Biol Blood Marrow Transplant 19 (2013) 888–892

Salivary Proteomic Analysis and Acute Graft-versus-Host Disease after Allogeneic Hematopoietic Stem Cell Transplantation

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Article history:
Received 20 December 2012
Accepted 19 March 2013

Key Words:
Salivary proteomic analysis
Allogeneic stem cell transplantation
Graft-versus-host disease
S100 proteins

A B S T R A C T

Graft-versus-host disease (GVHD) is the major life-threatening complication after allogeneic hematopoietic stem cell transplantation (allo-HSCT), developing in 35%-70% of all allo-HSCT recipients despite immunosuppressive prophylaxis. The recent application of proteomic tools that allow screening for differentially expressed or excreted proteins in body fluids could possibly identify specific biomarkers for GVHD. Whole saliva is highly attractive for noninvasive specimen collection. In the present study, we collected saliva specimens from 40 consecutives patients who underwent allo-HSCT between December 2008 and March 2011 at our institution. The specimens were analyzed by HPLC coupled to electrospray-ionization mass spectrometry. Variable expression of S100 protein family members (S100A8, S100A9, and S100A7) was detected. Fourteen of 23 patients with GVHD demonstrated the presence of S100A8, compared with only 2 patients without GVHD and 0 patients in the control group ($P = .001$). S100A7 was detectable in 11 of the 23 patients with GVHD but was absent in the other 2 groups ($P = .0001$). S100A9-short was detected in 20 patients with GVHD, in 9 patients without GVHD, and in 8 healthy volunteers ($P = .01$) Further studies are needed to clarify the role of these proteins as a marker of GVHD or as an index of mucosal inflammation.

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is a potential curative treatment for hematopoietic malignancies, hematopoietic failure, and autoimmune disease. Despite enormous advances in allo-HSCT practice, significant challenges remain that limit more widespread application of this treatment. Although nonmyeloablative conditioning regimens have decreased treatment-related mortality, major complications, such as graft-versus-host disease (GVHD) and severe infection, still occur [1]. Depending on the type of transplantation, the immunosuppressive treatment, and underlying diseases, between 35% and 70% of patients develop GVHD after allo-HSCT, and more than 35% of these patients require immunosuppressive therapy [2].

Currently, the diagnosis of acute GVHD (aGVHD) is based mainly on clinical parameters, including skin rash, diarrhea, elevated serum liver enzyme levels, and others. Differential diagnosis of GVHD depends on organ biopsy analyses to distinguish GVHD from other common complications after HSCT, including bacterial and viral infections and chemotherapy–induced toxicities [3]. To date, there is no validated diagnostic test for aGVHD, although several studies have identified multiple blood proteins as potential biomarkers; however, differences in any single protein lack sufficient specificity and sensitivity to be of clinical use, and so a GVHD diagnosis is still confirmed by invasive procedures [4-8].

The study of proteomics has evolved rapidly over the past decade. High-throughput MS approaches coupled to different separation techniques, including HPLC and bidimensional gel electrophoresis have been applied to several human diseases with the aim of replacing or complementing traditional tools for disease diagnosis and monitoring of disease activity or response to therapy. Proteomic techniques have specific application in the search for biomarkers and therapeutic targets, especially in oncologic and autoimmune diseases [9-15].

The study of proteomic patterns of biological samples in patients affected by GVHD has expanded rapidly over the last few years, with the aim of identifying specific and sensitive markers of GVHD with predictive and prognostic value. Plasma and urine are the biological fluids principally investigated for proteomic profiling in studies of patients with and without GVHD after allo-HCT [16-20]. Few studies have examined the analysis of salivary proteins, despite the fact that the oral cavity is often involved in the pathology of GVHD [21-24]. Chronic GVHD affects the oral mucosa with a frequency second only to that of skin, causing significant morbidity [25]. Major oral clinical manifestations include mucositis, xerostomia, gingivitis, erythema, inflammation, salivary gland dysfunction, pain and taste disorders; oral findings include lichen planus–like changes, hyperkeratotic plaques, mucosal atrophy, and pseudomembranous ulceration in patterns reminiscent of autoimmune diseases such as lichen planus, lupus systemic sclerosis, and Sjogren syndrome [26-28].

Financial disclosure: See Acknowledgments on page 892.
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1083-8791/$ – see front matter © 2013 American Society for Blood and Marrow Transplantation.
http://dx.doi.org/10.1016/j.bbmt.2013.03.011

Table 1
Patient Characteristics

Number of Patients	40
Males/females	17/23
Diagnoses	20 acute myelogenous leukemia, 6 acute lymphoblastic leukemia, 5 non-Hodgkin lymphoma, 2 Hodgkin lymphoma, 1 chronic lymphocytic leukemia, 2 myelodysplastic syndrome, 1 idiopatic myelofibrosis, 3 multiple myeloma
Disease status	17 complete remission, 5 partial remission, 2 progressive disease, 3 refractory disease, 11 relapsed disease, 2 stable disease
Donors	28 sibling HLA-matched donors; 12 unrelated HLA-matched donors
Conditioning regimens	13 myeloablative; 27 reduced-intensity
Hematopoietic stem cell source	37 peripheral blood, 1 bone marrow, 2 cord blood
$CD34^+$ cells, $\times 10^6$/kg, median (range)	7×10^6/kg ($1.47\text{-}17 \times 10^6$/kg)
GVHD prophylaxis	20 CSA + methotrexate, 14 CSA + mycophenolate mofetil, 3 CSA + Campath, 1 CSA, 2 no GVHD prophylaxis

Saliva is an attractive diagnostic fluid, providing several key advantages, including easy and noninvasive sample collection, ready processing, and fewer proteins than plasma. A body fluid of a very complex and specific composition devoted to the protection of the oral cavity, saliva includes organic and inorganic solutes and specific peptides and proteins containing components of both innate and adaptive immunity. Some of these proteins have specific antimicrobial, immunomodulatory, and anti-inflammatory activities [29]. Proteins with these functions are differently expressed in the saliva of patients affected by Sjogren syndrome; in particular, α-defensins and some members of the S100A protein family (S100A9 and S100A7) were found to be upregulated in both saliva and tears of patients with Sjogren syndrome [13,30-32].

Sjogren syndrome is a chronic inflammatory autoimmune disease characterized by functional inhibition of autonomic neurotransmission to lacrimal and salivary glands and infiltration of the exocrine glands by effector T cells, leading to qualitatively altered and diminished or absent glandular secretion with clinical analogies to the sicca syndrome affecting patients with GVHD, with xerostomia and xerophtalmia [33]. Thus, in our evaluation of changes in salivary proteins after allo-HSCT, particularly in patients with GVHD, we supposed that these changes would be similar to those seen in patients with Sjogren syndrome. Consequently, we performed salivary proteome analysis in patients undergoing allo-HSCT, with the aim of comparing those with GVHD and those without GVHD. A group of healthy subjects was also enrolled to serve as a control. Specifically, using a HPLC–electrospray-ionization mass spectrometry (ESI-MS)–based approach, we investigated the levels of the following proteins with known roles in inflammation, repair of tissue damage, and innate and adaptive immunity: α-defensins 1–4, thymosins β4 and β10, and S100A family proteins S100A8, 4 isoforms of S100A9 (short, long, and corresponding phosphorylated forms), 2 isoforms of S100A7 (D_{27} and E_{27}), and S100A12.

PATIENTS AND METHODS

Patients

Subjects enrolled for the study were 40 consecutive patients who underwent allo-HSCT at Hematology Department, Università Cattolica S. Cuore between December 2008 and March 2011. The group included 17 males (43%) and 23 females (57%), with a median age of 46 years (range, 14 to 65 years), affected by the following hematologic diseases: 20 (50%) with acute myelogenous leukemia, 6 (15%) with acute lymphoblastic leukemia, 5 (12.5%) with non-Hodgkin lymphoma, 2 (5%) with Hodgkin lymphoma, 1 (2.5%) with chronic lymphocytic leukemia, 2 (5%) with myelodysplastic syndrome, 1 (2.5%) with idiopatic myelofibrosis, and 3 (7.5%) with multiple myeloma.

Disease status at the time of allo-HSCT was 17 patients (42.5%) in complete remission, 5 (12.5%) in partial remission, 2 (5%) with progressive disease, 3 (7.5%) with refractory disease, 11 (27.5%) with relapsed disease, and 2 (5%) with stable disease. Thirteen of the patients (32.5%) received a myeloablative conditioning regimen before allo-HSCT, and 27 patients (67.5%) received a reduced-intensity conditioning regimen. Stem cell grafts were obtained from sibling donors for 28 patients and from unrelated donors for 12 patients. GVHD prophylaxis was provided with cyclosporine (CSA) and methotrexate in 20 patients, CSA and mycophenolate mofetil in 14 patients, CSA and Campath in 3 patients, and CSA in 1 patient; 2 patients received no GVHD prophylaxis (Table 1). Eighteen normal healthy subjects (6 males and 12 females), with a median age of 35 years (range, 25 to 50 years), were included as controls. Saliva samples were obtained after informed consent from each patient for use in biological studies.

Sample Collection

Whole human saliva was collected every 30 days and at the onset of GVHD in the first 100 days after allo-HSCT, in accordance with a standard study protocol. Donors did not eat or drink anything in the 30 minutes before collection, which was performed between 10:00 and 12:00 a.m. Whole saliva was collected with a soft plastic aspirator connected to a plastic tube. An acidic solution (0.2% trifluoroacetic acid [TFA]) was immediately added to the saliva samples at a 1:1 vol/vol ratio, and the resulting solution was centrifuged at 8000 × g for 5 minutes at 4 °C. The acidic supernatant was separated from the precipitate and either immediately analyzed with the HPLC–ESI-MS apparatus or stored at −80°C until analysis. In the GVHD group, samples were collected at the onset of complications, before the initiation of steroid therapy. All patients developed oral cavity manifestations of GVHD, such as xerostomia, erythema, lichen planus-like changes, hyperkeratotic plaques, mucosal atrophy, and pseudomembranous ulcerations. In the other group, saliva samples were collected every 30 days during the first 100 days after allo-HSCT.

HPLC–ESI-MS Analysis

The HPLC–ESI-MS apparatus was a Surveyor HPLC system (Thermo Fisher, San Jose, CA) equipped with a photodiode array detector and connected by a T splitter to a Thermo Fisher LCQ Deca XP Plus electrospray-ionization/ion-trap mass spectrometer (split ratio 2:1) with a Vydac C8 chromatographic column (Vydac, Hesperia, CA) with 5 μm particle diameter (column dimensions, 150 × 2.1 mm). The following solutions were used for reversed-phase HPLC–ESI-MS analysis: 0.056% (vol/vol) aqueous TFA (eluent A) and 0.05% (vol/vol) TFA in 80/20 acetonitrile/water (eluent B). The flow rate was 0.30 mL/min. Salivary proteins were eluted using a linear gradient from 0 to 55% of B in 40 minutes.

Qualitative and Quantitative Data Analysis

The following proteins were analyzed: α-defensins 1–4, thymosins β4 and β10, and S100A family proteins S100A8, 4 isoforms of S100A9 (short, long, and corresponding phosphorylated forms), 2 isoforms of S100A7 (D_{27} and E_{27} isoforms), and S100A12. These proteins have been identified and characterized in previous studies [34,35].

Label-free quantification of proteins was based on the measure of area of the reversed-phase HPLC–ESI-MS extracted ion current (XIC) peaks. Under constant analytical conditions, the area of the extracted ion current peaks is proportional to the peptide/protein concentration [34,36].

Reagents

All general chemicals and reagents were of analytical grade and were purchased from Farmitalia-Carlo Erba (Milan, Italy), Merck (Darmstadt, Germany), and Sigma-Aldrich (St Louis, MO).

Data Analysis

Data were analyzed with the Mann-Whitney *U* test and one-way ANOVA using SPSS 16.0 (SPSS, Chicago, IL) and Prism software (Graph-Pad, San Diego, CA). Statistical analysis was considered significant at a *P* value of $<.05$ (2-tailed tests).

Table 2
Experimental Average Mass Values, Swiss Prot Identification Codes, and Posttranslational Modifications of the Peptides and Proteins Analyzed in this Study

Protein	MAV exp, Da	Swiss Prot Code	Posttranslational Modification
α-defensin 1	3442.0 ± 0.5	P59665	3 S-S
α-defensin 2	3371.0 ± 0.5	P59665/6	3 S-S
α-defensin 3	3486.1 ± 0.5	P59666	3 S-S
α-defensin 4	3707.8 ± 0.5	P12838	3 S-S
Thymosin β4	4963.5 ± 0.5	P62328	Acetyl (N-term)
Thymosin β10	4934.5 ± 0.5	P63313	Acetyl (N-term)
S100A8 (calgranulin A)	10,834 ± 1	P05109	—
S100A9 (calgranulin B) short	12,689 ± 1	P06702	Acetyl, MTCKM missing (N-term)
S100A9 (calgranulin B) short P	12,769 ± 1	P06702	Acetyl, MTCKM missing (N-term), phosphorylation (T_{108})
S100A9 (calgranulin B) long	13,153 ± 1	P06702	Acetyl, M missing (N-term)
S100A9 (calgranulin B) long P	13,233 ± 1	P06702	Acetyl, M missing (N-term), phosphorylation (T_{112})
S100A12 (calgranulin C)	10,444 ± 1	P80511	M missing (N-term)
S100A7 (psoriasin D_{27})	11,368 ± 1	P31151	Acetyl, M missing (N-term), E_{27} → D
S100A7 (psoriasin E_{27})	11,382 ± 1	P31152	Acetyl, M missing (N-term)

MAV exp indicates experimental average mass value; S-S, disulfide bridge.

RESULTS

Patient Characteristics

Patients received grafts with a median $CD34^+$ stem cell dose of 7.0×10^6/kg (range, 1.47 to 17×10^6/kg). After allo-HSCT, the median time to neutrophil engraftment at a level of $>0.5 \times 10^9$/L was 16 days (range, 1 to 34 days), and that to a level of $>1.0 \times 10^9$/L was 18 days (range, 8 to 34 days). The median time to spontaneous platelet recovery at $>20 \times 10^9$/L was 12 days (range, 2 to 38 days), and that to $>50 \times 10^9$/L was 14 days (range, 1 to 185 days). During the first 100 days after allo-HSCT, sepsis was microbiologically documented in 14 patients (35%), whereas 13 patients presented with fever of unknown origin (32.5%). Twenty-five patients (62.5%) developed cytomegalovirus reactivation at a median time of 45 days (range, 5 to 200 days) after transplantation, with a median maximum number of 4130 viral genome copies/mL (range, 800 to 583.000 copies/mL). These included 15 of 23 patients (65%) in the GVHD group and 10 of 17 patients (58%) in the other group. All patients received preemptive antiviral therapy with gancyclovir.

Twenty-three patients (57%) developed aGVHD, at a median time of 50 days (range, 10 to 97 days) after allo-HSCT. In 5 of these patients, symptoms resolved after treatment. Nine patients died early, at a median time of 2 months (range, 1 to 3 months) after allo-HSCT, for a transplantation-related mortality of 23% by 100 days posttransplantation. Twenty patients (50%) had chronic GVHD, 8 with classic chronic GVHD developing at a median of 152 days (range, 100 to 927 days) after allo-HSCT, and the other 12 with an overlap syndrome. All patients exhibited oral cavity manifestations of GVHD. GVHD was diagnosed based on the Seattle criteria [37,38].

Proteomic Analysis of Salivary Samples

HPLC–ESI-MS analysis of whole human saliva detected and quantified the following peptides/proteins: α-defensins 1–4, thymosins β4 and β10, and S100A8, S100A9, S100A12, and S100A7 proteins [34,35]. Average mass values and the various posttranslational modification derivatives of the proteins investigated are reported in Table 2. S100A proteins analyzed included 2 isoforms of S100A7 presenting a residue of glutamic acid or aspartic acid in position 27 (E_{27} and D_{27} isoforms), and 4 isoforms of S100A9 corresponding to short and long phosphorylated and nonphosphorylated forms. The long form of S100A9 is generated from the pro-protein by loss of the N-terminal methionine residue followed by acetylation, and phopshorylation may occur at the penultimate threonine residue. In the short form of S100A9, removal of the 5 N-terminal amino acid residues is followed by acetylation. This form also may be phosphorylated at the penultimate threonine residue.

The XIC procedure allowed measurement of the relative amounts of the peptises/proteins in the study subjects, who were divided into 3 groups: a group of 23 patients who developed aGVHD after allo-HSCT, a group of 17 patients without GVHD, and a group of 18 healthy controls matched for age.

No differences in levels of the defensins and thymosin β4 were detected among the 3 groups. In contrast, statistically significant differences in the frequency and levels of S100A8, S100A9-short, and S100A7 were found among the groups. S100A8 was detected in 14 patients in the group with GVHD, with a mean XIC peak area value of 3.50×10^8 (SD, 9.9×10^8), compared with only 2 patients in the group without GVHD, with a mean value of 2.46×10^8 (SD, 9.9×10^8) and none in the control group. A statistically significant difference among the 3 groups in terms of frequency ($P = .001$) and levels of detection ($P = .0002$) was calculated (Figure 1). Sensitivity and specificity for S100A8 were 60% and 88%, respectively.

S100A9 short was detected in 20 patients in the group with GVHD (mean value, 9×10^8; SD, 1.6×10^9), in 9 patients in the group without GVHD (mean value, 6×10^8; SD, 1.7×10^9), and in 8 healthy controls (mean value, 6×10^7; SD, 7.9×10^7), with statistically significant differences in terms of frequency ($P = .01$) and levels of detection ($P = .002$)

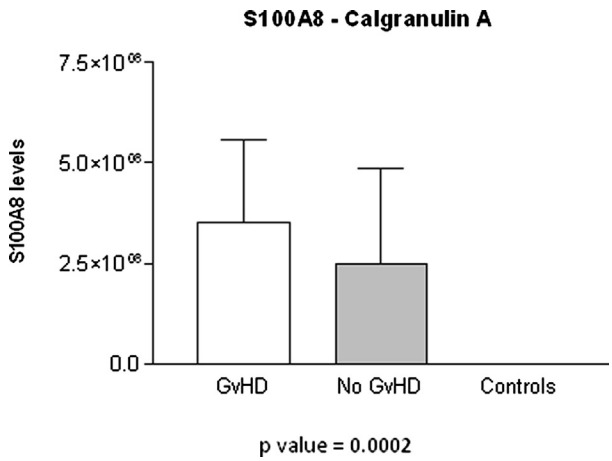

Figure 1. Levels of S100A8 for the 3 groups, expressed as XIC peak area.

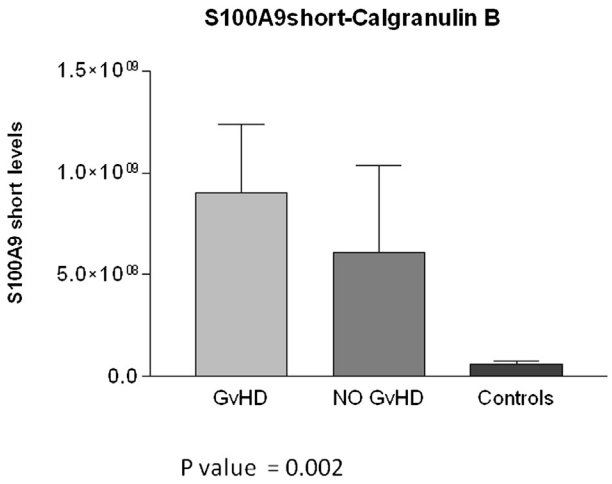

Figure 2. Levels of S100A9 short isoform for the 3 groups, expressed as XIC peak area.

(Figure 2). Sensitivity and specificity for S100A9 short were 87% and 47%, respectively.

S100A7 (varD27) was detected in 11 of the 23 patients with GVHD, with a mean XIC peak area value of 7.03×10^8 (SD, 1.4×10^8), but was not detected in any patients in the other 2 groups, for a statistically significant difference in terms of frequency ($P = .0001$). Sensitivity and specificity for S100A7 were 48% and 100%, respectively. No statistically significant differences in the S100A9 long and S100A12 proteins were seen among the 5 study groups.

DISCUSSION

Allo-HSCT is frequently complicated by severe infections and GVHD. Between 35% and 70% of patients develop GVHD after allo-HSCT, depending on the type of transplantation, immunosuppressive treatment, and underlying diseases. Currently, aGVHD is diagnosed based mainly on clinical parameters, such as skin rash, diarrhea, and elevated serum liver enzymes. To date, there is no validated diagnostic test for aGVHD; thus, differential diagnosis of GVHD depends on organ biopsy analysis to distinguish it from other common complications after allo-HSCT.

Saliva contains many components of adaptive and innate immune response crucial for local host defenses. Change in salivary constituents could reflect systemic processes occurring after allo-HSCT, such as immune reconstitution and development of GVHD. Imanguli et al. [23] reported increased levels of lactoferrin, secretory leukocyte protease inhibitor, and β2-microglobulin after allo-HSCT, whereas Dens et al. [22] found decreased levels of secretory IgA. Nagler and Nagler [21] reported higher Ig levels in patients with oral chronic GVHD. Bassim et al. [24] identified decreased levels of lactotransferrin and lactoperoxidase in the saliva of patients with oral GVHD.

Given the advantages of saliva over plasma, including low cost, noninvasiveness, easy sample collection, easy sample processing, and fewer proteins, we decided to investigate this biofluid for possible biomarkers of aGVHD. We performed a comparative analysis of saliva from 23 patients who developed aGVHD after allo-HSCT, from a group of 17 allo-HASCT recipients without aGVHD, and a group of 18 healthy volunteers. We found statistically significant differences among the 3 groups in terms of frequency and levels of the proteins S100A8 ($P = .001$ and .0002, respectively), S100A9 short ($P = .01$ and .002, respectively), and S100A7 (varD27) ($P = .0001$).

S100 proteins are calcium-binding proteins characterized by 2 calcium-binding EF (helix-loop-helix)-hand motifs connected by a central hinge region. S100A8 (also known as calgranulin A or myeloid-related protein 8) and S100A9 (calgranulin 9 or myeloid-related protein 9) are found in granulocytes, monocytes, and early-stage macrophages. S100A8 and S100A9 are able to form homodimers, but they also exist as heterodimers, better known as calprotectin [39].

In the extracellular compartment, S100A8 and S100A9 are known as damage-associated molecular pattern proteins because they are secreted by activated phagocytes during inflammatory processes. This secretion involves activation of protein kinase C and active metabolisms of the microtubule network. S100A8 and S100A9 induce a proinflammatory and thrombogenic response in endothelial cells, which is characterized by induction of proinflammatory cytokines and adhesion molecules such as VCAM-1 and ICAM-1, loss of cell–cell contacts and increased permeability of endothelium. S100A8 and S100A9 increased the binding affinity of the integrin receptors CD11c and CD18 on neutrophil granulocytes, favoring leukocyte extravasation during inflammation, and also acted as an endogenous Toll-like receptor-4 ligand involved in amplifying the effects of lipopolysaccharide on phagocytes [40].

There is evidence of the release of S100A8 and S100A9 in synovial fluid of patients affected by autoimmune diseases, such as rheumatoid arthritis; in stool of patients with inflammatory bowel disease, in which calprotectin is used as a biomarker for diagnosis and monitoring disease activity; and in the saliva of patients with Sjogren syndrome [31,32,41-43]. Our findings are interesting for several reasons. First, although the S100 protein family has been studied extensively, these proteins were detected in saliva samples in patients with periodontitis, head and neck squamous cell carcinomas, and Sjogren syndrome, as well as in preterm neonates, only recently [31,32,35,44,45]. Moreover, the discovery of elevated S100A protein levels in the saliva of patients with GVHD may be explained by the these proteins' contributions to amplification of inflammatory processes.

In this context, it is even more interesting that S100A7 was detected only in the group of patients who developed GVHD. S1000A7 was identified almost 2 decades ago, when it was named "psoriasin" because it is overexpressed by psoriatic keratinocytes [46]. Human psoriasin is a potent and selective chemotactic inflammatory protein for $CD4^+$ T lymphocytes [47], which are crucial in the pathogenesis of GVHD.

To confirm our data and to clarify the role of S100 proteins in both allo-HSCT and GVHD, evaluating their expression in a larger cohort of subjects will be necessary. In such studies, standardizing study populations according to disease, conditioning regimens, and GVHD prophylaxis will be useful to minimize possible confounding factors.

CONCLUSION

In view of the exclusive presence of S100A8 and S100A7 proteins in the saliva of patients with GVHD, we can hypothesize that these proteins can serve as an index of GVHD activity for use in early diagnosis, monitoring response to therapy, or prognosis. Obviously, more data on a larger number of patients are needed to validate this assay as a tool for these purposes.

ACKNOWLEDGMENTS

We thank Professor Valerio De Stefano for his valuable contributions to the revision of manuscript.

Financial disclosure: This work was supported by Associazione Italiana per la Ricerca sul Cancro (AIRC) funds and Linea D1- Fondi di Ateneo.

Conflict of interest: The authors declare no conflicts of interest.

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