancreatic duct secretions, sputum, and serum/plasma. This DNA circulating in serum/plasma is found in small amounts in healthy controls, but in cancer patients higher concentrations of DNA are present. The presence of nucleic acids (not only DNA but also RNA) in plasma or serum of cancer patients has been recognized since the 1970s [1,2], when Leon et al. reported the presence of DNA in the serum of patients with diverse neoplastic diseases. In this study they correlated the amount of circulating DNA with stage and response to treatment and they observed that the DNA levels in patients with metastatic disease were higher compared to those with non-metastatic disease. In 1989, Stroun et al., using a technique based on decreased strand stability of cancer cell DNA, recognized that part of the plasma DNA had the origin in cancer cells [3]. Five years later, Sorensen et al. reported the detection of tumor derived mutated k-ras DNA sequences in human blood from pancreatic cancer patients [4], and in 1994 Vasioukhin et al. reported N-ras mutations in plasma samples from patients with myelodysplastic syndrome [5]. Recently, an analysis published by Sozzi et al. [6] , they evaluated whether the amount of circulating DNA in plasma could discriminate between lung cancer patients and healthy individuals. In this work, they also

ADDRESS FOR CORRESPONDENCE:
Rafael Rosell, MD, Chief, Medical Oncology Service
Scientific Director of Oncology Research
Institut Català d'Oncologia, Hospital Germans Trias i Pujol
Ctra Canyet, s/n, 08916 Badalona (Barcelona), Spain
Tel.: 34-93-497-89-25 Fax: 34-93-497-89-50
e-mail: rrosell@ns.hugtip.scs

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determined the positive association between plasma levels and clinical progression. Moreover to date, several studies have reported the presence of free DNA concentrations in the serum of cancer patients, and molecular studies have provided evidence that this DNA has a tumoral origin. Thus, Chen et al. [7] analyzed the presence of microsatellite instability and loss of heterozygosity (LOH) in plasma DNA isolated from breast cancer patients and they found identical alterations in the corresponding plasma samples and tumor specimens. Anker et al. [8] commented in their review the detection of an increased concentration of DNA in plasma of cancer patients and how different molecular biological techniques, such as point mutation of the ras gene and the analysis of microsatellite instability, were used to establish the origin of this circulating tumoral DNA. De Kok et al. [9] investigated the fact that increased cell-free DNA in cancer patients was derived from tumor DNA as they describe the same point mutation, corresponding to the K-ras mutation in the primary tumor in the serum DNA. Furthermore, microsatellite instability in cell-free DNA has also been reported in lung cancer [10], breast carcinoma [11] in melanoma [12] and in head and neck squamous cell carcinomas [13]. All these studies suggest that tumor DNA is released into the circulation and is enriched in plasma and serum, on average, 3-4 times the amount of free DNA, compared to normal controls.

In addition to the molecular genetic alterations described above, transcriptional silencing of tumor suppressor genes by aberrant promoter methylation is a common feature in human cancer [14] and can contribute to oncogenesis. Hypermethylation of normally unmethylated CpG islands in the promoter region of many genes, including p16, p15, E-Cadherin, VHL, and hMLH1, correlate with loss of transcription in human tumors [15,16]. One interesting case that shows the importance of the analysis of aberrant methylation is the study performed by Tang et al. where they analyzed the hypermethylation of DAPK in patients with pathological stage I NSCLC who had undergone curative surgery. They found that patients whose tumors showed hypermethylation had significantly poorer overall survival compared to those without any alteration [17]. In cancer patients, aberrant promoter DNA methylation has been observed in a number of genes including several well-characterized candidate tumor suppressor genes including p16 [18] involved in cell cycle regulation, RAR-beta [19] involved in cell differentiation, RASSF1A [20] involved in signal transduction, FHIT [21], APC [22] also involved in aberrant signal transduction and TMS-1 gene [23] involved in apoptosis. Esteller et al. [24] reported a study in lung cancer patients that combined the analysis of aberrant promoter methylation of tumor suppressor genes in serum DNA comparing to tumor paired DNA. They found that aberrant promoter methylation of at least one of the genes p16, MGMT, GSTP1, and DAPK was identified in 68% of NSCLC patients [24]. The same alterations were found in serum/plasma of 71% of patients who presented methylated DNA in primary tumor [24]. These experiments were carried out using MSP, which is a sensitive and specific technique for methylation analysis. In this technique, DNA was amplified using primer pair sets in order to distinguish between methylated and non-methylated DNA by taking advantage of sequence differences resulting from sodium-bisulfite treatment, where unmethylated Cytosine are converted to Uracil, and methylated Cytosine remains as a cytosine [25].

However, one interesting conclusion from most of these studies is the fact that it is possible to find alterations in serum/plasma in the vast majority of cancer patients. This finding opened a lot of possibilities for the use of this serum/plasma as a tool for the detection of potential molecular markers to avoid invasive tools in order to obtain tumoral tissue [24,26]. To date, most of the studies published in the literature have analyzed the pattern of methylation only in serum/plasma or tissue, but in general they don't correlate gene promoter methylation status between serum and tumor DNA obtained at the time of surgery or biopsy. For this reason, the goal of our study is to try to answer two important questions. Firstly, are the alterations that appear in tumor DNA the same as the ones found in serum/plasma DNA? Secondly, do these alterations have a good correlation between tumor and serum DNA? To answer these questions, in the present study we examine the presence of genetic abnormalities in primary tumor and paired serum DNA (obtained at time of surgery) in two different tumor models (GBM and NSCLC), and their relationship with clinico-pathological characteristics and response to chemotherapy.

Using the sensitive MSP technique, we analyzed the methylation patterns of genes involved in apoptotic signalling molecules and cell cytoskeleton (TMS-1, DAPK) and in DNA repair and cell cycle control (RASSF1A), in non-microdisected tumor specimens and matching serum DNA from 51 resected NSCLC [27]. Our objective was to correlate the methylation status in paired tumor and serum samples, and to examine the impact of their aberrant methylation on survival. We also collected tumor and serum from 28 GBM patients treated prospectively with surgery, radiotherapy, and chemotherapy with BCNU or Temozolamide plus cisplatin [28]. Our objective was to confirm the presence of circulating serum DNA in these patients; to examine the concordance between methylation of MGMT (related with DNA repair capacity), p16 (cell cycle control), DAPK (apoptosis) and RASSF1A (ras signalling) in tumor and serum DNA; and finally, to correlate the methylation status of these genes with response and time to progression in GBM patients.

The study of serum or plasma DNA has opened up numerous areas of investigation and new possibilities for molecular diagnosis. It is very important to understand the study of these new areas of analysis due to the similarities of alterations found in serum DNA and primary tumor, the value as a prognosis tool to calculate the risk of local or distant recurrence and its relationship with survival; its value for patient follow-up or to evaluate response to therapy, and finally the possibility to use it as an early diagnostic tool in an asymptomatic high-risk population [29].

Material and methods

Patients. In this study we have analyzed methylation status in four different genes in tumor and paired serum DNA samples from 28 GBM patients and 51 NSCLC patients treated at the University Hospital Germans Trias i Pujol.

Table 1. PCR primer sequences, annealing temperatures and PCR length products.

Gene/ Marker	Forward primer	Reverse primer	MT (^{0}C)	Size (Bp)
MGMT M	5´-TTT CGA CGT TCG TAG GTT TTC GC-3´	5´-GCA CTC TTC CGA AAA CGA AAC G-3´	65	81
MGMT U	5´-TTT GTG TTT TGA TGT TTG TAG GTT TTT GT-3´	5´-AAC TCC ACA CTC TTC CAA AAA CAA AAC A-3´	63	93
P16 M	5´-TTA TTA GAG GGT GGG GCG GAT CGC GTG C-3´	5´-ACC CGA CCC CGA ACC GCG ACC GTA A-3´	65	150
P16 U	5´-TTA TTA GAG GGT GGG GTG GAT TGT-3´	5´-CAA CCC CAA ACC ACA ACC ATA A-3´	62	151
RASSF-1 M	5´-CGC GTT TAG TTT CGT TTT CG-3´	5´-AAC TTT AAA CGC TAA CAA ACG C-3´	63	165
RASSF-1 U	5´-TTT GTG AGA GTG TGT TTA GTT TTG-3´	5´-CCC AAT TAA ACC CAT ACT TCA-3´	55	200
TMS-1M	5´-TTG TAG CGG GGT GAG CGG C´-3´	5´-AAC GTC CAT AAA CAA CAA CGC G-3´	65	96
TMS-1U	5´-GGT TGT AGT GGG GTG AGT GGT-3´	5´-CAA AAC ATC CAT AAA CAA CAA CAC A-3´	65	98
DAPK-M	5´-GGA TAG TCG GAT CGA GTT AAC GTC -3´	5´-CCC TCC CAA CAG CGC A-3´	64	98
DAPK-U	5´-GGA GGA TAG TTG GAT TGA GTT AAT GTT-3´	5´-CAA ATC CCT CCC AAA CAC CA A-3´	60	108

M: methylated-specific primers; U: unmethylated-specific primers; MT: melting temperature

In GBM, DNA was obtained from 21 tumors and 28 serum samples that were collected at time of surgery. After pathologic review, 27 patients had confirmed GBM and one had gliosarcoma. The median age was 59 years (range, 30 to 76 years), 16 were men and 12 were women, Karnofsky performance status was over 70% in 16 patients and 70% in 12 patients. Twenty one patients underwent partial resection with measurable residual disease observed in post-operative brain magnetic resonance imaging (MRI), 2 patients had radical surgery and the remaining 5 patients, stereotactic biopsy. Frozen glioma tissue was available only for 21/28 patients. Sixteen patients were treated with Temozolamide 200 mg/m^2/day for 5 consecutive days plus cisplatin 100 mg/m^2 on day 1, every 28 days for 3 cycles before radiotherapy 60 Gy over the primary tumor and edema; 8 patients were treated with BCNU 200-250 mg/m^2 every 6 weeks and radiotherapy. One patient received only Temozolamide plus radiotherapy, and 3 patients received no treatment due to early progression.

In NSCLC, DNA was obtained from 51 tumors and the corresponding paired serum samples in patients who had undergone curative surgery between October 1998 and September 1999. Seven patients were stage I tumors (T1-2, N0), 14 patients had stage II (T1-2, N1), 24 patients had stage IIIA (T1-3, N2), and 6 patients had stage IV. According to histological criteria, 26 patients were classified as squamous cell carcinoma, 15 as adenocarcinoma and 10 as large cell undifferentiated carcinoma. Patients were enrolled on the basis of the availability of fresh-frozen stored tissue.

All these studies were approved by the Clinical Research Ethics Committee, and all patients gave informed consent for obtaining the blood and tumor samples and for the gene methylation assays.

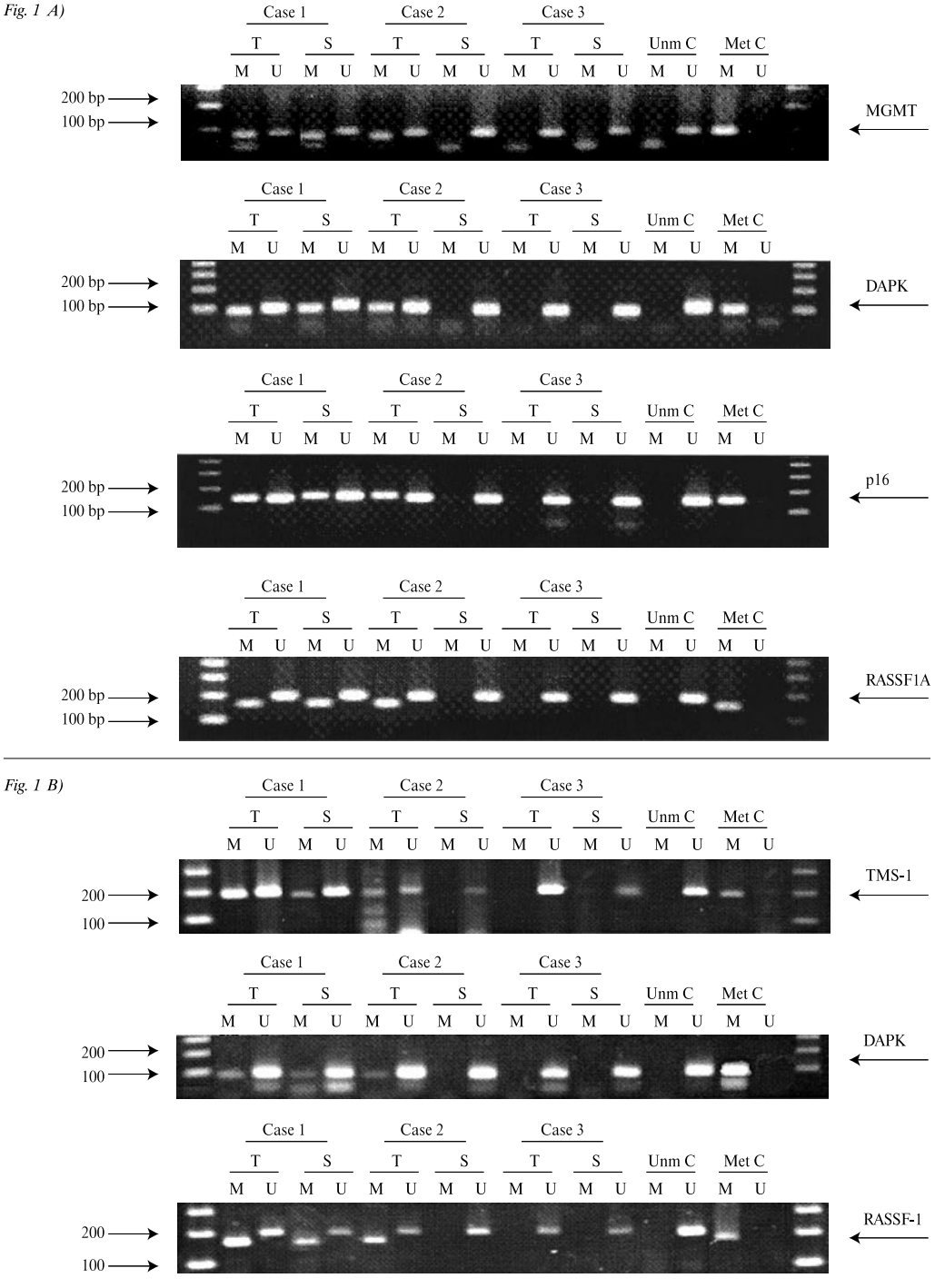
Sample collection and DNA extraction from tumor and serum DNA

We collected freshly resected surgical specimens and biopsies and paired peripheral venous blood drawn in the operating theater. Tumor samples were immediately snap-frozen in liquid nitrogen, and stored at -80^{0}C until DNA extraction was performed. Serum samples were collected before surgery or at the same time of surgery.

Five to ten mL of venous blood from each patient was withdrawn into vacutainer tubes containing SST gel and clot activator (Becton Dickinson, Plymouth, United Kingdom). Serum was isolated after centrifugation at 2500 rpm for 10 min and stored at -20^{0}C until use. Genomic DNA was obtained from 25 mg of each tissue sample, using DNAeasy Tissue kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Serum DNA was extracted using the QIAmp Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

MSP. DNA methylation patterns in the CpG islands of MGMT (GenBank accession No X_61657), p16 (GenBank accession No X_94154), RASSF1a (GenBank No. XM_011780), TMS-1 (GenBank accession no AF184072), and DAPK (GenBank accession No NM_004938) were determined by MSP. Primers are described in *Tab. 1*. Briefly, up to 1μg of genomic DNA was denatured by NaOH and modified by sodium bisulfite. DNA samples were purified using Wizard DNA purification resin (Promega), again treated with

Figure 1 A) Methylation-specific PCR of RASSF1A, TMS-1 and DAPK in primary resected NSCLC and paired serum DNA. *B)* MSP of MGMT, p16, DAPK and RASSF1A in Glioblastoma patients (GBM) comparing tumoral tissue vs. serum. Five 1μ of bisulfite-modified DNA was amplified by PCR using primers that were specific for methylated or unmethylated sequences of each of these genes. Pt: patient sample; T: tumor; S: serum; M: methylated; U: unmethylated; Unm C: unmethylated control (lymphocytes); Met C: methylated control (SW-48 for MGMT gene, modified placental for DAPK gene, HT-29 for p16 gene, MOLT-4 for RASSF1A gene and SKBR-3 for TMS-1 gene).



NaOH, precipitated with ethanol, and resuspend in water. Controls without DNA were performed for each set of PCR. Each PCR products (15μL) was loaded onto a 2% agarose gel, stained with ethidium bromide, and visualized under UV illumination. DNA from peripheral blood lymphocytes and placenta treated with SssI Methyltransferase (New England Biolabs) was used as a positive control for unmethylated (lymphocytes) and methylated PCR reaction (placenta). In addition, all of the amplification assays included positive control for methylated form; thus the MOLT-4 cell line as a methylated control for RASSF1A, the SW-48 cell line is used as a positive control for MGMT methylation and finally, the cell line SKBR3 is used for TMS-1 methylated control (*Fig. 1*).

Results

The results obtained for GBM and NSCLC patients were analyzed separately and are described in *Tab. 2*.

Epigenetic alterations in Serum DNA and Tumor DNA in GBM patients

Methylation status for patients was studied in DNA extracted from 28 serum samples and 21 available paired glioma samples. Methylation status did not differ according to age, gender or treatment approach.

The prevalence of MGMT, p16, DAPK, and RASSF1A promoter methylation was 8/21 (38.1%), 14/21 (66.7%), 11/21 (52.4%), 12/21 (57.1%), respectively, in glioma tissue, and 11/28 (39.3%), 15/27 (53.6%), 9/26 (34.3%), 13/26 (50%), respectively, in serum.

We found that the concordance between alterations in tumor versus serum DNA was 17/21 (80%) for MGMT, 17/20 (85%) for p16, 17/21 (80%) for DAPK and 16/21 (76%) for RASSF1A. *Tab. 3* summarizes the concordance between methylation status of these genes in tumor and serum. All correlation coefficients were highly significant (Spearman correlation <0.05).

We only found 1 patient without any alteration in the four genes analyzed. The rest of the populations have at least one alteration in either tumor or serum DNA. We observed that methylation status did not differ according to age, gender, or treatment approach.

Overall, complete or partial response, or stable disease was observed in 15 patients (60%) whereas 10 patients had progressive disease. When patients were divided into those with MGMT methylation (either in tumor or serum) and those without MGMT methylation, response defined as no progression was noted in 10 of 11 (90,9%) of methylated patients as opposed to 5 of 14 (35, 7%) unmethylated patients (Fisher's exact test P=0.01). In addition, when response was broken down by methylation status in serum only, response plus stable disease was seen in 8 of 8 (100%) patients with methylated bands (Fisher's exact test p=0.008), but for tumor tissue alone, the differences in response were not significant.

When patients were broken down by treatment group, in the 16 patients treated with Temozolamide plus Cisplatin, no significant correlation between MGMT methylation status and response was observed, whereas in BCNU-treated patients, a significant difference was observed in favour of those with methylated MGMT. No correlation was observed between response and p16, DAPK or RASSF1A methylation in tumor and serum (data not shown).

Finally, for the analysis of methylation status and time to progression we observed that for patients who present aberrant methylation in MGMT in either tumor or serum, time to progression was 29.9 weeks (95% CI, 24.3 to 35.4). Time to progression for patients without MGMT methylation was 15.7 weeks (95% CI, 14.3 to 17.2) (log-rank test P=0.006) (*Fig. 2*). Neither p16, DAPK nor RASSF1A methylation correlated with time to progression.

Table 2. A) Summary of methylation patterns in matching tumor and serum DNA in NSCLC

	Tumor DNA/Serum DNA				
Patient No	RASSF1A	DAPK	TMS-1	Stage(TNM)	Histology
Pt#1	M/M	U/U	U/U	IB (T2N0M0)	AC
Pt#2	U/U	U/M	U/U	IA (T1N0M0)	SCC
Pt#3	M/M	U/U	M/M	IIB (T3N0M0)	AC
Pt#4	U/U	M/M	M/M	IIIA (T1N2M0)	LCC
Pt#5	M/M	M/U	U/U	IA (T1N0M0)	AC
Pt#6	U/U	U/U	M/M	IIIA (T2N2M0)	SCC
Pt#7	U/U	M/M	U/U	IIA (T2N1M0)	SCC
Pt#8	U/U	M/M	U/U	IIB (T3N0M0)	AC
Pt#9	U/U	M/U	U/M	IB (T2N0M0)	SCC
Pt#10	U/U	U/U	U/U	IIB (T2N1M0)	SCC
Pt#11	M/M	U/U	M/M	IV (T3N0M0)	AC
Pt#12	M/M	U/U	M/M	IIIA (T3N2M0)	LCC
Pt#13	U/U	U/U	U/U	IIIA (T3N2M0)	LCC
Pt#14	U/U	U/U	M/U	IIB (T3N0M0)	SCC
Pt#15	M/U	M/M	M/M	IIIA (T3N2M0)	ACC
Pt#16	U/U	U/U	U/U	IV (T3N2M1)	SCC
Pt#17	U/U	U/M	U/M	IIIA (T1N2M0)	SCC
Pt#18	M/U	M/U	U/U	IIIA (T2N2M0)	SCC
Pt#19	U/U	M/U	M/M	IB (T2N0M0)	SCC
Pt#20	U/U	M/U	M/M	IA (T1N0M0)	SCC
Pt#22	M/M	M/U	M/U	IB (T2N0M0)	AC
Pt#23	U/U	M/U	U/M	IIB(T2N1M0)	SCC
Pt#24	U/U	M/U	M/M	IB (T2N0M0)	SCC
Pt#25	U/U	U/U	U/U	IIB (T2N1M0)	LC
Pt#26	U/U	M/U	U/M	IIIA (T3N1M0)	SCC
Pt#27	U/U	U/U	U/U	IB (T2N0M0)	LC
Pt#28	U/M	M/M	U/U	IB (T2N0M0)	AC
Pt#29	M/M	M/U	U/U	IB (T2N0M0)	LC
Pt#30	U/U	U/U	U/U	IIIA (T3N1M0)	SCC
Pt#31	U/U	U/U	M/U	IV (T2N1M0)	SCC
Pt#32	U/U	U/U	U/U	IIIA (T2N2M0)	AC
Pt#33	U/M	U/U	U/U	IIIA (T2N2M0)	SCC
Pt#34	U/M	U/U	M/U	IB (T2N0M0)	AC
Pt#35	M/M	U/M	M/M	IIIA (T2N2M0)	AC
Pt#36	M/U	U/U	M/U	IB (T2N0M0)	SCC
Pt#37	U/M	U/M	U/U	IIB (T3N0M0)	LCC
Pt#38	U/U	M/M	M/M	IIIA (T3N1M0)	LCC
Pt#39	U/U	M/U	U/U	IV (T2N0M1)	SCC
Pt#40	U/U	U/U	U/U	IA (T1N0M0)	LCC
Pt#41	M/M	U/M	U/U	IB (T2N0M0)	SCC
Pt#42	U/M	M/M	U/U	IV (T1N1M1)	AC
Pt#43	U/U	U/U	U/U	IIIA (T3N1M0)	SCC
Pt#44	M/M	M/M	U/M	IB (T2N0M0)	SCC
Pt#45	M/U	M/M	U/U	IV (T3N0M1)	LCC
Pt#46	M/M	M/M	U/U	IIIA (T2N2M0)	SCC
Pt#47	U/U	U/U	U/U	IIA (T1N1M0)	AC
Pt#48	U/U	U/U	U/M	IIIA (T2N2M0)	AC
Pt#49	U/U	U/M	M/M	IIIA (T3N2M0)	AC
Pt#50	M/U	M/M	U/U	IIA (T1N1M0)	SCC
Pt#51	M/M	U/M	U/U	IIIA (T2N2M0)	LCC

AC: Adenocarcinoma; SCC: Squamous Cell Carcinoma; LCC: Large Cell Carcinoma

Table 2. B) Summary of methylation patterns in matching tumor and serum DNA in Glioblastoma Multiforme

	Tumor DNA/Serum DNA			
Patient No.	MGMT	P16	RASSF1A	DAPK
Pt#1	M/U	M/M	U/U	M/U
Pt#2	M/M	M/M	M/U	U/U
Pt#3	U/U	M/M	U/U	M/M
Pt#4	-/M	-/M	-/M	-/U
Pt#5	-/M	-/U	-/U	-/U
Pt#6	U/U	M/M	U/U	M/M
Pt#7	-/U	-/M	-/U	-/U
Pt#8	U/U	M/M	U/M	U/U
Pt#9	U/U	M/M	M/M	M/M
Pt#10	U/U	U/U	M/M	U/U
Pt#11	-/M	-/U	-/U	-/U
Pt#12	U/U	U/U	U/U	U/U
Pt#13	U/U	M/M	M/M	U/U
Pt#14	-/M	-/M	-/M	-/U
Pt#15	U/U	M/M	M/M	M/M
Pt#16	U/M	U/U	M/M	U/U
Pt#17	M/M	-/M	U/U	M/M
Pt#18	M/U	M/M	U/U	U/U
Pt#19	M/M	U/U	M/U	M/M
Pt#20	-/M	-/U	-/M	U/M
Pt#21	M/M	M/M	M/M	M/M
Pt#22	U/U	M/U	M/M	U/U
Pt#23	M/U	U/U	U/U	M/U
Pt#24	-/U	-/U	-/U	-/U
Pt#25	U/U	M/U	U/M	M/U
Pt#26	U/U	M/M	U/U	U/U
Pt#27	M/M	U/M	M/M	M/M
Pt#28	U/U	M/M	M/U	M/U

Theses tables represent alterations in tumoral tissue DNA vs. serum DNA, (T/S). U for Unmethylated gene and M for Methylated gene. –/S indicates Tumor DNA not available.

Table 3. Concordance between methylation of MGMT, p16, DAPK and RASSF1A in tumor and serum DNA from glioblastoma patients.

Markers	Assessment in TISSUE	Assessment in SERUM		Spearman Correlation
		Unmethylated No (%)	Methylated No (%)	
MGMT	Unmethylated	12 (57.1)	3 (14.3)	0.005
	Methylated	1 (4.8)	5 (23.8)	
DAPK	Unmethylated	10 (47.6)	3 (14.3)	0.00001
	Methylated	–	8 (38.1)	
p16	Unmethylated	6 (28.6)	2 (9.5)	0.00001
	Methylated	1 (4.8)	12 (57.1)	
RASSF1A	Unmethylated	8 (38.1)	2 (9.5)	0.00001
	Methylated	1 (4.8)	10 (47.6)	

Figure 2. Time to progression according to MGMT methylation pattern in serum or tissue.

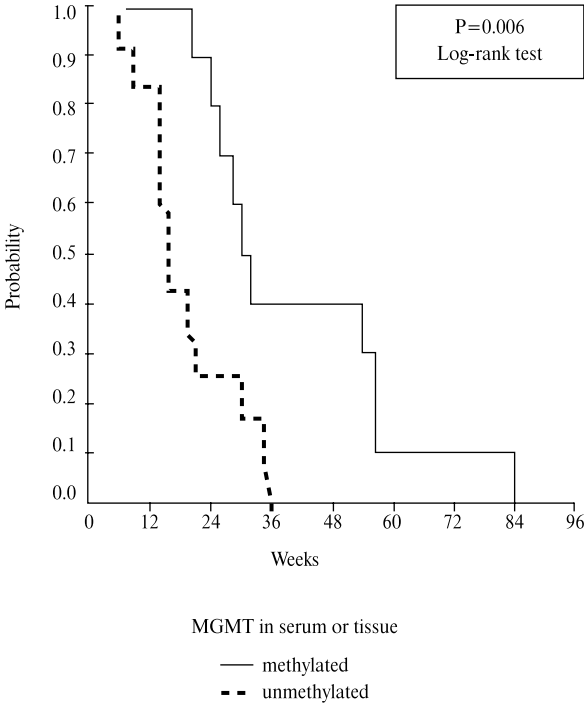


Table 4. Methylation patterns of RASSF1A, DAPK, and TMS-1 found in tumor and serum DNA from NSCLC patients.

Markers	Assessment in TISSUE	Assessment in SERUM		Spearman Correlation
		Unmethylated No (%)	Methylated No (%)	
TMS1	Unmethylated	27 (54)	6 (12)	0.0001
	Methylated	5 (10)	12 (24)	
DAPK	Unmethylated	19 (39.6)	9 (18.7)	0.05
	Methylated	8 (16.7)	12 (25)	
RASSF1A	Unmethylated	28 (56)	5 (10)	0.0001
	Methylated	5 (10)	12 (24)	

Gene promoter Hypermethylation Profiles in NSCLC patients

The analysis for methylation status in NSCLC was developed in DNA extracted from 51 patients (49 male smokers and two female non-smokers). Neither gender, age nor stages were associated with the promoter methylation status of the genes.

The prevalence of RASSF1A, DAPK and TMS-1 methylation was 17/51 (34%), 23/51 (45%) and 18/51 (35%), respectively, in tumor tissue, and 17/51 (34%), 20/51 (40%) and 17/51 (34%), respectively, in serum (*Tab. 4*).

For RASSF1A gene we found that 41/51 (80%) of patients had the same alteration in tumor and serum DNA; for DAPK was 33/51 (65%); and for TMS-1 was 40/51 (78%). In other words, a good correlation was found between alterations found in tumor and serum (Spearman correlation <0.05).

Overall, 74.5 % of patients had at least one genetic alteration in tumor, and 76.5 % had at least one in serum. Eighty-two percent of patients had at least one alteration in either tumor or serum. Stage I NSCLC patients had at least two genetic alterations in either tumor or serum in 87.5 % of cases.

We observed no relationship between the number of genetic alterations and tumor size. We found no correlation between methylation status of RASSF1A, TMS-1 or DAPK, either in tumor or serum, and survival.

Discussion

The most important of the studies published in the literature that analyze aberrant methylation have been performed in DNA isolated from tumor tissue, and few studies on aberrant DNA methylation have been done in cell free circulating serum DNA [24,26]. In addition, these studies do not analyze the correlation of gene promoter methylation between serum and tumor DNA at time of surgery or biopsy. For these reasons, we have assessed methylation of MGMT, p16, DAPK and RASSF1A, TMS-1 in tumor and matching serum of GBM patients and NSCLC patients, and we have found that the concordance between methylation of these genes in tumor and serum DNA is highly significant.

Furthermore, we demonstrate, for the first time, that in GBM patients it is possible to detect aberrant promoter hypermethylation in serum DNA and that these alterations have the origin in the tumoral tissue. As we know, when malignant gliomas are transplanted into subcutaneous tissues, the glioma cells grow locally but do not metastasize, indicating that primary brain tumors are fundamentally different from tumors originating elsewhere [30]. This was one of the reasons that it is particularly intriguing to screen methylation in serum of GBM patients. The only previous research along these lines was the seminal work by Leon et al. [1] examining the concentration of free serum DNA in several tumors including four gliomas. Another reason for analyzing MGMT status was the study performed by Esteller et al. [31], where they found an increased responsiveness to BCNU and cisplatin in MGMT-deficient anaplastic astrocytomas and GBM. In our study, MGMT methylation, especially in serum DNA, was significantly associated with response and time to progression in all patients, as had previously been suggested [32,33]. However, when patients were broken down by treatment group, no significant correlation between MGMT methylation and response was observed in patients treated with Temozolamide plus Cisplatin, suggesting a role for Cisplatin, as previous in vitro experience suggests that Cisplatin may enhance the activity of Temozolamide by the inhibition of MGMT [34]. Although there are other DNA repair pathways that are important in chemoresistance, the examination of these factors involved in these pathways requires primary tumor tissue, whereas the MSP assay of MGMT methylation in serum DNA can be easily performed in samples obtained by non-invasive tools. The importance of this study lies in the findings that serum MGMT methylation predicted response and time to progression in BCNU-treated GBM patients and that, moreover, serum DNA is a feasible alternative source for testing methylation.

We also analyzed aberrant promoter methylation in tumoral and serum DNA from NSCLC patients, obtained at the time of surgery. The prevalence of RASSF1A and DAPK methylation found in the study was along the same lines as that previously reported [17,10,35]. In this study, we have detected a similar frequency of methylation of all three genes in the paired serum DNA and a good correlation was found between alterations found in tumor and serum (Spearman test $P=0.0001$) (*Tab. 4*).

For DAPK gene there are previous studies that also detected this gene methylated in serum DNA from NSCLC patients [14]. Although DAPK methylation was found in 59/135 (44%) of resected NSCLC patients and correlated significantly with worse survival [35], in another study, DAPK methylation was not significantly associated with worse survival [17]. In our study, no differences in survival were observed according to DAPK methylation status. A growing list of reports indicates that methylation in serum DNA correlates with methylation in primary tumors, including DAPK in gastric carcinoma [36].

For the first time, TMS-1 has been examined in lung cancer and found in 18/50 (35%) primary tumors, which concurs with the frequency described in breast cancer [23] .We have found TMS-1 methylated in 35% of primary tumors and 34% of serum samples, similar to the 40% found in breast cancer [23]. The good correlation between tumor and serum methylation status makes the examination of TMS-1 in serum DNA particularly interesting since it has been postulated that methylation-mediated silencing of TMS-1 could confer resistance to cytotoxic drugs [23].

Finally, RASSF1A methylation in either tumor or serum did not correlate with survival. The importance in this study is the fact that the high concordance of methylation patterns in tumor and serum DNA makes it particularly attractive to use peripheral blood for methylation assessment.

In summary, in this report we demonstrate the feasibility of finding the presence of alterations in tumor DNA and in serum DNA and the possible origin of these free circulating DNA. This evidence is supported by the fact that a good correlation exists between methylation in serum and primary tumor tissue. Moreover, the molecular techniques most commonly used to detect the neoplastic DNA in serum/plasma DNA are based on PCR assays. These PCR techniques can detect very few copies in tumor DNA containing a specific alteration among an excess background of normal DNA; for this reason, we decided to use the MSP protocol, which is a highly sensitive technique that allows approaching 1 methylated gene copy in 1000 unmethylated copies in dilution experiments [25]. The results obtained in different cancers open a new research area indicating that serum/plasma DNA could be used for the development of non-invasive tests for cancer patients in diagnosis, prognosis and follow-up. Since promoter methylation of different genes has been shown to be related to chemotherapy response or survival, the analysis in serum or plasma might be a useful tool for tailoring chemotherapy.

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