**137Cs γ Ray and 28Si Irradiation Induced Murine Hepatocellular Carcinoma Lipid Changes in Liver Assessed by MALDI-MSI Combined with Spatial Shrunken Centroid Clustering Algorithm: A Pilot Study**

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**ABSTRACT:** Characterization of lipids by matrix-assisted laser desorption ionization mass spectrometry imaging (MALDI-MSI) is of great interest because not only are lipids important structural molecules in both the cell and internal organelle membranes, but they are also important signaling molecules. MALDI-MSI combined with spatial image segmentation has been previously used to identify tumor heterogeneities within tissues with distinct anatomical regions such as the brain. However, there has been no systematic study utilizing MALDI-MSI combined with spatial image segmentation to assess the tumor microenvironment in the liver. Here, we present that image segmentation can be used to evaluate the tumor microenvironment in the liver. In particular, to better understand the molecular mechanisms of irradiation-induced hepatic carcinogenesis, we used MALDI-MSI in the negative ion mode to identify lipid changes 12 months post exposure to low dose 28Si and 137Cs γ ray irradiation. We report here the changes in the lipid profiles of male C3H/HeNCrl mice liver tissues after exposure to irradiation and analyzed using the spatial shrunken centroid clustering algorithm. These findings provide valuable information as astronauts will be exposed to high-charge high-energy (HZE) particles and low-energy γ-ray irradiation during deep space travel. Even at low doses, exposure to these irradiations can lead to cancer. Previous studies infer that irradiation of mice with low-dose HZE particles induces oxidative damage and microenvironmental changes that are thought to play roles in the pathophysiology of hepatocellular carcinoma.

**INTRODUCTION**

The primary goal of NASA’s space irradiation research is to assess the health effects of deep space irradiation on astronauts, to provide risk prediction, and to develop preventive measures against diseases that would further slow space exploration. The irradiation encountered in space is a form of galactic cosmic rays, which include low-energy gamma rays (137Cs γ), energetic protons (a large component), helium nuclei, and particle nuclei of high-charge high-energy (HZE, Z > 13), and other ions that are created during interactions within the spacecraft. The majority of the most harmful space irradiation dose is due to HZE particles, such as 56Fe, 28Si, 16O, and 12C. Among HZE particles, 28Si contributes more than 10% to the HZE dose in the space irradiation environment. It has been shown that 28Si total body irradiation causes injury to hematopoietic stem cells via induction of cellular apoptosis; however, the lipid changes in response to 28Si irradiation in the pathogenesis of hepatocellular carcinoma (HCC) have not been explored. Understanding these changes is needed to develop new strategies and mitigate or prevent irradiation-induced liver injury. Even at low doses, exposure to HZE, such as 28Si, as well as low-energy γ rays can lead to cancer. Previous studies have shown that irradiation of mice with low-dose HZE significantly increases the incidence of HCC. Current literature supports that irradiation of mice induces oxidative damage and microenvironmental changes that are thought to play roles in the pathophysiology of HCC. Tumor heterogeneity is now considered a critical factor in the prognosis and clinical outcomes of patients and the way they respond to chemotherapy. Understanding the molecular basis and characterization of tumor heterogeneity is a crucial...
nonirradiated specimens show upregulation of lower as well as early detection and recognition of microenvironmental also been exploited for the identi

Figure 1. Segmentation result of the MALDI-MSI dataset using the SSCCA. (a) There are two biological replicates from the tumor sections of 237Cs γ-ray-irradiated, two biological replicates from the tumor sections of 24Si-irradiated, and three biological replicates from healthy nonirradiated control murine liver tissues. The segmentation with spatial shrunken centroids and structurally adaptive distance selected five tissue segments that are color-coded. The colors correspond to numbers in the bottom figure legend (b), which represent segments 1 (orange), 2 (green), 3 (light blue), 4 (pink), and 5 (dark blue). Segment 1 (orange) shows the core tumor/HCC region on each tumor specimen, where the disease-looking part of the specimen was observed both histologically (H&E staining) and visible by the naked eye on the MALDI slide. Segment 2 (green), segment 3 (light blue), and segment 4 (pink) show a healthy looking part of the tumor sections as well as in control sections. Segment 5 (dark blue) shows a transition looking part of the specimens in which the tissue looks neither completely healthy nor tumor looking, and (b) t-statistics quantified the relative importance of the peaks in each segment. The spectra show that segment 1 (orange/core tumor) is enriched (upregulated) mainly in higher m/z values (1400) and downregulated in lower m/z values (<600). Conversely, healthy parts of the tumor sections from irradiated and healthy nonirradiated specimens show upregulation of lower m/z values (<600). (c) H&E slide corresponding to the slide shown in panel (a).

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step to shed light on how HZE and γ ray irradiation induce liver tumors (HCC). It has been reported there is abnormal lipid accumulation in the livers of irradiated mice compared to control, regardless of strain or irradiation exposure duration.10,11 Similarly, dysregulation of several lipid pathways such as loss of retinoids from the hepatic stellate cell lipid droplets, activation of peroxisome proliferator-activated receptor alpha pathways related to glycerophospholipid and sphingolipid metabolism, increased lipid metabolism, fatty acid metabolism, lipid processing, and localization have been observed in animals exposed to spaceflight.12−14 Additionally, spaceflight has been shown to significantly increase corticosterone and changes in some of the components of glutathione synthesis, as well as increases in glycerophosphorylcholine and glycerol.15

Matrix-assisted laser desorption ionization mass spectrometry imaging (MALDI-MSI) is a powerful tool that has been extensively utilized in cancer research to characterize heterogeneities because of its exceptional advantage of maintaining the spatial organization of cells when analyzing tissue specimens. MALDI-MSI allows researchers to conduct an in-depth analysis of the molecular content of their specimens. MALDI-MSI has indeed been able to characterize relevant tumor populations within cancer tissues.16−22 It has also been exploited for the identification of novel biomarkers as well as early detection and recognition of microenvironmental changes in lipid profiles.23−27 The high abundance of various lipids in biological tissues has rendered MALDI-MSI a suitable method to detect these changes. Lipids are the main constituents of membranes in cells as well as of internal organelles. Most membranes are lipid bilayers composed of different classes of lipids such as phospholipids, cholesterol, sphingolipids, triglycerides, and so forth. The method has been shown to identify and quantify (relative to control) polar lipids per sample, providing information on the structural integrity of cells and the potential state of cellular communication.17−21 Changes in lipid expression have been shown to be involved in numerous pathological states.32−36

Each MALDI-MSI experiment produces a distribution of complex lipids in the tissue, a wealth of information that requires extensive effort and time to analyze. The complexity and size of this data require the use of data reduction methods and more automated approaches to provide useful information. Various tools and image segmentation methods have been used to segment MALDI-MSI images, based on the characteristic masses for specific tissue regions.37−40 Here, we use the Cardinal R package,41 an open-source R-based software package developed specifically for MALDI-MSI, to segment liver tissues, based on their characteristic masses, and to further detect the significant lipid differences between HCC tumor liver sections of irradiated and healthy liver sections of nonirradiated controls in mice. The Cardinal R package has been used in the context of brain tissue as a tool to segment different anatomical regions with different spectral characteristics.42 However, multivariate statistical techniques and model-based image segmentation have not found application in the liver tissue because of its homogeneous nature, as
opposed to the brain, which has distinct anatomical regions. Analyzing liver spectra is often quite tedious because of the large and homogeneous nature of this tissue and the biological and technical variations in the intensities of spectral features. Using statistical inference is crucial for differentiating the systematic signals in the spectra from the noise and for further molecular mechanisms of low-energy experimental conditions. To better understand the irradiated control were measured using MALDI-MSI and nontumor liver slices from biological replicates of non-

radiation Laboratory (NSRL) (power #s were calculated for the experiments) and the low number of mice with liver tumors one year post irradiation, the image segmentation analysis was oriented to qualitatively identify significant mass signatures in the irradiated tumor regions compared to healthy nonirradiated control that would otherwise go undetected if one were to exclusively focus on the m/z peak magnitudes. Figure 1 shows the different segmentations that resulted from spatial heterogeneities within the specimens. In particular, in tumor specimens (γ ray- and 25Si-irradiated), segment 1 (orange) originated from the sections of tumor/HCC that represented as the most disease-looking segment histologically [hematoxylin and eosin (H&E) staining], also visible on the slide prepared for MALDI by naked eyes. This observation possibly represents the tumor initiation region (core tumor) where the initial cells transformed by a switching event possibly represents the tumor initiation region (core tumor) slide prepared for MALDI by naked eyes. This observation possibly represents the tumor initiation region (core tumor) where the initial cells transformed by a switching event where the initial cells transformed by a switching event

# RESULTS

Screening of Lipid Biomarkers in γ Ray-Irradiated Tumor and 25Si-Irradiated Tumor Compared to Healthy Nonirradiated Control Tissues. Two tumor liver slices from biological replicates of 25Si-irradiated, two tumor liver slices from biological replicates of γ ray-irradiated, and three healthy nontumor liver slices from biological replicates of non-irradiated control were measured using MALDI-MSI and evaluated using the SSCCA as shown in Figure 1. Even though the number of biological replicates is low due to the lower number of total animals being irradiated at the NASA Space Radiation Laboratory (NSRL) (power #s were calculated for the experiments) and the low number of mice with liver tumors one year post irradiation, the image segmentation analysis was oriented to qualitatively identify significant mass signatures in the irradiated tumor regions compared to healthy nonirradiated control that would otherwise go undetected if one were to exclusively focus on the m/z peak magnitudes. Figure 1 shows the different segmentations that resulted from spatial heterogeneities within the specimens. In particular, in tumor specimens (γ ray- and 25Si-irradiated), segment 1 (orange) originated from the sections of tumor/HCC that represented as the most disease-looking segment histologically [hematoxylin and eosin (H&E) staining], also visible on the slide prepared for MALDI by naked eyes. This observation possibly represents the tumor initiation region (core tumor) where the initial cells transformed by a switching event common to all malignancies to malignant cells. Table 1 shows the identified signature m/z values with their corresponding t-scores in the irradiated core tumor segments (segment 1/orange) and the healthy segments, which included non-irradiated control segments and the healthy looking part of the tumor segments (segment 3/green). Table 2 shows the parent ions and MS/MS fragments that were used to identify the corresponding lipids.

Upregulation of Higher m/z Values and Down-regulation of Lower m/z Values from Mice Livers in Irradiated Tumor/HCC Versus Healthy Segments. Our findings show that irradiation-induced HCC results in the upregulation of lipids with higher masses and downregulation of lipids with lower masses in the liver. In particular, most upregulated peaks (Table 1) correspond to a mouse analogue

| m/z-detected | t-score in healthy | t-score in irradiated core tumor/HCC | up/downregulated in tumor/HCC vs healthy | identified lipid |
|--------------|--------------------|--------------------------------------|------------------------------------------|-----------------|
| 533.48       | 42.33              | −77.41                               | down                                     | PA(14:1(9Z)/10:0) or isomer PA(10:0/14:1(9Z)) |
| 535.11       | 43.67              | −76.21                               | down                                     | PA(25:0/0:0)    |
| 537.09       | 26.77              | −53.50                               | down                                     | PG(15:1(9Z)/4:0) or PG(P-16:0/4:0) |
| 551.06       | 47.62              | −81.27                               | down                                     | PE(23:0/0:0)    |
| 553.11       | 51.72              | −88.72                               | down                                     | PG(10:0/10:0) or isomer PG(12:0/8:0) and PS(20:0/0:0) |
| 567.1        | 32.33              | −59.24                               | down                                     | PS(16:0/4:0)    |
| 569.1        | 54.51              | −94.01                               | down                                     | PS(22:6(4Z,7Z,10Z,13Z,16Z,19Z)/0:0:0) |
| 579.07       | 25.83              | −50.63                               | down                                     | PG(18:1(11E)/4:0) |
| 595.11       | 33.57              | −60.59                               | down                                     | PS(23:0/0:0)    |
| 611.11       | 11.09              | −30.47                               | down                                     | PS(2:0/22:6(4Z,7Z,10Z,13Z,16Z,19Z)) |
| 613.13       | 29.62              | −59.52                               | down                                     | mixture of PA(20:3(8Z,11Z,14Z)/10:0) and PS(20:5(9Z,11Z,14Z,17Z)/4:0) |
| 705.12       | 7.77               | −19.68                               | down                                     | PE(21:0/12:0) or isomer PE(12:0/21:0) |
| 885.38       | −39.73             | 92.24                                | up                                       | PI(18:0/20:4(5Z,8Z,11Z,14Z)) |
| 1370.52      | −63.40             | 177.15                               | up                                       | mouse analogue of ganglioside GM2 GalNAcβ1-4(NeuGc2-3)Galβ1-4Glcβ3-Cer (undetermined ceramide tail) |
| 1454.73      | −72.08             | 116.29                               | up                                       | mouse analogue of ganglioside GM2 GalNAcβ1-4(NeuGc2-3)Galβ1-4Glcβ3-Cer (d18:1/22:0) |
| 1482.76      | −75.94             | 143.95                               | up                                       | mouse analogue of ganglioside GM2 GalNAcβ1-4(NeuGc2-3)Galβ1-4Glcβ3-Cer (undetermined ceramide tail) |
| 1484.74      | −76.40             | 144.59                               | up                                       | mouse analogue of ganglioside GM2 GalNAcβ1-4(NeuGc2-3)Galβ1-4Glcβ3-Cer (undetermined ceramide tail) |
| 1497.71      | −121.51            | 304.79                               | up                                       | mouse analogue of ganglioside GM2 GalNAcβ1-4(NeuGc2-3)Galβ1-4Glcβ3-Cer (undetermined ceramide tail) |

*The identified lipid column shows lipids that were identified by manual searching of on-tissue MS/MS fragmentations (100 ppm accuracy) of the corresponding m/z values.

Table 1. Identified Signature m/z Values with Their Corresponding t-Scores in Segment 1/orange, Which Represented the Core Tumor in Irradiated Specimens vs Healthy Segments 2/green Which Was Identified in All Control Specimens As Well As Healthy Parts of the Tumor Specimens.
Table 2. Parent Ion and MS/MS Fragments Which Are Used to Identify Lipids Listed in Table 1

| m/z-detected | identified lipid | MS/MS fragments used to identify lipids | MS/MS fragment name |
|--------------|-----------------|----------------------------------------|---------------------|
| 533.48       | PA(14:1(9Z)/10:0) or isomer PA(10:0/14:1(9Z)) | 533.3249 precursor ion [M – H]⁻ | loss of sn2 acyl chain as ketene (RCH=C=O) from \([M – H]⁻\) |
|              |                 | 379.1891 neutral loss of sn1 RCOOH group from \([M – H]⁻\) | glycerol-3-phosphate ion with loss of H₂O |
|              |                 | 152.9958 glycerol-3-phosphate ion with loss of H₂O | 152.9958 neutral loss of sn1 RCOOH group from \([M – H]⁻\) |
| 535.11       | PA(25:0/0:0)    | 535.3769 precursor ion [M – H]⁻ | neutral loss of sn1 RCOOH group from \([M – H]⁻\) |
|              |                 | 381.3738 sn1 RCOO⁻ ion | glycerophosphoglycerol ion |
|              |                 | 152.9958 glycerol-3-phosphate ion with loss of H₂O | 152.9958 neutral loss of sn2 RCOOH group from \([M – H]⁻\) |
| 537.09       | PG(15:1(9Z)/4:0) or PG(P-16:0/4:0) | 537.2834 precursor ion [M – H]⁻ | neutral loss of sn1 RCOOH group from \([M – H]⁻\) |
|              |                 | 449.2310 neutral loss of sn1 RCOOH group from \([M – H]⁻\) | glycerophosphoglycerol ion |
|              |                 | 245.0432 glycerol-3-phosphate ion with loss of H₂O | 152.9958 glycerol-3-phosphate ion with loss of H₂O |
| 551.06       | PE(23:0/0:0)    | 550.3878 precursor ion [M – H]⁻ | neutral loss of ethanolamine (C₂H₆N) from \([M – H]⁻\) |
|              |                 | 506.3378 neutral loss of ethanolamine (C₂H₆N) and H₂O from \([M – H]⁻\) | 353.3425 sn1 RCOO⁻ ion |
|              |                 | 488.3272 neutral loss of ethanolamine (C₂H₆N) and H₂O from \([M – H]⁻\) | glycerophosphoglycerol ion |
| 551.11       | PG(10:0/10:0)   | 552.3147 precursor ion [M – H]⁻ | neutral loss of sn1 acyl chain as ketene (RCH=C=O) from \([M – H]⁻\) |
|              |                 | 399.1790 neutral loss of sn1 acyl chain as ketene (RCH=C=O) from \([M – H]⁻\) | glycerophosphoglycerol ion |
|              |                 | 152.9958 glycerophosphoglycerol ion | 152.9958 glycerol-3-phosphate ion with loss of H₂O |
| 555.11       | PS(20:0/0:0)    | 552.3007 precursor ion [M – H]⁻ | neutral loss of sn1 acyl chain as ketene (RCH=C=O) from \([M – H]⁻\) |
|              |                 | 465.2987 neutral loss of sn1 acyl chain as ketene (RCH=C=O) from \([M – H]⁻\) | glycerophosphoglycerol ion |
|              |                 | 152.9958 glycerophosphoglycerol ion | 152.9958 glycerol-3-phosphate ion with loss of H₂O |
| 567.1        | PS(16:0/4:0)    | 566.3100 precursor ion [M – H]⁻ | neutral loss of sn1 acyl chain as ketene (RCH=C=O) from \([M – H]⁻\) |
|              |                 | 479.2779 neutral loss of sn1 acyl chain as ketene (RCH=C=O) from \([M – H]⁻\) | glycerophosphoglycerol ion |
|              |                 | 152.9958 glycerophosphoglycerol ion | 152.9958 glycerol-3-phosphate ion with loss of H₂O |
| 569.1        | PS(22:6(4Z,7Z,10Z,13Z,16Z,19Z)/0:0) | 568.2681 precursor ion [M – H]⁻ | neutral loss of sn1 acyl chain as ketene (RCH=C=O) from \([M – H]⁻\) |
|              |                 | 481.2361 neutral loss of sn1 acyl chain as ketene (RCH=C=O) from \([M – H]⁻\) | glycerophosphoglycerol ion |
|              |                 | 327.2330 neutral loss of sn1 acyl chain as ketene (RCH=C=O) from \([M – H]⁻\) | glycerophosphoglycerol ion |
|              |                 | 152.9958 glycerophosphoglycerol ion | 152.9958 glycerol-3-phosphate ion with loss of H₂O |
| 579.07       | PG(18:1(11E)/4:0) | 579.3304 precursor ion [M – H]⁻ | neutral loss of sn1 acyl chain as ketene (RCH=C=O) from \([M – H]⁻\) |
|              |                 | 491.2779 neutral loss of sn1 acyl chain as ketene (RCH=C=O) from \([M – H]⁻\) | glycerophosphoglycerol ion |
|              |                 | 417.2412 neutral loss of sn1 acyl chain as ketene (RCH=C=O) from \([M – H]⁻\) | glycerophosphoglycerol ion |
| 595.11       | PS(23:0/0:0)    | 594.3777 precursor ion [M – H]⁻ | neutral loss of sn1 acyl chain as ketene (RCH=C=O) from \([M – H]⁻\) |
|              |                 | 507.3456 neutral loss of sn1 acyl chain as ketene (RCH=C=O) from \([M – H]⁻\) | glycerophosphoglycerol ion |
|              |                 | 353.3425 neutral loss of sn1 acyl chain as ketene (RCH=C=O) from \([M – H]⁻\) | glycerophosphoglycerol ion |
| 611.11       | PS(2:0/22:6(4Z,7Z,10Z,13Z,16Z,19Z)) | 610.2787 precursor ion [M – H]⁻ | neutral loss of sn1 acyl chain as ketene (RCH=C=O) from \([M – H]⁻\) |
|              |                 | 523.2466 neutral loss of sn1 acyl chain as ketene (RCH=C=O) from \([M – H]⁻\) | glycerophosphoglycerol ion |
|              |                 | 152.9958 glycerophosphoglycerol ion | 152.9958 glycerol-3-phosphate ion with loss of H₂O |
| 613.13       | PA(20:3(8Z,11Z,14Z)/10:0) | 612.2936 precursor ion [M – H]⁻ | neutral loss of sn2 acyl chain as ketene (RCH=C=O) from \([M – H]⁻\) |
|              |                 | 459.2517 neutral loss of sn2 acyl chain as ketene (RCH=C=O) from \([M – H]⁻\) | glycerophosphoglycerol ion |
|              |                 | 305.2486 neutral loss of sn2 acyl chain as ketene (RCH=C=O) from \([M – H]⁻\) | glycerophosphoglycerol ion |
|              |                 | 152.9958 glycerophosphoglycerol ion | 152.9958 glycerol-3-phosphate ion with loss of H₂O |
| 613.13       | PS(20:5(5Z,8Z,11Z,14Z,17Z)/4:0) | 612.2936 precursor ion [M – H]⁻ | neutral loss of sn2 acyl chain as ketene (RCH=C=O) from \([M – H]⁻\) |
|              |                 | 525.2623 neutral loss of sn2 acyl chain as ketene (RCH=C=O) from \([M – H]⁻\) | glycerophosphoglycerol ion |
|              |                 | 301.2173 neutral loss of sn2 acyl chain as ketene (RCH=C=O) from \([M – H]⁻\) | glycerophosphoglycerol ion |
| 705.12       | PE(21:0/12:0) or isomer PE(12:0/21:0) | 704.5326 precursor ion [M – H]⁻ | neutral loss of sn2 acyl chain as ketene (RCH=C=O) from \([M – H]⁻\) |
|              |                 | 522.3565 neutral loss of sn2 acyl chain as ketene (RCH=C=O) from \([M – H]⁻\) | glycerophospholipid |
|              |                 | 504.3460 neutral loss of sn2 acyl chain as ketene (RCH=C=O) from \([M – H]⁻\) | glycerophospholipid |
Table 2. continued

| m/z-detected | identified lipid | MS/MS fragments used to identify lipids | MS/MS fragment name |
|--------------|-----------------|----------------------------------------|---------------------|
| 396.2157     |                 | loss of sn1 acyl chain as ketene (RCH=CO) from [M – H]− |                    |
| 152.9958     |                 | glycerol-3-phosphate ion with loss of H2O |                    |
| 885.5499     |                 | precursor ion [M – H]−                    |                    |
| 723.4970     |                 | loss of inositol from [M – H]−            |                    |
| 619.2889     |                 | loss of sn1 acyl chain as ketene (RCH=CO) from [M – H]− |                    |
| 601.2783     |                 | neutral loss of sn1 RCOOH group from [M – H]− |                    |
| 599.3202     |                 | loss of sn1 acyl chain as ketene (RCH=CO) from [M – H]− |                    |
| 581.3096     |                 | neutral loss of sn2 RCOOH group from [M – H]− |                    |
| 439.2255     |                 | neutral loss of sn1 RCOOH group and inositol from [M – H]− |                    |
| 437.2674     |                 | loss of sn2 acyl chain as ketene (RCH=CO) and inositol from [M – H]− |                    |
| 419.2568     |                 | neutral loss of sn2 RCOOH group and inositol from [M – H]− |                    |
| 259.0225     |                 | inositol phosphate ion |                    |
| 152.9958     |                 | glycerol-3-phosphate ion with loss of H2O |                    |
| 96.9696      |                 | H2PO4− ion (from phosphate) |                    |
| 78.9591      |                 | PO4− ion (from phosphate) |                    |

1370.52 all of the versions of the mouse analogue of ganglioside GM2 GalNAcβ1-4(NeuGc) Galβ1-4Glcβ-Cer were identified by the characteristic fragmentation of the sugar head group, as seen in Figure 3. The characteristic [M – H]− fragments that were monitored were m/z 201.98 Gal NAc (B,α), m/z 255.2 N-acyl, and m/z 306.06 NeuGC (B,β).

1454.73 1482.76 1484.74 1497.71

of human GM2 as determined by m/z matches to lipid masses in the Lipid Maps Database, Swiss Lipids Database, and MS/MS fragmentations. The human GM2 and the mouse analogue of GM2 gangliosides only differ in their terminal sialic acid residue. Human GM2 has a terminal N-acetyl neuraminic acid (Neu5Ac), and the mouse analogue has a terminal N-glycolyl neuraminic acid (Neu5Gc). The first GM2 analogue identified was m/z 1454.73, which was identified first in the Swiss Lipids Database as GalNAcβ1-4(Neu5Gcα2-3)Galβ1-4Glcβ-Cer (d18:1/22:0) based on accurate mass. Subsequent MS/MS validated the correct sugar head group on this glycosphingolipid (GSL) (data not shown). The other proposed lipids that fall into the GM2 family are m/z 1370.52, 1482.76, 1484.74, and 1497.71. Each of these m/z ions shows the characteristic fragmentation pattern of the sugar head group of GM2. Our MS/MS fragmentation did not produce fragment ions of the ceramide tail; thus, we cannot predict an accurate ceramide tail for the other m/z ions of the GM2 family. It is worth mentioning that the mass difference between m/z 1454.73 and 1482.76 is 28.03, which suggests the addition of two CH2 to the ceramide tail. The other mass differences between ions within this family can be attributed to the addition or subtraction of CH2 and double bonds. The increased levels of the mouse analogue of GM2 post exposure to low doses of HZE irradiation, in particular 28Si (0.2 Gy), and low-energy γ ray (1.0 Gy) irradiation appear to be a novel finding, as we know of no reports in the literature identifying higher levels of these GM2 analogues in mice in association with HCC.

Additionally, downregulation of phosphatidylycerol (PG), phosphatidylglycerol (PG), phosphatidylserine (PS), and phosphatidylethanolamine (PE) lipids was observed. Specifically, PA, which can have many different combinations of fatty acids of varying lengths and saturation, was downregulated. PAs are important for the biosynthesis of triacylglycerols and phospholipids and many other lipids, and their physical properties influence the membrane curvature. They are maintained at low concentrations in order to act as a signaling molecule.45–47 PG indirectly activates lipid-gated ion channels;48 PS, a component of the cell membrane, plays a key role in cell cycle signaling and apoptosis;47,48 and finally, PE, a class of phospholipids found in biological membranes, plays a role in the secretion of lipoproteins in the liver.49,50

**DISCUSSION**

Our study demonstrates for the first time that MALDI-MSI combined with unsupervised image segmentation can qualitatively identify (due to the low number of biological replicates) m/z changes in a homogenous tissue, specifically, liver, where the distinct anatomical structures are challenging to identify as opposed to tissues such as brain, where different anatomical features are recognized relatively easily. Although unquestionably advantageous, the routinely used gene-expression profiling and proteomics methods not only require considerable amounts of tissue material but also use tissue homogenates, which would negatively influence the diagnostic characterization based on tissue heterogeneity. On the other hand, MALDI-MSI segmentations of the liver would allow for more specific identification of the location of biological regions, leading to a better definition of m/z signatures obtained to describe tumor heterogeneity. The different segments identified in the tumor sections (Figure 1) demonstrate the distinct heterogeneities and chemical properties within the tumor microenvironments. These heterogeneities are crucial to identify different subtypes of irradiation-induced HCC, each of which could have potentially different
prognoses and require different approaches in clinical management.

Alterations in lipid metabolism and regulation are hallmarks of cancer that affect cellular function and growth. These alterations in the setting of radiation exposure during spaceflight remain largely unexplored. A previous study has shown that radiation exposure in the spaceflight environment enhances fat accumulation in the livers of mice as compared with ground controls. Genes involved in triglyceride biosynthesis were observed to be upregulated in the irradiated mice. The authors postulated that lipid turnover is increased due to radiation exposure, with an enhanced anabolic mechanism and decreased catabolic mechanism, such as lipolysis, which will lead to an overall accumulation of lipids. Our study is in line with previously shown increased lipid turn over. Specifically, we have observed an upregulation of lipids with higher masses (~1300–1500 m/z) and downregulation of smaller lipids (~500–700 m/z). It is likely that the observed upregulation of lipids with higher masses contributes to the pathogenesis of fatty liver at the expense of decreased synthesis of smaller lipids leading to altered dynamic lipid metabolism in the livers of irradiated mice compared to nonirradiated control.

The MALDI-MSI analysis of the livers in this study identified a 1454.73 m/z peak (Table 1) that corresponds to a GSL belonging to the ganglioside subfamily, as shown in Figure 2. The MS/MS fragmentations for the GSL family, in particular, 1454.73, 1484.74, and 1497.71 m/z peaks, are shown in Figure 3 for reference. This GSL is upregulated in irradiated HCC in contrast to healthy control specimens. This GSL is analogous to human GM2 (molecules only differ by an OH or H on a terminal sugar). Several studies have described an association between increased levels of GM2 and HCC in humans. One such study reported 20–100× increased levels of GM2 in sera from patients with HCC. This increased level of GM2 might be due to the elevated synthesis of complex gangliosides and/or decreased levels of GM2 cleaving enzymes. GM2 has been found to have an immunogenic activity similar to other tumor-associated antigens. The immunological significance of GM2 makes it a strong candidate for irradiation-induced biomarkers.

Additionally, peaks 551.06 and 705.12 identified as PEs were downregulated in core tumor regions. Previous studies have demonstrated a decrease in the PC/PE ratio to be associated with hepatocyte nodules in rat livers. This might be due to the decreased synthesis of PC or decreased conversion of PE to PC following inactivation of the enzyme phosphatidylethanolamine N-methyltransferase, which is responsible for the conversion of PE to PC. The observation suggests that PE synthesis might be downregulated locally in tissues, regardless of this ratio. The decreased observed ratio could also potentially be due to the decreased PC synthesis through pathways (Kennedy pathway) not dependent upon PE to PC conversion. Moreover, peaks 533.48, 535.11, and 613.13 identified as PAs were also downregulated in core tumor regions. Since PAs can act as lipid ligands that gate ion channels, this finding may guide our understanding of lipid signaling cascades and their roles in the carcinogenic process of irradiation-induced HCC.

These novel findings on the lipid species post exposure to low doses of 25Si and 26Ca irradiation, support the strong potential of exploring these lipid species as early biomarkers of HCC risk in astronauts during deep space travel. In future works, the incorporation of targeted gene expression, proteomics, and comprehensive ultrahigh-resolution lipidomic arrays are planned to allow for the identification of enzymes involved in the synthesis of these lipids, validate the potential identified biomarkers, and possibly determine targets for mediation of irradiation-induced HCC.

**CONCLUSIONS**

This investigation shows that even though MALDI-MSI datasets are complex and high-dimensional and especially difficult to interpret quantitatively in homogenous tissues such as liver, segmentation can provide a unique way to depict the complex lipid heterogeneity of liver tissues in one image. Utilizing the SSSCRA enabled us to extract meaningful information from the raw measurements by reducing the dimensionality and identifying region-specific m/z changes in irradiated versus nonirradiated control mice liver tissues. We collected MS/MS data and refined the identification of the
lipid species found in the study and have assigned lipid species identifications based on the Lipid Maps Database and Swiss Lipids Database search of MS/MS fragmentations. This approach has allowed us to reliably identify a number of lipid ion species (Table 1) whose levels were significantly changed in irradiated tumor/HCC compared to healthy nonirradiated controls, based on both signal intensity and spatial distribution. In particular, we have confirmed the identity of Neu5Gc, GalNAcβ1-4(NeuGcα2-3)Galβ1-4Glcβ-Cer (d18:1/22:0). Its upregulation is of special interest because human GM2 ganglioside has been reported to be elevated in patients with HCC. Comparative analysis of this data enabled us to get a better systematic picture of the oncogenic transformation and cancer progression induced by HZE and γ ray irradiation in mice livers. Understanding this process and its mechanisms are critical to the safety of future manned spaceflights. Since lipids serve as an excellent source of information on various cellular signaling processes, including cancerous growth, our assigned lipid species can further be explored in future studies as potential biomarkers of HCC risk in astronauts during deep space travel. An attractive medical translation would be using lipid antibody arrays in which antibodies to the aforementioned lipids (esp. GM2) are immobilized and probed with astronauts’ sera for the presence of increased levels of these lipids during their time in space as a biomarker for irradiation-induced HCC.

## METHODS

### Animal Experiment
C3H/HeNCrl mice purchased from Charles River (Wilmington, MA) were used in this experiment. Mice were used for this study because they have been shown in the past to be a good experimental model for liver carcinogenesis based on previous studies, which demonstrated that C3H/HeNCrl mice are sensitive to the induction of HCC after exposure to a dose of 0.2 Gy of 600 MeV/n 56Fe.5 The dosage of 28Si (0.2 Gy) and 137Cs gamma (1 Gy) were based on the work of Weil et al., where they saw ∼51% of the C3H/HeNCrl mice developing HCC at 800 days post exposure.6 Tumor induction studies and studies of molecular changes in the irradiated tissues can only be conducted in whole animals. In vivo studies are necessary to study the microenvironmental effects of HZE exposure because computer models or cell culture are inadequate based on extensive literature searches. Conducted studies were approved by the Institutional Animal Care and Use Committees that are charged with evaluating the appropriateness of the use of animals in specific experiments and the number of animals requested for each group. The numbers of animals used were based on the expected numbers of irradiation-related tumors that would develop if animals were allowed to live out their lifespan. Power calculations for numbers in this study are based on the chi-square test for comparing two proportions, controlling a two-sided significance level at 0.05 or with 95% confidence about our results and with 80% power.

The serial sacrifice study consisted of seven (two per treatment and three control) male C3H/HeNCrl mice 360 days post exposure to HZE 28Si irradiation (0.2 Gy) (n = 2) and 137Cs γ rays (1.0 Gy) (n = 2) and nonirradiated/sham-irradiated control (n = 3). Eight- to ten-week-old male C3H/HeNCrl mice were purchased from Charles River Laboratories and shipped to the Brookhaven National Laboratory (BNL) and housed at the BNL animal facility until the time of irradiation at the NSRL. Following irradiation, the animals were shipped to the University of Texas Medical Branch (UTMB) Animal Resources Center (ARC), quarantined for 1 month, and then maintained in the ARC for the duration of the experiment. Animals were housed in sterile cages with free access to food (PicoLab Rodent Diet 20, Labdiet, St. Louis, MO) and water. Facilities at both BNL and UTMB are fully AAALAC accredited, which ensured adequacy of all animal care issues at both animal facilities. At 12 months, animals from each treatment group and control were randomly selected and euthanized by using CO2 asphyxiation as per current AVMA guidelines for euthanasia. Prior to euthanasia, animals were weighed and weight recorded. Post euthanasia, tissues of the left lobe of livers were collected, snap-frozen on either dry ice or liquid nitrogen, and stored at −80 °C until tissues could be extracted for MALDI-MSI. Livers were sampled by taking two 10 μm slices using a cryotome at −20 °C.

### MALDI-MSI Experiment

#### Sample Preparation and Histological Annotation.
In the present study, two tumor samples from biological replicates of C3H/HeNCrl mice irradiated with 137Cs γ rays (1.0 Gy), two tumor samples from biological replicates of C3H/HeNCrl mice irradiated with 350 MeV/n 28Si (0.2 Gy), and three healthy samples from biological replicates of C3H/HeNCrl mice in nonirradiated/sham-irradiated control were collected 12 months post irradiation. Left lobes of the livers were harvested, frozen in liquid nitrogen vapor, and stored at −80 °C. All tissue samples were sectioned at 10 μm thickness using a Leica CM1950 cryostat (Leica Microsystems GmbH, Wetzlar, Germany) at −20 °C and thaw mounted onto indium tin oxide (ITO)-coated glass slides (ITO glass, type II, 0.7 mm) [Hudson Surface Technology, Inc., West New York, NJ; cat. # PL-IC-000010-P100 (formerly PSI 1207000)]. Each tissue slice for MALDI imaging was followed by a slice collected for morphological/immunocytochemical staining (H&E staining) and mounted on a regular Superfrost Plus microscope slide (Fisher Scientific, Pittsburgh, PA; cat. # 12-550-15). Each slide was stored in a 50 mL plastic conical tube at −80 °C. A detailed histological analysis of the cryosections was performed by an experienced pathologist (HSL) post H&E staining to verify that tumors in irradiated mice livers were HCC positive. Before the matrix application, the slides were transferred to −20 °C for 20 min and then placed into a vacuum desiccator for approximately 25 min.

#### MALDI Matrix Application and MSI
1,5-Diaminonaphthalene (DHB) (MilliporeSigma, St. Louis, MO; cat.# 149357) was applied by sublimation under vacuum using a procedure similar to that described by Wildburger (2017).61 Sublimation of DHB was performed for 1 min at 120 °C under 10−15 mTorr. Matrix coating density was approximately 500 μg/cm². After that, the slide was placed in a 50 mL plastic conical tube at −80 °C. Prior to MALDI imaging, the slides were transferred to −20 °C for 20 min and then quickly transferred into a vacuum desiccator and brought to room temperature under vacuum for approximately 10 min. An ultraflexXtreme MALDI-time-of-flight (TOF)/TOF mass spectrometer (Bruker Daltonics, Billerica, MA) equipped with a smart beam Nd:YAG 355 nm laser was utilized for the MALDI analysis. The laser was operated at 250 Hz in the negative ion reflector mode. Mass spectra were collected with a pulsed ion extraction time of 80 ns, an accelerating voltage of 20.0 kV, an extraction voltage of 17.90 kV, a lens voltage of 7.80 kV, and a reflector voltage of 21.15 kV. The laser spot size was set at medium focus (∼50 μm laser spot diameter), and laser power
was optimized at the start of each run and then fixed for the whole experiment. The mass spectral data were acquired over a mass range of m/z 400–1600 Da. Mass calibration was performed prior to data acquisition using Bruker Peptide Calibration Standard II (Bruker Daltonics, Billerica, MA) as an external standard. For MS analysis, spatial imaging resolution was set to 100 μm. Each spectrum was a sum of 500 laser shots. Regions of interest were manually defined in the flexImaging 3.0.54 software using a scan of the slide with the tissue sections acquired at 1200 dpi using an Epson 3170 photo scanner. MALDI mass spectra were processed with the total ion current (TIC) normalization, and the signal intensity of each imaging data was represented as the normalized intensity.

Data Analysis. Image Preprocessing. It is necessary to use appropriate preprocessing steps in order to derive reliable conclusions and to obtain maximal biological information from MALDI-MSI data. In order to improve signal quality and compress the raw data acquired into a list of valuable peaks associated with relevant m/z values for further analysis, the Cardinal R package41 was used to perform all preprocessing data. This includes normalization based on the total ion current (TIC), spectra smoothing, baseline correction, peak detection, and spectra alignment.

Image Segmentation. The SSCCA was used, which is provided in the Cardinal R package,41 a model-based unsupervised image segmentation technique using adaptive weights that preserve edges between segments. The SSCCA uses a statistical regularization to enforce feature sparsity. As a result, the analysis automatically identifies informative m/z values and partitions the observations within a dataset into segments. The pixels in the dataset are partitioned according to their m/z similarity, expressed by their chemical and/or biological properties.62 In particular, the parameters are r (the neighborhood smoothing radius), k (the initial number of segments/clusters), and s (the shrinkage parameter). We selected a set of parameters to get sharp (not pixelated) segments with sharp edges (r = 6, k = 5, s = 3). We then exported each segment with its corresponding list of m/z and t-score, which is an enrichment score that is calculated based on the abundance of specific m/z values in the corresponding segment compared to other segments. In this analysis, the segmentation was performed on one slide/image with seven different specimens on it partitioned together. In particular, the samples were two biological replicates from the liver tumor section in 28Si-irradiated, two biological replicates from the liver tumor section in 29Si-irradiated, two biological replicates from the liver tumor section in γ-ray-irradiated, and three biological replicates from the nontumor liver section in nonirradiated/sham-irradiated control mice.

Statistical Comparison and MALDI-MSI Visualizations. After image segmentation, we picked the highly enriched upregulated m/z as well as downregulated m/z features in segment 1 (core tumor regions) and compared to segment 3 (healthy nonirradiated control) using their t-score enrichment values. Evaluation of t-scores from core tumor regions in irradiated liver sections compared to nonirradiated healthy control and healthy-looking noncore tumor regions revealed peaks that could discriminate between different segments based on their corresponding t-scores. These peaks were also confirmed using flexImaging 3.0.54 (Bruker Daltonics, Billerica, MA) by visualizing the hotspots and differences between the segments. Despite the unquestionable advantages of visualization software in visualizing the spatial distribution and relative abundance of m/z directly, the visualized features are often complex and easy to miss, which makes analysis and interpretation challenging. Therefore, using a model-based approach and t-score enrichment values allowed us to detect different features in a more systematic manner, as opposed to purely relying on the qualitative visualization of hot spots.

MS/MS Validation of Selected m/z Values. On-tissue MS/MS fragmentation spectra of the lipid species of interest were acquired using the ultrafleXtreme mass spectrometer in the negative ion LIFT mode. The laser was operated at 1000 Hz; each MS/MS spectrum was a sum of 1500 shots for precursor ions and 5000–10,000 shots for product ions. MS/MS spectra in the reflector mode were acquired with a pulsed ion extraction time of 80 ns, an accelerating voltage of 7.5 kV, an extraction voltage of 6.8 kV, a lens voltage of 3.5 kV, a reflector voltage of 29.5 kV, a lens voltage of 19.00 kV, and a lift 2 voltage of 3.7 kV. Preliminary assignments for the detected lipid species and their identification by MS/MS spectra were performed using the LIPID MAPS Structure Database,63 Human Metabolome Database,64 and Swiss Lipids.65

Lipid Identifications. The selected parent ions with significant differences between irradiated core tumor region compared to healthy nonirradiated control were analyzed via direct MS/MS and were then manually searched for in the LIPID metabolites and pathway strategy structure database using the negative ion mode (LIPID MAPS Structure Database) Search results were further filtered by eliminating matches outside a mass error range of 100 ppm, thus reducing the potential for false positives.

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A.M.N. analyzed the data, developed the methodology, and wrote the manuscript. A.M.N. and M.R.E. conceived and designed the experiment and methodology. R.L.U. participated in the design of the biological study. A.S. performed the experiments. All authors participated in the analysis and interpretation of the results. All authors read and approved the final manuscript.

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Notes
The authors declare no competing financial interest.

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ABBREVIATIONS
GCR, galactic cosmic rays
HCC, hepatocellular carcinoma
HZE, high-charge high-energy ions
MALDI-MSI, matrix-assisted laser desorption ionization-mass spectrometry imaging

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