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Research Article

Gangliosides of Human Glioblastoma Multiforme: A Comprehensive Mapping and Structural Analysis by Ion Mobility Tandem Mass Spectrometry

Mirela Sarbu, Ligia Petrica,* David E. Clemmer, Željka Vukelić, and Alina D. Zamfir*

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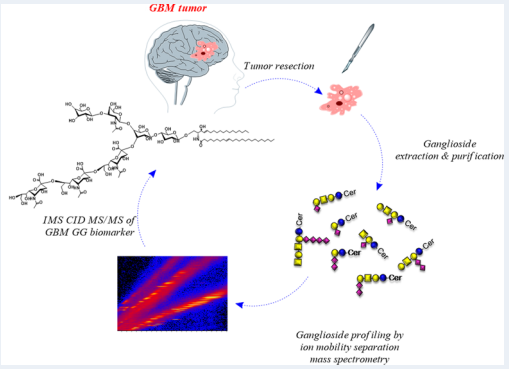
ABSTRACT: Glioblastoma multiforme (GBM), a malignant, highly aggressive, grade IV brain tumor, which rapidly infiltrates into the nearby tissue, has drawn a significant amount of attention because of its poor prognosis and the limited treatment options available. In GBM, nearly all tumor cells exhibit aberrant cell-surface glycosylation patterns due to the alteration of their biosynthesis or postsynthesis modification process. Since gangliosides (GGs) are acknowledged as tumor-associated antigens, we have carried out here a comprehensive profiling of native ganglioside mixtures extracted and purified from GBM specimens. For this purpose, high performance ion mobility separation mass spectrometry (IMS MS) was thoroughly optimized to allow the discovery of GBM-specific structures and the assessment of their roles as tumor markers or possible associated antigens. GG separation by IMS according to the charge state, carbohydrate chain length, degree of sialylation, and ceramide composition led to the identification of no less than 160 distinct components, which represents 3-fold the number of structures identified before. The detected GGs and asialo-GGs were found characterized by a high heterogeneity in their ceramide and glycan compositions, encompassing up five Neu5Ac residues. The tumor was found dominated in equal and high proportions by GD3 and GT1 forms, with a particular incidence of C24:1 fatty acids in the ceramide. By the occurrence of only one mobility feature and the diagnostic fragment ions, the IMS tandem MS conducted using collision-induced dissociation (CID) disclosed for the first time the presence of GT1c(d18:1/24:1) newly proposed here as a potential GBM marker.



KEYWORDS: *human glioblastoma multiforme, gangliosides, biomarker, ion mobility separation/spectrometry mass spectrometry (IMS MS), collision-induced dissociation*

1. INTRODUCTION

An important class of molecules, widely distributed throughout the cells of the body and abundantly expressed in brain tissue, is represented by the sialic acid-containing glycosphingolipids, known as gangliosides (GGs).[1,2] Under normal physiological conditions, GGs play important roles in the development of the central nervous system (CNS),[3,4] promoting the growth of neurons and axons, and the regeneration of nerves, influencing the differentiation of neural cells and protecting the function of nerves.[1]

Simple species with a short glycan chain, such as GM3, GD2, and GD3, are expressed mainly in early stages of embryogenesis, where they are involved in cell signaling, recognition, migration, and regulation of membrane-bound signaling proteins.[1,5] Following CNS maturation, their expression diminishes significantly, and complex glycoforms, such as GM1, GD1a, GD1b, and GT1b, characterize the later stages of brain formation, that is, neurite extension and synaptogenesis.[6−9]

Besides, GGs, especially the disialogangliosides, exhibit a great importance also under pathological conditions, being directly related to the occurrence and development of cancers.[6,8] As a result of initial oncogenic transformation, GD2 and GD3 are upregulated in neoplastic cells, where they may play a role in enhancing tumor cell proliferation, motility, migration, adhesion, angiogenesis, apoptosis, invasion, and preventing immunosuppression of tumors.[1,8−12] Recently, an abnormal increase of GD3 and GD2 was reported to be closely related to a number of neuro-ectoderm-derived cancers and sarcoma, such as melanomas,[13−16] neuroblastomas,[17−19] and soft tissue sarcomas,[20,21] or to small cell lung carcinomas,[22−24] osteosarcomas,[25,26] and breast cancers.[27,28] In human gliomas, the expression levels of GD3 and GD2 were found to increase along with increased malignant properties.[7,29] However, the roles of GD3/GD2 in the malignant properties of human glioma

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were not demonstrated to date, at least to the best of our knowledge.

Among CNS primary tumors, gliomas are the most frequent ones, while glioblastoma multiforme (GBM) accounts for about 65% of all gliomas.[6] Classified by the World Health Organization as a grade IV glioma, the highest malignancy grade, GBM exhibits not just a highly infiltrative growth pattern,[7] but also a heterogeneous nature, showing differences in epigenetic regulation and differentiation status.[8,30] Since neural tissues have no regenerative capacity, and removing healthy brain tissue together with invading tumor cells may lead to disorders in normal brain functions,[6] a total removal of GBM by surgical resection is not so efficient. Moreover, GBM is highly refractory to radiation therapy and chemotherapy, as the brain−blood barrier impedes the effects of various anticancer reagents.[7] Therefore, the patients diagnosed with GBM have a very poor prognosis: 51.6%, 1 year survival rate; only 7.8%, 5 year survival rate.[7,31−33]

In this context, the research is focused now on the determination of the molecular mechanisms related to GBM tumor invasion and the discovery of innovative approaches for invasiveness suppression. Given their reduced expression in normal adult human tissues and the known extensive expression in various malignant cancers, including gliomas, GD2/GD3, and their related molecules on the cell surface were reported to be tumor-associated antigens,[24,29,34,35] and potential targets for the next-generation antitumor vaccination therapy.[1,7,36,37]

In view of the GG structural diversity, an accurate mapping of their expression in GBM is mandatory for understanding the role of GD3/GD2 in the tumor cell proliferation and invasion. Up to date, there is only one study focused on ganglioside profiling in GBM.[38] One of the most resourceful methods for achieving a highly accurate, sensitive and reproducible mapping of GGs is mass spectrometry (MS),[29,38−46] most often coupled with efficient separation techniques, such as electrophoresis, thin layer chromatography (TLC), high-performance liquid chromatography (HPLC), or ion mobility separation (IMS). Platforms based either on high capacity ion trap (HCT), quadrupole time-of-flight (QTOF), Orbitrap, or Fourier transform ion cyclotron resonance (FTICR) mass spectrometers were also successfully implemented for a thorough investigation of GGs in various human matrices, such as fetal cerebellum,[47] occipital and frontal lobes,[46,48] fetal frontal neocortex,[43,48] fetal and adult hippocampus,[49] anencephalic residual brain tissue,[45,50] and brain tumors.[29,38−41] Besides the correlation of GG expression and specificity with a certain brain region or disorder, the combination with tandem MS allowed the structural characterization of individual species in complex native mixtures, highlighting and connecting the incidence of a particular isomer with the function of the respective brain area or the pathological state investigated.

In the latest years, traveling-wave IMS MS (TWIMS MS) demonstrated a superior potential in discrimination and identification of GG glycoforms in highly complex mixtures from healthy fetal and adult brain tissues,[51−53] cerebrospinal fluid[54] and human melanoma cells[55] due to the possibility to separate ions not only according to the differences in their size, but also in analyte collision cross section. The previously published studies using ESI QTOF IMS MS on native GG extracts[51−55] revealed an efficient separation of species based on the charge state, the carbohydrate chain length, the number of sialic acid (Neu5Ac) residues, and the ceramide composition. Hence, the discovery of a high number of glycoforms, mainly novel species characterized by an elevated degree of sialylation or modified by noncarbohydrate type of attachments, could be achieved for the first time through IMS MS.

Unlike the IM resolution of TWIMS MS, which is about 40, the unique design of the recently developed trapped ion mobility spectrometry MS (TIMS MS) technology combines the high IM resolution ($R > 200$), with the performance of QTOF analyzer, enabling nearly 100% duty cycle and an unprecedented collisional cross section (CCS) reproducibility (<0.5% RSD) at high sensitivity. Hence, given the elevated complexity and heterogeneity of GGs in biological samples, the newly emerging TIMS MS might be a powerful tool for generating data on GGs at a deeper level. Considering (i) the poor prognosis of GBM, (ii) the need for development of more advanced methods able to offer an early GBM diagnostic through molecular markers, (iii) the successful use of disialo-gangliosides as tumor-associated carbohydrate antigens in various types of cancer, and (vi) the state-of-the-art in research oriented toward the study of GGs as potential targets for development of anticancer vaccines, the current study aims to provide a comprehensive mapping of GG expression in GBM and the discovery of potential biomarkers, which might be further used for clinical applications.

The combination of the straightforward and highly sensitive IMS MS and collision-induced dissociation (CID) MS/MS, provided here a reliable separation, leading to the discovery in GBM of no less than 160 distinct GG molecules, more than triple the number of species previously detected in this tumor with no separation prior to MS.[38] The GBM was found dominated by GD3 species, which represent 36% of the total number of discovered glycoforms. Surprisingly, in addition to the GD3, IMS MS revealed for the first time the incidence of a similar number of GT1 forms. Since, up to now, no relation between GT1 structures and GBM was considered, the next step of the present research was focused on the structural characterization by CID MS/MS performed in the transfer cell, after ion mobility separation, of a GT1 species exhibiting an unusual ceramide composition. The generated diagnostic fragment ions together with the occurrence of a single mobility feature exposed the presence of the GT1c isomer bearing (d18:1/24:1) ceramide moiety.

2. MATERIALS AND METHODS

2.1. Glioblastoma Sampling. A brain tumor localized in the frontotemporal cortex of the right hemisphere in a male patient, age 47, was clinically diagnosed using computerized tomography and magnetic resonance. The surgical procedure and histopathological diagnosis using specific silver stain, of glioblastoma multiforme, grade IV, were performed in the Department of Neurosurgery and Clinical Department of Pathology "Ljudevit Jurak", University of Zagreb, Croatia.

Permission for experiments with human tissue for scientific purposes was obtained from both the Ethical Committee of the School of Medicine, University of Zagreb, and the Ethical Committee of the University Hospital Center "Sestre milosrdnice", Zagreb, under the Project No. 108-1081870-2415 to Z.V. supported by the Croatian Ministry of Science, Education and Sports. All procedures on the human tissues were in agreement with the 1964 Helsinki declaration and its later amendments. Prior to the ganglioside extraction procedure, the tissue sample was weighed and stored at −20 °C.

2.2. Ganglioside Extraction and Purification. GG extraction was performed according to the method developed by Svennerholm and Fredman[56] and modified by Vukelić et al.[57]

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Fresh tissue sample was weighed and homogenized in ice-cold distilled water (W) to obtain the 10% homogenate. Lipids extraction was performed twice using a solvent mixture of chloroform (C): methanol (M) (1:2, by volume). In order to separate GGs from other lipids, a phase partition followed by repartition was carried out by adding to the combined supernatant resulted after centrifugation M and W to a final volume ratio 1:1:0.8. Upper phases containing polar GSLs (GG) were collected. The crude GG extract was purified in several steps, including precipitation of coextracted protein-salt complexes followed by centrifugation, gel-filtration on Sephadex-G25 (Sigma-Aldrich, St Louis, SAD) column and dialysis against water in an overnight procedure at 4 °C for removing low molecular weight contaminants. For IMS MS analysis, a stock solution of the native GG extract (~1 mg/mL) was prepared by dissolving the dried material in pure methanol.

2.3. Chemicals. The GBM GG mixture was dissolved in pure methanol to the final working concentration of 5 pmol/μL, calculated for an average relative molecular mass (M_r) of 2000. Analytical grade methanol was purchased from Merck (Darmstadt, Germany). Prior to nanoESI MS infusion, to further eliminate any potential clogging of the emitter by residual particles in the solution, the sample dissolved in methanol was centrifuged for 2 h at 5000 rpm in a SIGMA2-16 model centrifuge from Sartorius GmbH (Göttingen, Germany), using a fixed-angle rotor 12148 for 2 mL Eppendorf tubes.

2.4. Ion Mobility Mass Spectrometry. The ion mobility mass spectrometry experiments, data acquisition, and processing were conducted on a Synapt G2S mass spectrometer (Waters, Manchester, U.K.) equipped with a nanoelectrospray (nanoESI) source, tuned in the negative ion mode, and interfaced to a PC computer running the Waters MassLynx (version V4.1, SCN 855) and Waters Driftscope (version V2.7) software.

Ten μL of the working GBM GG solution was introduced into the back of a 10 cm long pulled emitter (ID 1.2 mm, OD 1.5 mm, 10 μm tip size, taper length 4 mm), and a 0.25 mm platinum wire was inserted into the solution. An efficient ionization of the molecules and a continuous spray was observed at 1.5 kV and 45 V potential for capillary and cone, respectively.

To enhance the GG separation, the MS and IMS parameters were set as follows: source block temperature, 100 °C; desolvation gas flow rate, 800 L/h; desolvation temperature, 150 °C; IMS gas flow, 90 mL/min; IMS wave velocity, 650 m/s; IMS wave height, 40 V. All mass spectra were acquired in negative ion mode using a scan range from m/z 100 to 2500. For CID fragmentation experiments, collision energies of 40−65 eV enhanced the production of relevant diagnostic fragment ions and provided high sequence coverage.

Under the same nanoESI IMS MS and MS/MS conditions, the in-run reproducibility of the experimental data in terms of sensitivity, number of detected molecular/fragment ions, relative intensity, and charge state was 100%, while the day-to-day reproducibility was 95%. For an experiment, the number of replicates was at least three.

2.5. Ganglioside Abbreviation and Assignment of the Spectra. For gangliosides assignment, the abbreviation system introduced by Svennerholm[58] and the recommendations of IUPAC-IUB Commission on Biochemical Nomenclature (IUPAC-IUB 1998)[59] were applied as follows: *GM1-II^3-α-Neu5Ac-Gg$_4$Cer*; *GM2-II^3-α-Neu5Ac-Gg$_3$Cer*; *GM3-II^3-α-Neu5Ac-LacCer*; *GM4-II^3-α-Neu5Ac-GgCer*; *GD1-II^3-α-(Neu5Ac)$_2$-Gg$_4$Cer*; *GD2-II^3-α-(Neu5Ac)$_2$-Gg$_3$Cer*; *GD3-II^3-α-(Neu5Ac)$_2$-LacCer*; *GT1-II^3-α-(Neu5Ac)$_3$-Gg$_4$Cer*; *GT2-II^3-α-(Neu5Ac)$_3$-Gg$_3$Cer*; *GT3-II^3-α-(Neu5Ac)$_3$-LacCer*; *GT4-II^3-α-(Neu5Ac)$_3$-GgCer*; *GQ1-II^3-α-(Neu5Ac)$_4$-Gg$_4$Cer*.

Additionally, the nomenclature introduced by Domon and Costello[60] and revised by Costello et al.[61] was applied for the assignment of the glycan backbone sequence ions generated within CID fragmentation experiments. Ceramide fragment ions were assigned according to the nomenclature of Ann and Adams.[62]

3. RESULTS AND DISCUSSION

3.1. IMS MS Mapping of GBM Gangliosides. A total of 10 μL of 5 pmol/μL purified native ganglioside mixture solution from GBM were analyzed by nanoESI IMS MS in negative ion mode. After 2 min of signal acquisition, the obtained two-dimensional (2D) data set of GBM GGs is presented in Figure 1.

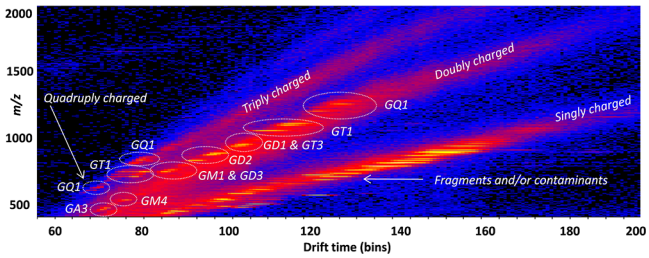

Figure 1. Driftscope display (drift time vs m/z) of the total distribution of GG ions from GBM. In the drift cell, GG ions were separated based on the charge state, the carbohydrate chain length, the degree of sialylation, and ceramide composition.

Next to the clear distribution of the chemical noise across a wide range of drift times, the evaluation of Figure 1 reveals the separation of GG components into mobility families according to their charge state, carbohydrate chain length and degree of sialylation, a pattern characteristic for this type of glycoconjugates.

Following the drift time retention of each narrow region indicated in Figure 1, by combining the scans of the extracted ion chromatograms (XICs), the mass spectra for each doubly to quadruply charged GG classes were generated as illustrated in Figures 2 and 3.

Unlike the experiments with direct infusion, yielding the total ion chromatogram (TIC) and a highly complex mass spectrum of GBM GGs, IMS MS separation gave the possibility to obtain individual mass spectra for each GG class indicated in Figure 1, allowing the detection and identification of a high number of species, listed in Table S1 (Supporting Information). Hence, the low abundant ions, frequently of biological relevance, which, due to ion suppression, would have remained undetected without separation, could be for the first time identified in GBM extract. The detailed inspection of Table S1 (Supporting Information) and Chart 1 discloses an unexpected structural diversity, a highly rich molecular ion pattern dominated by doubly and triply deprotonated GG and asialo-GG components. Obviously, the GBM GG mixture contains species bearing up to five sialic acids and exhibits a high heterogeneity in both their ceramide and glycan architecture. No less than 215 ions, corresponding to 160 distinct ganglioside species, were detected and identified with an average mass accuracy of 6.3 ppm.

Two earlier studies on (i) native GBM GGs conducted on an (−)ESI ion trap instrument[38] and (ii) GGs expressed in a specimen of gliosarcoma, the most aggressive form of all gliomas, using (−)nanoESI Fourier-transform ion cyclotron

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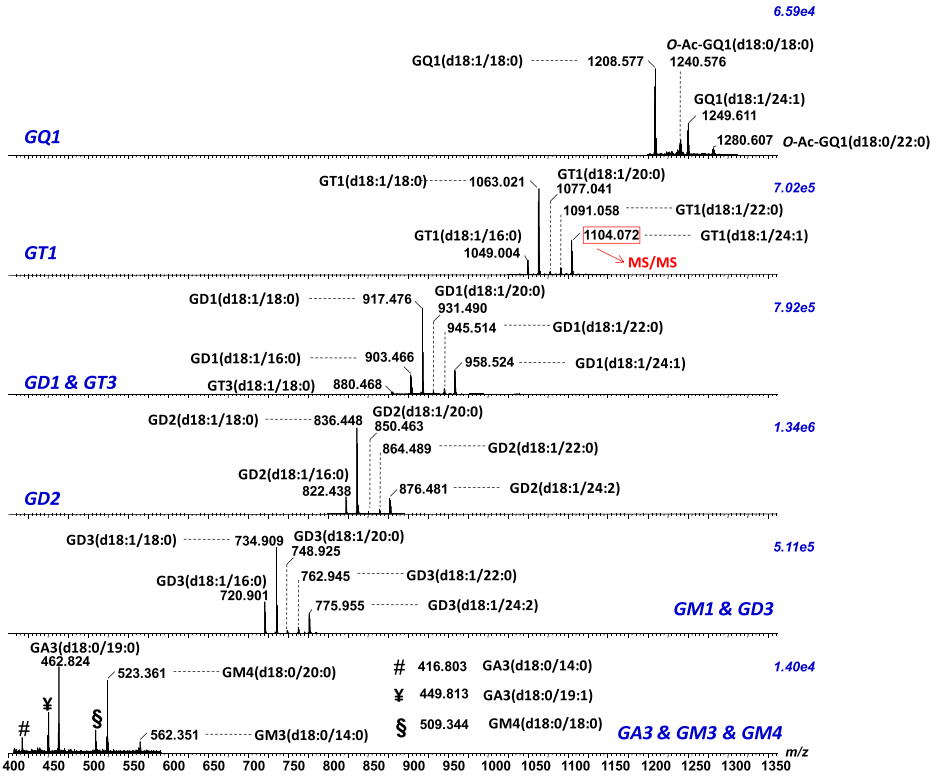

Figure 2. Extracted (−)nanoESI IMS mass spectra of doubly charged GA3 and GM3 and GM4, GM1 and GD3, GD2, GD1 and GT3, GT1 and GQ1 from the corresponding areas indicated in Figure 1. Solvent, methanol; sample concentration, 5 pmol/μL; acquisition time, 2 min; spray voltage, 1.5 kV; cone voltage, 45 V.

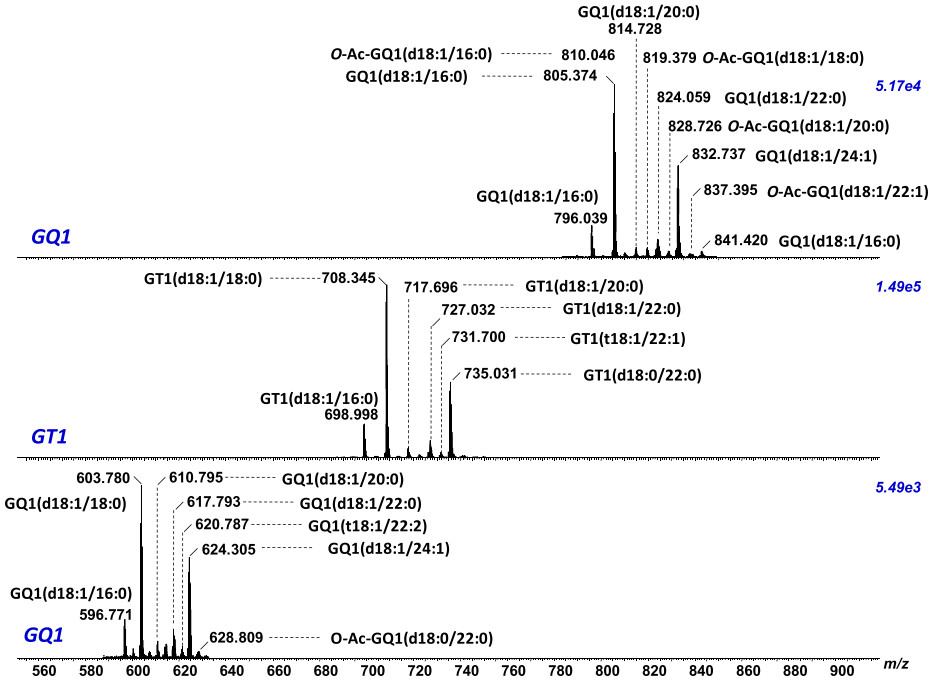

Figure 3. Extracted (−)nanoESI IMS mass spectra of triply charged GT1 (middle), GQ1 (up), and quadruply charged GQ1 (down) from the corresponding areas indicated in Figure 1. Solvent, methanol; sample concentration, 5 pmol/μL; acquisition time, 2 min; spray voltage, 1.5 kV; cone voltage, 45 V.

resonance (FT-ICR) and quadrupole time-of-flight (QTOF) MS,[29] reported the discovery of 50 and 73 species, respectively. This lower number of compounds identified as compared to the data in the current study is certainly attributable to the lack of separation prior to MS screening. The major GG fraction discovered in both samples was GD3, accounting for only 20%[38] and 17%[29] of the total number of ganglioside species identified in the samples. In contrast to these results, the current method,

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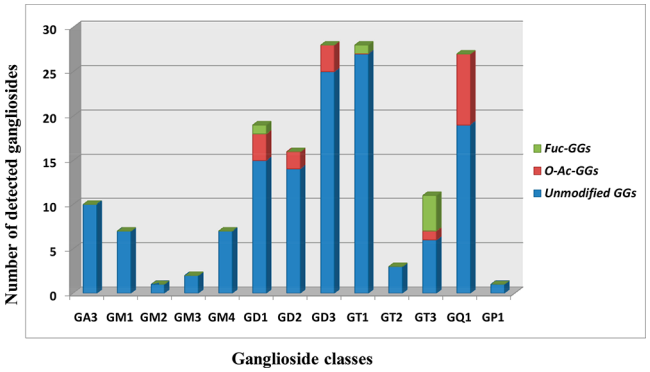
Chart 1. Chart Plotting the Number of the Identified Ganglioside Species in GBM According to the Composition of Their Glycan Core and the Sialylation Degree



based on ion mobility separation, followed by high resolution mass spectrometry analysis, revealed 28 different GD3 glycoforms and 28 GT1 forms in GBM (Table S1, Supporting Information), which, from the total number of components, represent 36% GD3 and 36% GT1, in equal proportions.

Correlated with previous findings, these results, documenting for the first time such a high expression of GD3 and GT1 species in GBM, have a particular biological significance. The expression of GD3 fraction, actually a minor constituent of the adult brain, however, with a crucial role in brain development, is markedly increased in a variety of malignant cancers,[1,7] being directly connected with tumor cell proliferation, invasion, and implicitly with the degree of malignancy.[7,9,41,63,64] GD3 and GD2 types of gangliosides were found to exert important functions in mediating cell proliferation, migration, adhesion, invasion, and angiogenesis and in preventing immunosuppression of tumors.[1,8] Since (i) no GD3 species were discovered in the sera of healthy donors and astrocytoma grade 2 patients, (ii) only smaller amounts in the sera of astrocytoma grade 3 patients,[62] (iii) 4−5% of total GGs in normal brain, and (iv) 20% of total GGs in GBM,[2] GD3 seems to be involved in the mechanism of normal cell transformation into cancerous cells.[2,63,64] Besides, the ratio between GD3 and *O*-acetyl GD3 represents another interesting parameter, which influences the proliferation of GBM and its ability to evade the immune response.[8] It was shown that cleaving off the acetyl group from *O*-Ac GD3 triggers tumor cell death via mitochondrially mediated apoptosis.[8] In the same context, it is worth mentioning that, as a key enzyme, GD3 synthase (GD3S), which regulates the biosynthesis of GD3 and GD2, is involved in the development of the nervous system, the preservation and repair of neural stem cells and nerves, and the development, proliferation, infiltration and metastasis of cancers.[1,63] The elevated expression of GD3S in GBM stem cells is responsible for the high abundance of GD3 and GD2. Considering that GD3S inhibition blocks the biosynthesis of GD3 and GD2 and, therefore, it determines the decrease of the proliferation, migration, and invasion of cancer cells, GD3 and GD3S might be used as potential drug targets against GBM.[1,63]

On the other side, given the GT1b expression in both the primary and brain metastasized tumors, Taki et al.[62] propose GT1b as a brain metastasis-associated species. Consequently, in view of our present findings and the earlier connection of GT1 with highly proliferative primary and secondary brain tumors, the elevated incidence of GT1 species found here in GBM support the biomarker role of GT1, along with GD3 glycoforms.

By IMS MS, a relatively high number of GQ1, GD1, and GD2 glycoforms were also detected in GBM and assigned with high mass accuracy. The elevated sensitivity of the instrument and the possibility to integrate the data over narrow regions and reduce the background noise contribution, allowed the identification and assignment of unusual and low abundant species, such those exhibiting a short glycan chain and one or no Neu5Ac, such as GM4, GM3, and GA3 specimens. Although high and ultrahigh resolution instruments were previously used for GG mapping in glioblastoma,[29] in the absence of the online separation prior to the MS screening, none of the analyzers was able to discriminate GQ class, despite the evidence upon GQ1 fraction provided by an off-line HPTLC profiling.[29]

Our current approach revealed also a high variability of GG fatty acid patterns in human GBM. Some of our data, collected by IMS MS, confirm earlier reports on GBM and the gliosarcoma GG pattern,[38,29] which was found characterized by a higher expression of species encompassing ceramides with shorter (C16) and longer (C22, C24) fatty acid residues. The longest fatty acid chain discovered before contained 24 carbon atoms. In contrast with these reports, here, a wider range of structures with fatty acid chains containing from 12 to 28 carbon atoms were identified; no less than 50 of the 160 glycoforms, having ceramides with C22 up to C28 fatty acid residues, were discovered by IMS MS (Table S1, Supporting Information). Fatty acid chains with C24 were mainly found in GD1, GD2, and GD3 species. GT1 and GT3 glycoforms with C24 were detected here and in gliosarcoma,[29] while GA3 and GQ1 classes bearing saturated and (poly)unsaturated C24 fatty acids residues were discovered only in GBM by using the present IMS MS approach.

Interestingly, over a half of the GGs detected here in GBM (n = 82) are characterized by unsaturated and polyunsaturated fatty acid residues, while no less than 12 species contained ceramides with an odd number of carbon atoms, from C17 to C23, mainly with C17 and C19. These findings corroborate the previously published data[38] according to which, the incidence of unsaturated fatty acid residues, such as C24:1, as well as of fatty acids with an odd number of carbon atoms, such as C17, C19 is a characteristic pattern of GGs in glioma tumors.

The (−)nanoESI IMS MS approach has also revealed the presence in GBM tissue of highly sialylated GGs and of species modified by noncarbohydrate type of attachments, which are known to be involved in certain biological events. Hence, (i) no less than 27 tetrasialylated and one pentasialylated gangliosides were evidenced; (ii) 17 species of which three GD1, two GD2, three GD3, one GT3, and eight GQ1 were found *O*-acetylated; and (iii) six species of which one GD1, one GT1, and four GT3 were found *O*-fucosylated.

3.2. Structural Characterization of GBM-Associated Species by IMS CID MS/MS. The elevated abundance and relevance of GD3 species in glioblastoma multiforme is a well-known fact, confirmed here by IMS MS as well. Nevertheless, the current study revealed novel information regarding the incidence of a relatively high number of trisialylated species in the investigated GBM. Hence, the next experimental step was focused on the structural investigation through (−)nanoESI IMS CID MS/MS of a glycoform detected at m/z 1104.072 in Figure 2, which according to mass calculation corresponds to GT1(d18:1/24:1). For the fragmentation spectrum depicted in Figure 4a, the signal was acquired for 2 min, while the collision energy was ramped within 40−65 eV range to enhance glycosidic bond cleavages and generate diagnostic fragment ions for the assignment of both carbohydrate and ceramide

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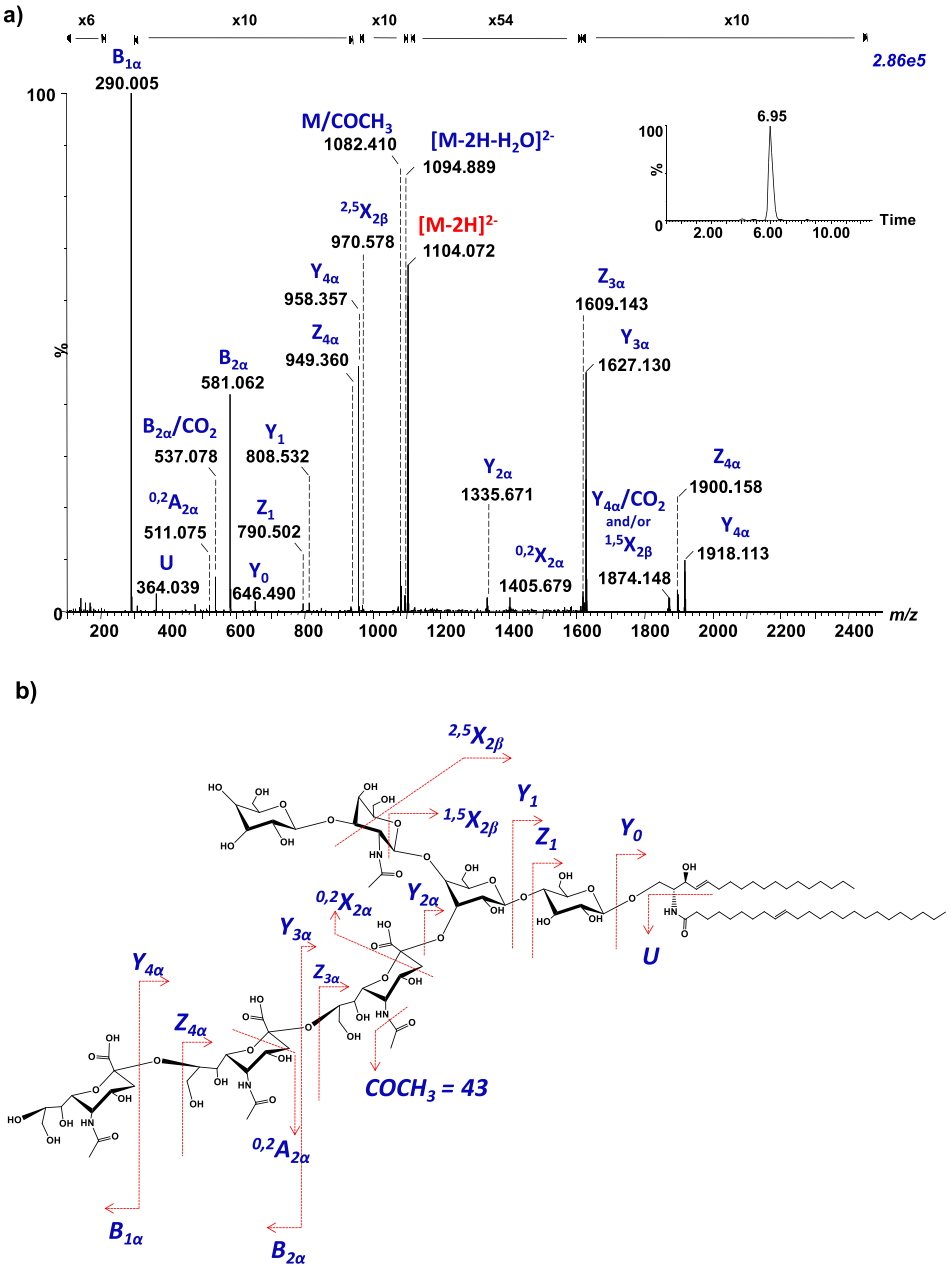

Figure 4. (a) (−)nanoESI IMS CID MS/MS of the $[M-2H]^{2-}$ at m/z 1104.072 corresponding to GT1(d18:1/24:1) species isolated and fragmented from a GBM sample. Inset: drift time distribution for the ion at m/z 1104.072 fragmented by CID. Cone voltage, 45 V; Capillary voltage, 1.5 kV; Acquisition, 120 scans. CID at variable collision energy within 40−65 eV. (b) Fragmentation scheme of GT1c(d18:1/24:1).

moieties. The scheme illustrating the fragmentation pathway experienced by this parent ion during the IMS CID MS/MS event is presented in Figure 4b.

No less than seven fragment ions, namely, $B_{1\alpha}^-$ and $B_{2\alpha}^-$, together with their counterparts $Y_{4\alpha}^-$ and $Y_{3\alpha}^-$, respectively, and $Z_{4\alpha}^-$, $Z_{3\alpha}^-$, and $Y_{2\alpha}^-$, detected at m/z 290.005, m/z 581.062, m/z 1918.113, m/z 1627.130, m/z 1900.158, m/z 1609.143, and m/z 1335.671, were diagnostic for the complete desialylation of the GT1 molecule. Although exhibiting a lower intensity, the signals detected at m/z 646.490, m/z 790.502, and m/z 808.532 and assigned to Y_0^-, Z_1^-, and Y_1^- represent the evidence of the Glc-Cer sequence, while U^-, diagnostic for the C24:1 fatty acid and detected at m/z 346.039, confirms the (d18:1/24:1) composition of the ceramide.

The detailed assessment of the spectrum in Figure 4a reveals that several internal cross-ring cleavages also occurred. $^{2,5}X_{2\beta}^-$ and $^{1,5}X_{2\beta}^-$ resulted following the internal fragmentation of GalNAc, upholding the concept of a free nonreducing end (Figure 4b) with the attachment of all three Neu5Ac residues at the inner Gal, which documents that the sequenced species is a *c*-type isomer. Due to the symmetry of the Gal-GalNAc-Gal chain, no product ions that are exclusively specific for GT1a, GT1b, and GT1d forms may be generated by CID MS/MS.[39] Therefore, it was shown before[39] that solely by fragmentation analysis it is not possible to exclude the presence of these isomers. However, in the current study, IMS provided hard evidence of only one mobility feature, documenting a single conformation (Figure 4a, inset), and ruling out the existence of several isomers/isobars. According to the detected diagnostic

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fragment ions, this species was identified as the GT1c(d18:1/24:1).

4. CONCLUSIONS

Only in the U.S.A., over a million people are diagnosed every year with cancer, of which more than 25000 are diagnosed with brain or other nervous system malignancies. In the case of invasive brain tumors like gliomas, the combined therapeutic scheme involving resection, radiation, and chemotherapy is still not effective. For this reason, nowadays, the research is conducted toward the development and refinement of high performance analytical approaches to acquire knowledge in the field of early diagnosis and monitoring of cancer progression through novel and more relevant molecular fingerprints. In this context, the goal of this study was the application of IMS MS to identify the ganglioside pattern uniquely developed in GBM, the most aggressive brain tumor with the poorest prognosis among all brain cancers. IMS in combination with highly sensitive (−)nanoESI and tandem MS by CID provided an exhaustive structural and compositional investigation of GBM gangliosides due to the advantages of the platform to (i) induce GG separation according to the charge state, m/z ratio, carbohydrate chain length, degree of sialylation and ceramide composition, prior MS detection; (ii) separate background chemical noise; (iii) reduce spectral congestion; and (iv) extract the MS for each GG class, allowing the detection and identification of no less than 160 distinct glycoforms with a high diversity of their glycan and ceramide constitutions. From a methodological point of view, for identical instrumental parameters in terms of ionization mode, applied voltages, desolvation temperature, collision energy, gas pressure, acquisition time, and the preset resolution, the current method provided excellent reproducibility: 100% in-run, around 98% run-to-run, and 95% day-to-day reproducibility of the results for the number of replicates of at least three.

The major outcome of this study is that, by IMS MS and CID MS/MS, various novel species could be identified and added to the currently existing panel of glioblastoma tissue-associated structures, since the number of the GGs identified here is 3× higher than ever discovered in this tumor type.

If, so far, the studies related to the glycosphingolipid markers of GBM were focused on GD3 due to the elevated expression of this ganglioside category in GBM biopsies, the IMS MS approach has revealed for the first time that GT1 has a similarly high expression. For this reason, next to GD3, trisialo tetraoses, characterized by heterogeneity in ceramide compositions, are potential glioma markers and should be more thoroughly investigated for understanding their involvement in tumor progression and invasiveness, for application in early diagnosis of GBM, or development of effective vaccines.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jasms.1c00088.

Table S1 (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

Ligia Petrica − *Department of Internal Medicine II − Division of Nephrology, County Emergency Hospital Timisoara and Centers for Molecular Research in Nephrology and Vascular Diseases, Translational Research and Systems and Cognitive Research in Neuropsychiatric Pathology, Faculty of Medicine, "Victor Babes" University of Medicine and Pharmacy, 300041 Timisoara, Romania*; Phone: +40-356-433433; Email: petrica.ligia@umft.ro

Alina D. Zamfir − *Department of Condensed Matter, National Institute for Research and Development in Electrochemistry and Condensed Matter, 300224 Timisoara, Romania; Department of Technical and Natural Sciences, "Aurel Vlaicu" University of Arad, 310130 Arad, Romania*; orcid.org/0000-0002-7711-0852; Phone: +40-256-494413; Email: alina.zamfir@uav.ro

Authors

Mirela Sarbu − *Department of Condensed Matter, National Institute for Research and Development in Electrochemistry and Condensed Matter, 300224 Timisoara, Romania; Faculty of Physics, West University of Timisoara, 300223 Timisoara, Romania*

David E. Clemmer − *Department of Chemistry, The College of Arts and Science, Indiana University, Bloomington, Indiana 47405-7102, United States*; orcid.org/0000-0003-4039-1360

Željka Vukelić − *Department of Chemistry and Biochemistry, Faculty of Medicine, University of Zagreb, Zagreb 10000, Croatia*

Complete contact information is available at: https://pubs.acs.org/10.1021/jasms.1c00088

Notes

The authors declare no competing financial interest.

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