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¹H NMR Metabolomics Study of Metastatic Melanoma in C57BL/6J Mouse Spleen

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Abstract

Melanoma is a malignant tumor of melanocytes. Although extensive investigations have been done to study metabolic changes in primary melanoma in vivo and in vitro, little effort has been devoted to metabolic profiling of metastatic tumors in organs other than lymph nodes. In this work, NMR-based metabolomics combined with multivariate data analysis is used to study metastatic B16-F10 melanoma in C57BL/6J mouse spleen. Principal Component Analysis (PCA), an unsupervised multivariate data analysis method, is used to detect possible outliers, while Orthogonal Projection to Latent Structure (OPLS), a supervised multivariate data analysis method, is employed to find important metabolites responsible for discriminating the control and the melanoma groups. Two different strategies, i.e. spectral binning and spectral deconvolution, are used to reduce the original spectral data before statistical analysis. Spectral deconvolution is found to be superior for identifying a set of discriminatory metabolites between the control and the melanoma groups, especially when the sample size is small. OPLS results show that the melanoma group can be well separated from its control group. It is found that taurine, glutamate, aspartate, O-Phosphoethanolamine, niacinamide, ATP, lipids and glycerol derivatives are decreased statistically and significantly while alanine, malate, xanthine, histamine, dCTP, GTP, thymidine, 2'-Deoxyguanosine are statistically and significantly elevated. These significantly changed metabolites are associated with multiple biological pathways and may be potential biomarkers for metastatic melanoma in spleen.

Keywords

¹HNMR metabolomics, B16-F10 melanoma; spleen; metabolomics; multivariate analysis; OPLS, and PCA

1 Introduction

As a malignant tumor of melanocytes, melanoma has now become one of the most common cancers in the United States and many other western countries. Melanoma is the most

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Conflict of interest

The authors declare no conflict of interest.

dangerous form of skin cancer due to its high capability of invasion and rapid metastasis to other organs, which causes 90% of skin cancer mortality and is increasingly popular in white populations. Every one person out of 75 in the United States suffers from this aggressive disease (Bailet et al. 2009; Orlov et al. 2012; Bourne et al. 2005; Maire et al. 2013). Early-stage melanoma can be treated by surgical resection, but, once metastasis occurs, few patients can survive longer than 5 years due to poor prognosis (Lee et al. 2011; Abaffy et al. 2013). Therefore, early detection is the key for increased chance of longtime survival. NMR-based metabolomics, a quantitative and highly reproducible method that has the largest dynamic range, i.e. 10^5 , and the ability of simultaneously detecting multiple metabolites (Keun et al. 2002), has emerged as a powerful tool for metabolite biomarker discovery for early detection of various diseases (Rocha et al. 2011; Weljie et al. 2007; Constantinou et al. 2005). NMR metabolic profiling has been extensively used for studying primary melanoma in vivo and in vitro. However, little attention has been paid to metabolic profiling of metastatic tumors in organs other than lymph nodes (Morvan et al. 2002; Morvan et al. 2006; Li et al. 2009; Morvan et al. 2003; Fages et al. 2010; Guitera et al. 2010; Triba et al. 2010; Bourne et al. 2005; Stretch et al. 2005), where animal models have been frequently used.

As an integrated part of metabolomics, statistical analysis methods, in particular the multivariate data analysis (both unsupervised and supervised methods), are often used for exploring the latent data structure imbedded within a set of complex data (Enot and Draper 2007), including pattern recognition, disease and non-disease classification, etc, and for identifying potential metabolite biomarkers (Qiu et al. 2008). Currently, there are three multivariate data analysis models that have been widely used in the field of Metabolomics, i.e. principal component analysis (PCA) (Xia et al. 2007; Mounet et al. 2007), partial least-squares to latent structures (PLS) (Szymanska et al. 2012; Kristensen et al. 2010) and orthogonal projections to latent structures (OPLS) (Ali et al. 2013; Lu et al. 2012). OPLS (Trygg and Wold 2002), a modification of PLS, has received much attention recently due to its superior capability of separating groups. In OPLS, variation in X -matrix is separated into two parts, i.e. predictive components and orthogonal components, thereby greatly facilitates the model interpretation (Cloarec et al. 2005; Wiklund et al. 2008). Based on OPLS model, Susanne Wiklund proposed a powerful visualization tool named S-plot in 2008 for interpretation of multivariate classification models, enabling direct inspection and extraction of statistically and potentially biochemically important variables (Wiklund et al. 2008). Usually, the OPLS model (in the case of NMR-based metabolomics) is constructed using a reduced data set obtained by binning of the original NMR spectra. This spectral binning method is very efficient for large scale sample sets and can be easily automated (Craig et al. 2006). However, to identify biochemically significant metabolites, 2D NMR spectra are needed for the purpose of peak assignment, a time consuming process for both data acquisition and data analysis/interpretation.

In this study, we are interested in evaluating the metabolic changes in spleen after metastasis of primary melanoma cells implanted at the flank location on the legs of a B16-F10 melanoma mouse model. ^1H NMR metabolomics is utilized for global metabolic profiling on both the water soluble (polar) and lipid soluble (lipophilic) tissue extracts from the

excised spleens of the control and the melanoma mice. Multivariate data analysis (both PCA and OPLS) are carried out for pattern recognition and for identifying a set of metabolites whose concentrations are statistically and significantly changed as a result of the melanoma metastasis in the spleen. Based on the findings, the metabolite pathways/networks that are affected by the disease are discussed. In addition, for PCA and OPLS analyses, both spectral binning and spectral deconvolution are used for data reduction before statistical analysis and the advantages and disadvantages of these two strategies are discussed.

2 Materials and Methods

2.1 Animal and Sample Preparation

A total of 12 six-week-old C57BL/6J male mice were purchased from Jackson Labs (Maine, USA) and were housed at the Pacific Northwest National Laboratory (PNNL) animal facility. After acclimation at the facility for one week, at the age of week-7 the mice were randomly divided into two groups, an experimental group (n=7) that received subcutaneous injection with suspended 10^5 B16-F10 tumor cells at four flank locations on each leg, and a control group (n=5) that were injected with the same amount of PBS at same places. The animals were fed a standard diet and housed one mouse per cage and maintained in a temperature-controlled room (22-25°C, 45% humidity) on a 12:12h dark-light cycle. All mice, each of which was weighed weekly, were allowed free access to water and food before being sacrificed by CO₂ asphyxiation at the age of fourteen weeks. Two mice from the experimental group died during the feeding process resulting in five mice per group. Spleens were dissected and snap-frozen at -80°C before NMR spectroscopy and morphology analysis. The presence of malignant melanoma in spleen can be observed by histological images (Figure S1 in Supporting Information), which is consistent with prior literature reports (Gheorgheosu et al. 2011; Roomi et al. 2008). All animal work was approved by the Institutional Animal Care and Use Committee (IACUC) at Pacific Northwest National Laboratory.

Polar and lipophilic metabolites were extracted from tissues using a modified Folch method by following published protocol (Beckonert et al. 2007), which was considered to generate the highest yields in mind condition (Washburn 1989; Iverson et al. 2001). It has been generally accepted that about 95% of tissue lipids can be extracted during the first step (Akoh 2008). The recipes based on tissue weight are provided in Table S1 in Supporting Information. In our case, the weight of spleen used for each sample was between 30 to 60 mg. The extraction steps are briefly summarized below.

Step 1: The pre-weighted intact frozen tissue was homogenized in ice bath after adding 0.25 ml MeOH and 0.053 ml H₂O for each sample containing 30 to 60 mg of tissue, followed by vortexing the mixture and then adding 0.125 ml chloroform, vortexing again. Step 2: 0.125 ml chloroform and 0.125 ml water was added to the sample and then vortexed again, followed by transferring different layers into glass vials separately with syringes after the mixture being left on ice for 15 min and centrifuged at 8000rpm for 2 min at 4°C. Finally, the solvents were removed by employing lyophilizer (MeOH/H₂O layer, hydrophilic metabolites) or by evaporating under dry nitrogen gas (CHCl₃ layer, hydrophobic

metabolites). The extracts were then stored at -80°C before performing NMR measurements.

2.2 ^1H NMR Spectroscopy

The hydrophilic metabolites were reconstituted in 600 μL of D_2O containing 0.5 mM 3-(trimethylsilyl)-2,2',3,3'-tetra deuteriopropionate (TSP- d_4) as chemical shift reference and internal concentration standard. About 550 μL of the prepared sample was loaded into a standard 5 mm NMR tube (Wilmad, Buena, NJ). 0.2% sodium azide (w/v) was added into the mixture to prevent biodegradation. All ^1H NMR spectra were recorded on a Varian 600 MHz NMR spectrometer equipped with a Z axis-gradient 5 mm HCN probe at 20°C . One-dimension ^1H NMR spectra were acquired from each sample using the standard Varian PRESAT pulse sequence with a single pulse excitation and 2 s low power pre-saturation at the water peak position to suppress the residual H_2O signal. A total of 8192 transients were accumulated. The acquisition time and recycle delay (RD) of a single scan were 1.9 s and 2 s, respectively, resulting in a total pulse sequence duration of about 4 s. The total experimental time for acquiring each spectrum was about 9.1 hours. Such a long data acquisition time was used to ensure that a high quality ^1H NMR spectrum was obtained with sufficient signal to noise ratio for metabolites with concentration as low as about 0.5 μM or lower in the NMR tube.

Similarly, the hydrophobic metabolites were reconstituted in 600 μL of deuterated chloroform (CDCl_3) containing 0.03% (v/v) tetramethylsilane (TMS) shortly before the NMR experiments. About 550 μL of the prepared sample was loaded into a standard 5 mm NMR tube. 1D NMR spectra were acquired at 20°C using a single pulse sequence with 1024 transients for each spectrum. The acquisition time was 1.9 s and the recycle delay was 2 s, resulting in a total experimental time of about 1 h.

2.3 NMR Data Processing and Multivariate Data Analysis

All free induction decays were multiplied by an exponential function with 0.5 Hz Lorentz line broadening prior to Fourier transformation. And, all ^1H NMR spectra were manually phased and baseline corrected using the Processor module of Chenomx (NMR suite 7.6, Professional) and referenced to the chemical shift of TSP- d_4 at 0 ppm. Then, two different strategies, i.e. spectral binning and spectral deconvolution, were used to reduce the original spectral data before statistical analysis.

2.3.1 Spectral binning—Spectral binning is a widely used method for producing a reduced data set with manageable proportion, which is efficient and easily automated for large sample sets and usually involves integration of peak intensities of specific spectral width within user defined spectral regions (Craig et al. 2006; Cloarec et al. 2005; Jiang et al. 2012; Huang et al. 2014). In this study, for hydrophilic extracts the spectral regions at δ 0.5 ~ 9.0 are segmented into discrete bins with equal width of 0.004 ppm using the Profiler module of Chenomx (NMR suite 7.6, Professional). Spectral regions at δ 3.34 ~ 3.38 and δ 4.7 ~ 5.1, containing MeOH and residual water signals, respectively, are excluded. For hydrophobic extracts, the binned spectral region is δ 0.65 ~ 5.95. It has been shown in our previous publication (Feng 2013) that normalization to a constant sum, i.e. setting each

individual spectrum to have unit total spectral intensity by expressing each data point as a fraction of total spectral integral, could sometimes result in false or biased conclusion of metabolites alterations, in particular for those metabolites with low concentration, due to large spectral perturbations caused by significant increase or decrease of some major metabolites with high concentration (Feng 2013). In order to reliably access the real metabolite concentration alternations in spleens, in particular for those metabolites with low concentration, the integral areas of all bins in this study are first divided by the area of TSP-d4 whose concentration is constant across the sample studied and then normalized to per unit weight of spleen tissue measured before extraction. Then, the normalized NMR data in terms of spectral bins is imported into SIMCA (Version 13.0.3 64-bit, Umetrics, Umea, Sweden) for multivariate data analysis, i.e. principal component analysis (PCA) and orthogonal projection to latent structure (OPLS). PCA is performed first using the mean-centered and unit-variance scaled NMR data to obtain an overview and detect possible outliers. Subsequently, OPLS is conducted using the autoscaled data as X -matrix (with each row representing a sample, each column representing a binned chemical shift range) and class information as Y -matrix to find significant variables, i.e. metabolites, responsible for the discrimination of two different classes (Cloarec et al. 2005; Trygg and Wold 2002). To simplify the interpretation of the results, $y = 0$ is assigned to the control group and $y = 1$ to the tumor group before building an OPLS model, so that positive loadings mean up regulated while negative loadings mean down regulated (Wiklund et al. 2008). Both PCA and OPLS models are constructed using the non-linear iterative partial least squares (NIPALS) algorithm and model complexity (number of components) is determined by a 7-fold cross-validation method. Model quality can be evaluated from parameters such as R^2 , revealing the interpretability of the model, and Q^2 , indicating the predictability of the model. Finally, the model significance is further assessed by the well-known CV-ANOVA test at the level of $p < 0.05$ (Eriksson et al. 2008). S plot (named S-line plot in SIMCA-13 for NMR spectral data), a useful visualization tool for interpretation of multivariate classification, is employed to help identify statistically significant and therefore potential biomarkers (Wiklund et al. 2008). In this plot, loadings obtained from the OPLS model are plotted with color-coded correlation coefficients denoting the variable importance for class separation with warm colored (e.g. red) metabolites being more significant than cold colored (e.g. blue) ones. A cutoff value, depending on sample number in each group, is chosen to select metabolites responsible for between group variation based on the discrimination significance ($p < 0.05$) (An et al. 2013; Jiang et al. 2012; Dong et al. 2013). Also based on the prior reports (An et al. 2013; Jiang et al. 2012; Dong et al. 2013), for a sample size of 5 (the actual sample size in this study), i.e. the number of samples in each group, metabolites with correlation coefficients $|r| > 0.811$ are considered statistically and significantly important.

2.3.2 Spectral deconvolution—A major drawback of spectral binning is reduction of spectral resolution. But this can be overcome with rapid development of high performance desktop computers enabling full resolution spectra being used directly for chemometric data analysis. However, at least 20 samples per group are required to properly model the peak position variation. Otherwise, the OPLS model will lose its prediction ability and interpretability (Cloarec et al. 2005). A significant amount of time is also still needed to

assign spectral peaks with the help of traditional two-dimensional NMR spectra such as ^1H - ^1H correlation spectroscopy (COSY), ^1H - ^1H total correlation spectroscopy (TCOSY), ^1H J -resolved spectroscopy (JRES), ^1H - ^{13}C heteronuclear single-quantum correlation spectroscopy (HSQC), and ^1H - ^{13}C heteronuclear multiple bond correlation spectroscopy (HMBC) (Dong et al. 2013; Jiang et al. 2012). Another even more important shortcoming associated with spectral binning is that there is not an established way of handling peak overlapping from different metabolites. Peak overlapping can dramatically weaken correlation of bins containing information of different functional groups from the same metabolite, resulting in dramatically declined performance of statistical analysis using the two most popular methods, i.e. PCA and OPLS. To address the problems associated with peak overlapping, in this study we decide to use spectral deconvolution in addition to the conventional spectral binning. Spectral deconvolution takes full advantage of the fact that a metabolite molecule often has several groups of ^1H NMR peaks located at different chemical shift positions corresponding to different functional groups in the same molecule, where one or more of these peaks may be identifiable, or do not overlap with peaks from other metabolites. Since ^1H NMR is quantitative, all the peaks belonging to a same molecule will be scaled the same during spectral deconvolution. In this way, the overlapped peaks that have peaks from different metabolites will be deconvoluted with high confidence. Spectral deconvolution is carried out using the Profiler module of Chenomx (NMR suite 7.6, Professional) which contains a database of more than 300 common metabolites associated with mammals and bacteria. Using the Profiler module of Chenomx, a ^1H NMR metabolite spectrum is first manually phased and baseline corrected and then deconvoluted to individual metabolites. A new but simple strategy (detailed in the Supporting Information) is used to filter out the broadline features arising from macromolecules exhibiting slow molecular motion such as high density lipoprotein (HDL) and low density lipoprotein (LDL), etc. Filtering out the broadline features in a ^1H metabolite NMR spectrum is important to ensure that accurate metabolite concentrations are obtained using the method of spectral deconvolution. The absolute concentration of each metabolite in the NMR tube is determined using the well-established method provided by Chenomx (NMR suite 7.6, Professional) with TSP-d4 as internal concentration standard. The metabolite concentrations are then normalized to per milligram of spleen tissue before extraction followed by multivariate data analysis and by applying exactly the same strategy aforementioned for spectral binning data.

3 Results and Discussion

3.1 NMR spectra of spleen tissue extracts

Examples of typical ^1H NMR spectra of hydrophilic extracts obtained from a control mouse and a melanoma mouse are shown in Figure 1. The peak intensities are normalized to per unit weight of spleen tissue before extraction, so that the concentrations of a given metabolite in the tumor group and the control group can be directly compared visually according to the peak intensities in the corresponding spectrum. Peak assignments are listed in Table 1. A total of 73 metabolites have been identified with good confidence based on spectral deconvolution using Chenomx. Chemical shift identities of metabolites are assigned according to both literatures (Lindon et al. 1999; Martin et al. 2007a; Martin et al. 2007b;

Nicholson et al. 1995; Jiang et al. 2012), and the metabolites library of Chenomx. A wide range of amino acids, carbohydrates, glycolysis and tricarboxylic acid cycle (TCA cycle) intermediates are detected. Other observed metabolites include choline metabolites, ethanolamine metabolites, organic bases. “*” denotes an unsigned peak which fortunately shows no significant statistical difference between the tumor group and the control group as confirmed by the OPLS model analysis, to be discussed below, where a correlation coefficient of 0.562, i.e. with $|r| < 0.811$, is obtained (see Table S2 in Supporting Information). Visual inspection of the ^1H NMR spectra reveals apparent metabolic alterations induced by melanoma. For example, the tumor cell treated mice have higher level of lactate (peak 11), alanine (peak 14), glutathione (peak 22), asparagine (peak 30), glycine (peak 54) and inosine (peak 60) in the top trace spectrum (red) in Figure 1, while the bottom trace spectrum (black) from the control group shows evidently higher level of glutamate (peak 19), aspartate (peak 29), choline (peak 41), O-phosphocholine (peak 42), O-phosphoethanolamine (peak 43), sn-glycero-3-phosphocholine (peak 44), glucose (peak 47), taurine (peak 48), myo-inositol (peak 50) and formate (peak 73). To identify statistically and significantly changed metabolites, in the following both PCA and OPLS are performed on the entire spectral set (i.e. five from tumor group and another five from control group).

3.2 Statistical results based on spectral binning

Unsupervised (or exploratory) data analysis, i.e. PCA in this case, is conducted firstly to get an overview of ^1H NMR spectral binning data set of hydrophilic extracts and detect possible outliers. PCA scores plot (Figure S2 (a) in Supporting Information) shows clear classification of the control and tumor cell treated mice without any outlier. Therefore all 10 samples are kept for further OPLS modeling. In order to maximize the correlation between X -matrix (the ^1H NMR spectral data set, i.e. the integral intensities of spectral bins) and Y -matrix (the class information, i.e. the control versus melanoma groups) as well as the variation in X -matrix, OPLS is performed to assess variable importance and determine discriminatory variables (i.e. the spectral bins) responsible for separation of different groups (Trygg and Wold 2002). Values of the resulting model parameters, i.e. R^2 and Q^2 , show good quality of the generated OPLS model (Figure 2 (a)). CV-ANOVA results further confirm the model validity ($p < 0.05$). The key variables showing significant differences between the control group and the melanoma (or the tumor) group are extracted from the correlation coefficients-coded loadings plot of the OPLS model constructed using the spectral binning data (Figure 2 (a)).

Evidently, spectral binning is an efficient and easily automated data reduction method, especially for large sample sets, that is highly applicable in assessing changes between two groups where decisions have to be made within a short period of time. However, there is an intrinsic shortcoming associated with this method. As shown in Figure 2 (a), instead of providing the chemical identities of specific metabolites, the selected discriminatory variables are just chemical shift (δ) values (or a small chemical shift region) of the corresponding “bins” that often cannot be assigned to a specific metabolite due to spectral peak overlap from different metabolites. Additionally, since the loadings plot is derived from binned spectra data, resolution of the loadings plot is reduced compared with the original NMR spectra, making the assignment of statistically significant changed variables

(peaks) more difficult in heavily overlapped peak regions. For example, in Figure 2 (a), variables with δ around 1.48 are considered important because the absolute values of their corresponding correlation coefficients are larger than the cutoff threshold ($|r| > 0.811$), and they can be easily assigned as alanine (14) due to no overlap with other high intensity peaks. However, peaks are heavily overlapped in most regions of the spectra, such as variables with δ 2.34 ~ 2.37 (glutamate and malate with metabolites key 19 and 24, respectively, in Table 1), and variables with δ 6.29 ~ 6.32 (Thymidine, dCTP and 2'-Deoxyguanosine with metabolites key 16, 23 and 27, respectively). These discriminatory variables cannot be assigned to any individual metabolites due to severe peak overlap.

3.3 Statistical results based on spectral deconvolution

Given the intrinsic shortcomings associated with the spectral binning data reduction method, spectral deconvolution (detailed in Section 2.3.2) is used to identify specific metabolites responsible for separating the melanoma group from the control group. PCA and OPLS are carried directly on the absolute concentrations of 73 metabolites (72 identifiable and one unsigned, Methanol is excluded) that are normalized to per milligram of spleen tissue before extraction. Statistical analysis procedures are exactly the same as mentioned above in spectral binning section. Metabolites assignments and the averaged concentrations, including the standard deviations, are summarized in Table 1. PCA scores plot (Figure S2 (b) in Supporting Information) shows clear classification of the control and the tumor mice without any outlier. Thus, all 10 samples are kept for OPLS modeling. OPLS scores plot and coefficients-coded loadings plot are illustrated in Figure 2 (b). Separation of the two different groups in Figure S2 (b) in Supporting Information from PCA on spectral deconvolution data is much better than that shown in Figure S2 (a) from PCA on spectral binning data. Also, model parameters such as $R^2X(\text{cum})$, i.e. the explained variance, and $Q^2(\text{cum})$, i.e. the predictive performance in cross-validation, show better statistical performance of PCA when spectral deconvolution data reduction method is used. Additionally, p values of CV-ANOVA results indicate that the OPLS model built from spectral deconvolution reduced data set shows statistically more significance than that from the spectral binning reduced data set. Based on these results, we conclude that spectral deconvolution is a better method than spectral binning for identifying discriminatory metabolites between the control and the melanoma groups. Based on the results from spectral deconvolution reduced data set, a group of metabolites are identified to be responsible for the discrimination of the tumor group from the control group and these metabolites are tabulated in Table 2. It is known from Tables 1 and 2 that compared with the control group, in the tumor group the concentrations of taurine (48), glutamate (19), aspartate (29), O-Phosphoethanolamine (43), niacinamide (69) and ATP (63) are decreased statistically and significantly (with negative loadings in Figure 2 (b)), while the concentrations of alanine (14), malate (24), xanthine (71), histamine (33), dCTP (23), GTP (65), thymidine (16), 2'-Deoxyguanosine (27) are increased statistically and significantly (with positive loadings in Figure 2 (b)). All these significantly changed metabolites can be considered as potential biomarkers of metastatic melanoma in spleen tissue.

^1H NMR spectra of hydrophobic extracts of spleens from control (black line) and tumor cell treated (red line) mice are shown in Figure 3, where peak assignment is based on previous

published papers (Lindon et al. 1999; Nicholson et al. 1995). The peak intensities are normalized to per unit weight of spleen tissue before extraction for direct comparison of lipids levels in two different groups. Visual inspection of Figure 3 reveals much lower level of linear lipids in tumor group, indicating significant alternation in lipids oxidation. OPLS scores plot in Figure 2 (c) shows clear classification of the control group (blue dots) and the tumor group (green dots), i.e. all samples from the control group (blue dots) have negative scores while samples from the tumor group (green dots) have positive scores. Not that, only one predictive component is extracted from the data set with no orthogonal component. To build the OPLS model, spectral region of δ 0.65 ~ 5.95 is segmented into discrete bins (0.004 ppm each in width). The integral areas of all bins are first divided by the area of TMS and then normalized to per unit weight of spleen tissue measured before extraction, followed by multivariate data analysis in SIMCA (Version 13.0.3 64-bit, Umetrics, Umea, Sweden) using exactly the same strategy aforementioned in *Section 2.3.1*. PCA scores plot is shown in Figure S2 (c) in Supporting Information. CV-ANOVA test of the model validity indicates that the OPLS model is of only marginal significance with a p -value of 0.117 (Eriksson et al. 2008). Discriminatory variables ($|r| > 0.811$), i.e. peaks, between the control and the tumor groups are extracted from the coefficients-coded loadings plot as illustrated in Figure 2 (c), which shows significantly decreased level of lipids in the tumor group. Assignments of peaks in Figure 2 (c) are listed in the caption of Figure 3.

3.4 Molecule pathway discussion

The major results from the combined ^1H NMR metabolic profiling and statistical analysis of spleen tissue extracts are summarized as follows. The tumor group is well separated from the control group based on either the metabolites found from the hydrophilic extracts or on metabolites from the hydrophobic extracts. A set of discriminatory metabolites that play a major role in separating the groups have been found. From the hydrophilic spleen extracts, we found that the concentrations of alanine, malate, xanthine, histamine, dCTP, GTP, thymidine and 2'-Deoxyguanosine are increased statistically and significantly while the concentrations of taurine, glutamate, aspartate, O-Phosphoethanolamine, niacinamide and ATP are decreased significantly in melanoma group. From the hydrophobic spleen extracts, we found that linear lipids and glycerol derivatives are in much lower concentrations in melanoma group when compared with the control group. These findings allow us to dissect the metabolic pathways that are affected as a result of metastatic melanoma in the spleen of mouse.

The decreased level of linear lipids and glycerol derivatives in melanoma mouse spleen implies increased consumption of energy for tumor growth and the accompanying DNA/RNA syntheses associated with the lipids metabolism pathway, a result consistent with that found in the liver of a metastatic melanoma mouse model (Feng 2013) where we have reported that β -oxidation of fatty acids plays an important role in the metastasis of melanoma. Previous literatures have also demonstrated that tumor metastasis can be activated during the process of cell migration, invasion and angiogenesis by modulating phosphorylation and acetylation of proteins, a process controlled by specific genes, such as MMP-2, MMP-9, uPA, ZNF580. It has been recently found that sphingolipids of cells can mediate the expression of these specific genes (Patwardhan and Liu 2011). O-

Phosphoethanolamine plays a central role in sphingolipids metabolism since the only route by which sphingolipids are converted to non-sphingolipids through sphingosine-1-phosphate lyase will result in the formation of O-Phosphoethanolamine (Frolkis et al. 2010). Strong phospholipid metabolism alterations have already been reported previously in melanoma tumors as a mechanism that could participate in tumor cell redifferentiation and/or survival (Morvan et al. 2002; Morvan et al. 2003). A lower concentration of ATP in melanoma group reveals the elevated energy demands for tumor growth, consistent with the decreased level of linear lipids and glycerol derivatives in melanoma mouse spleen.

It has been reported (Yu and Kim 2009) that taurine can stimulate detoxification within cells and significantly inhibit the production of reactive oxygen species, which can cause lipid peroxidation, massive protein oxidation and degradation and eventually lead to the development of cancer. Also, taurine affects the activities and gene expression of the antioxidant enzymes in melanoma cell. Thus, taurine is evaluated as a chemopreventive agent against cancer. A much lower level of taurine found in the tumor group of our present study reveals the cell damage by metastasis melanoma.

Glutamate plays multiple roles in the body and is involved in a variety of metabolic pathways as depicted in the Small Molecule Pathway Database (SMPDB) (<http://www.smpdb.ca/>). Through the actions of different enzymes, glutamate can interconvert with many other metabolites. For instance, glutamate can be generated from alanine or aspartate in combination with 2-Oxoglutarate catalyzed by transaminase, the resulting byproducts (pyruvate and oxaloacetate) of which are key components in glycolysis, gluconeogenesis and the TCA cycle. Via the action of enzyme glutamine synthetase, glutamate can also be converted to glutamine and then, further catalyzed by GMP synthase, to produce GMP, an important intermediate in purine metabolism. Additionally, glutamate-cysteine ligase will, combined with glycine and cysteine, convert glutamate into glutathione, a key redox regulatory molecule which plays important roles in gene expression, DNA and protein synthesis, cell proliferation, signal transduction and so on (Frolkis et al. 2010). A significant decreased level of glutamate in melanoma group suggests that the above mentioned pathways may have been profoundly disturbed. Glutamate signaling in cancer has stimulated extensive research interest recently and has shown great importance in human melanoma development (Namkoong et al. 2007; Choi et al. 2011; Stepulak et al. 2005; Rzeski et al. 2001). The blockade of glutamate receptors has been proposed as a promising novel therapy for treating melanoma (Song et al. 2012). Suppression of glutamate release can result in decreased cell proliferation in ex vivo and tumorigenesis in vivo as well as migration and invasion of human melanoma (Namkoong et al. 2007; Lee et al. 2011).

Niacinamide can be interchanged with nicotinamide-adenine dinucleotide (NAD) and nicotinamide-adenine dinucleotide phosphate (NADP) with the help of ADP-ribosyl cyclase 2 in nicotinate and nicotinamide metabolism and eventually contributes to the metabolism of pyruvate which sits at an intersection of key pathways of energy metabolism. Depending on the cell energy charge, pyruvate has different fates, e.g., it can either be converted by the pyruvate dehydrogenase complex to produce acetyl-CoA in fatty acid synthesis, or carboxylated by pyruvate carboxylase in gluconeogenesis to produce oxaloacetate which participates the TCA cycle (Frolkis et al. 2010). Niacinamide, also known as nicotinamide,

is the amide form of vitamin B3 and has exhibited the ability to inhibit the formation of vasculogenic mimicry which describes functional vascular channels composed only of tumor cells and has been observed in highly aggressive melanoma cells (Itzhaki et al. 2013; Maniotis et al. 1999). Through unique cellular pathways, niacinamide can directly impact normal physiology, influence oxidative stress, block pro-inflammatory signal transduction mediators *in vitro* and act as an epigenetic gene regulator as well as activate cellular repair to prevent cancer (Maiese et al. 2009; Jacobson and Jacobson 1993). Aspartate is a precursor to many other cofactors or compounds involved in cellular signaling. Furthermore, aspartate carries the reducing equivalents in the mitochondrial malate-aspartate shuttle, which utilizes the ready interconversion of aspartate and oxaloacetate catalyzed by aspartate aminotransferase that has shown the ability to influence overall survival of metastatic melanoma patients (Cybulska-Stopa et al. 2012). Measurements of serum levels of aspartate aminotransferase and alanine aminotransferase are used to assay metastatic tumor burden (Alizadeh et al. 2003). Malate is an intermediate metabolite of TCA cycle for the generation of NADH which is then passed to the respiratory chain for ATP production (Chiu et al. 2007). Accumulation of malate observed in this study indicates an alteration of energy metabolism caused by metastatic melanoma in spleen. Since malate is a substrate of mitochondrial solute carrier family 25 member 10 (Slc25a 10), accumulation of malate in melanoma mice also suggest the suppression of Slc25a 10, which has been found to down-regulate fatty acid synthesis and lead to a reduction of lipids level (Mizuarai et al. 2005), a consistent result as observed in this study. Histamine is the product of histidine decarboxylation and, once generated, can be converted to several other breakdown products. Histidine catabolism begins with release of the α -amino group and ends up with glutamate being the ultimate product, making histidine one of the glucogenic amino acids (Frolkis et al. 2010). A recent research conducted by Noelia and coworkers has concluded the exhibition of antitumor effect of histamine *in vitro* and *in vivo* on human melanoma, suggesting histamine as a potential therapeutic compound for the treatment of malignant melanoma (Massari et al. 2013). Significant elevation of histamine in melanoma group observed in this study may be a result of up-regulation of histidine decarboxylase expression caused by metastatic melanoma in spleen, which is consistent with previous reports where B16F10 mouse melanoma cells show a rising level of histamine compared with normal melanocytes (Pos et al. 2005; Davis et al. 2011; Gruetter et al. 2009; Gruetter et al. 2008). Alanine is most commonly produced from pyruvate via alanine transaminase. So, elevation of alanine in melanoma mice may be due to perturbed expression of alanine transaminase the level of which is used to evaluate tissue damage and adverse events in preclinical and clinical researches (Bernardo et al. 2013; Sato et al. 2013). Enhanced production and excretion of alanine has been reported previously as a metabolic signature of melanoma (Filipp et al. 2012). dCTP, GTP and thymidine are involved in the biosynthesis of DNA and RNA in pyrimidine metabolism as depicted in Figure 4. Guanine is one of the most important purines, which, including substituted purines, are the most widely distributed kind of nitrogen-containing heterocycle in nature. By phosphorylation or phosphoribosylation, guanine is either converted to its corresponding nucleotides, i.e. 2'-Deoxyguanosine or GMP catalyzed by purine nucleoside phosphorylase and adenine phosphoribosyltransferase (or hypoxanthine-guanine phosphoribosyltransferase), respectively. Further phosphorylation by transferase enzymes will result in the conversion of GMP to GTP. Xanthine, another notable

purine, can be directly generated from guanine via the action of guanine deaminase (Frolkis et al. 2010). GTP not only participates in RNA polymerization but also regulates diverse critical aspects of cellular functioning, such as protein synthesis, activation of CTP synthetase and signal transduction, resulting in the profound impact on cell growth and division which may have been caused by a relatively small alteration of intracellular GTP level (Sikora-Borgula et al. 2002; Kizaki et al. 1980). An unbalanced dCTP/dTTP ratio may affect the fidelity of DNA replication (Pontarin et al. 2007). Increased level of thymidine may be attributed to thymidine kinase deficiency in melanoma bearing mice. A recent clinical study of malignant melanoma has shown a metastatic site dependent serum level of thymidine kinase 1 with adjacent metastases having significantly lower levels of thymidine kinase 1 than those distant metastases (Wu et al. 2013). Significantly increased level of dCTP, thymidine in pyrimidine metabolism and 2'-Deoxyguanosine, GTP, xanthine in purine metabolism to provide pyrimidine and purine substrates for DNA/RNA biosynthesis denote an altered nucleotide metabolism, i.e. enhanced pyrimidine and purine degradation, caused by metastatic melanoma in spleen, which plays an important role in malignant transformation, tumor cell differentiation and has been observed in many cancers (Hatse et al. 1999).

Based on the results from our metabolomics study of metastatic melanoma mice and the metabolite fates described in the Small Molecule Pathway Database (<http://www.smpdb.ca/>) as mentioned above, we propose the molecule pathways/networks (shown in Figure 4) that are affected by metastatic melanoma in spleen. As shown in the figure, metabolites pathways significantly affected by metastatic melanoma are pyrimidine metabolism, purine metabolism, lipids metabolism, nicotinate & nicotinamide metabolism, pyruvate metabolism, glutamate metabolism, histidine metabolism, taurine metabolism and TCA cycle. Therefore, our ¹H NMR metabolomics study clearly show that multiple biological pathways are affected by the metastatic melanoma in spleen.

4 Concluding Remarks

We have shown that the combined use of ¹H NMR metabolomics on tissue extracts and multivariate statistical analysis (i.e. PCA and OPLS) is a powerful tool for studying metastatic melanoma in mouse spleen. As many as 73 metabolites with estimated concentrations in spleen ranged from as low as 6 μ M to as high as 24.6 mM are identified in the hydrophilic extracts of spleen. Both PCA and OPLS show that the tumor group is well separated from the control group based on either the metabolites found from the hydrophilic extracts or on metabolites from the hydrophobic extracts. However, a better separation is obtained with OPLS. Spectral deconvolution is recommended for determination of discriminatory metabolites between the control and the tumor cell treated groups, especially for small sample sets. Melanoma causes obvious metabolic changes in mouse spleen tissue, resulting in statistically and significantly down regulated taurine, glutamate, aspartate, O-Phosphoethanolamine, niacinamide, ATP and linear lipids as well as glycerol derivatives, accompanied by elevation of alanine, malate, xanthine, histamine, dCTP, GTP, thymidine, 2'-Deoxyguanosine. These significantly changed metabolites are associated with multiple biological pathways (i.e. pyrimidine metabolism, purine metabolism, lipids metabolism, nicotinate & nicotinamide metabolism, pyruvate metabolism, glutamate metabolism,

histidine metabolism, taurine metabolism and TCA cycle) and may be potential biomarkers for metastatic melanoma in spleen.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. 600 MHz liquid state ^1H NMR metabolite spectra of the hydrophilic extracts of spleens excised from the control and the melanoma mice. The peak intensities were normalized to per unit weight of spleen tissue before extraction. In the plot, spectral region between 5.0 to 9.0 ppm is vertically expanded by 7 times while the 0.5 to 5 ppm spectral region is vertically expanded by one time. “*” indicates an unsigned peak. A total of 73 metabolites have been identified with metabolite numbers, i.e. the metabolite keys shown in Table 1

Fig. 2.

OPLS scores (left) and coefficients-coded loadings plot (right) of the model discriminating the control (blue dots) and the tumor (green dots) groups. (a) Using the data derived from binning results of ^1H NMR spectra from hydrophilic extracts. (b) Using the metabolites concentrations obtained by spectral deconvolution (only metabolites with correlation coefficients $|r| > 0.811$ are plotted, correlation coefficients of all metabolites used for OPLS analysis are listed in Table S2 in Supporting Information). (c) Using the data derived from binning results of ^1H NMR spectra from hydrophobic extracts. CV-ANOVA results gave p values of 0.046, 1.37×10^{-3} and 0.117 for models (a), (b) and (c), respectively. Regions are vertically expanded as denoted in the figure. For example, “×75” means the values of the loading are vertically expanded by 75 folds when compared with “×1” for the loading plots illustrated. The relationship between the metabolites keys in (a) and the names of the metabolites are given in Table 1. Peak assignments of hydrophobic extracts in (c) are listed in the caption of Figure 3

Fig. 3. 600 MHz ^1H NMR spectra of the hydrophobic extracts of mouse spleens from the control group (black line) and the tumor cell treated group (red line). For generating the stacked plot, the relative peak intensities of the spectra are normalized to per unit weight of spleen tissue before extraction so that the spectral peak intensities can be directly compared. Regions are vertically expanded as denoted in the figure. Peak assignments: 1, $\text{CH}_3\text{CH}_2\text{CH}_2\text{C}=\text{C}$; 2, $(\text{CH}_2)_n$, $\text{CH}_3\text{CH}_2(\text{CH}_2)_n$, $\text{CH}_2\text{CH}_2\text{CH}_2\text{CO}$, CH_2 ; 3, $\text{CH}_2\text{CH}_2\text{C}=\text{C}$; 4, $\text{CH}_2\text{C}=\text{C}$; 5, CH_2CO ; 6, $\text{C}=\text{CHCH}_2\text{CH}=\text{C}$; 7, CH_2OCOR (Glycerol of lipids); 8, $-\text{CH}=\text{CHCH}_2\text{CH}=\text{CH}-$ (Unsaturated lipid), $=\text{CHCH}_2\text{CH}_2$; 9, $=\text{CHCH}_2\text{CH}_2$ (Unsaturated lipid)

Fig. 4.

Proposed molecular pathway networks associated with the significantly altered metabolites during melanoma development based on the findings from this work and the diverse metabolic fates depicted in the Small Molecule Pathway Database (SMPDB) (<http://www.smpdb.ca/>). Solid line indicates one-step process, while dash line indicates multi-steps process. Metabolites colored green are not detected. Molecular pathways under the numbered shadow squares are parts of metabolism networks of particular metabolites: ① pyrimidine metabolism, ② purine metabolism, ③ pyruvate metabolism, ④ glutamate metabolism. Other significantly affected metabolites pathways are histidine metabolism, lipids metabolism, nicotinate & nicotinamide metabolism, taurine metabolism and TCA cycle

Table 1

Peak assignments and average concentrations of Metabolites identified from hydrophilic tissue extracts

Key	Metabolites	$\delta^1\text{H}$ (ppm) and multiplicity	Concentration ($\mu\text{g}/\text{mg}$) Mean $\pm$ SD		Estimates of absolute concentrations in tissue (mM) Mean $\pm$ SD	
			Tumor	Control	Tumor	Control
1	Isoleucine	0.94(t), 0.99(d), 1.27(m), 1.46(m), 1.95(m), 3.64(d)	4.74 $\pm$ 2.21	2.79 $\pm$ 0.53	2.99 $\pm$ 1.40	1.76 $\pm$ 0.33
2	Alloisoleucine	0.96(d), 0.97(t), 1.37(m), 1.43(m), 2.05(m), 3.73(d)	2.35 $\pm$ 0.97	1.83 $\pm$ 0.43	1.48 $\pm$ 0.61	1.16 $\pm$ 0.27
3	Leucine	0.96(d), 0.97(d), 1.67(m), 1.71(m), 1.73(m), 3.74(t)	1.97 $\pm$ 0.62	1.07 $\pm$ 0.23	1.24 $\pm$ 0.39	0.68 $\pm$ 0.15
4	2-Aminobutyrate	0.96(t), 1.91(m), 3.68(t)	1.15 $\pm$ 0.35	0.70 $\pm$ 0.14	0.73 $\pm$ 0.22	0.44 $\pm$ 0.09
5	Valine	0.97(d), 1.02(d), 2.27(m), 3.6(d)	2.47 $\pm$ 1.16	1.52 $\pm$ 0.29	1.56 $\pm$ 0.73	0.96 $\pm$ 0.18
6	Isobutyrate	1.05(d), 2.38(m)	2.05 $\pm$ 0.96	1.17 $\pm$ 0.23	1.29 $\pm$ 0.61	0.74 $\pm$ 0.15
7	3-Hydroxyisobutyrate	1.08(d), 2.48(m), 3.53(m), 3.69(m)	0.07 $\pm$ 0.02	0.06 $\pm$ 0.03	0.04 $\pm$ 0.01	0.04 $\pm$ 0.02
8	Ethanol	1.19(t), 3.66(q)	2.90 $\pm$ 1.17	2.66 $\pm$ 1.77	1.83 $\pm$ 0.74	1.68 $\pm$ 1.12
9	3-Hydroxybutyrate	1.2(d), 2.3(dd), 2.39(dd), 4.14(dd) 1.2(m), 3.4(dd), 3.63(dd),	0.17 $\pm$ 0.05	0.2 $\pm$ 0.03	0.11 $\pm$ 0.03	0.13 $\pm$ 0.02
10	Fucose	3.8(m), 4(m), 4.19(m), 4.55(d), 5.22(m)	0.28 $\pm$ 0.15	0.24 $\pm$ 0.02	0.18 $\pm$ 0.09	0.15 $\pm$ 0.01
11	Lactate	1.33(d), 4.11(q)	17.04 $\pm$ 2.31	14.42 $\pm$ 3.49	10.76 $\pm$ 1.46	9.11 $\pm$ 2.20
12	Threonine	1.33(d), 3.58(d), 4.26(m) 1.43(m), 1.51(m), 1.72(m),	4.07 $\pm$ 1.54	2.35 $\pm$ 0.58	2.57 $\pm$ 0.97	1.48 $\pm$ 0.37
13	Lysine	1.89(m), 1.91(m), 3.03(t), 3.75(t),	5.16 $\pm$ 2.19	3.04 $\pm$ 0.67	3.26 $\pm$ 1.38	1.92 $\pm$ 0.42
14	Alanine	1.48(d), 3.78(q)	11.38 $\pm$ 2.62	6.22 $\pm$ 1.17	7.19 $\pm$ 1.65	3.93 $\pm$ 0.74
15	Arginine	1.65(m), 1.74(m), 1.9(m), 1.92(m), 3.23(t), 3.77(t), 1.87(s), 2.37(dd), 3.76(dd),	4.24 $\pm$ 1.81	3.62 $\pm$ 0.69	2.68 $\pm$ 1.14	2.29 $\pm$ 0.44
16	Thymidine	3.84(dd), 4.01(m), 4.46(dt), 6.29(t), 7.66(s)	0.09 $\pm$ 0.03	0.04 $\pm$ 0.01	0.06 $\pm$ 0.02	0.03 $\pm$ 0.01
17	Acetate	1.92(s) 1.92(s), 2.38(m), 4.19(m),	1.35 $\pm$ 0.61	1.27 $\pm$ 0.49	0.85 $\pm$ 0.39	0.80 $\pm$ 0.31
18	dTTP [#]	4.22(m), 4.62(m), 4.74(m), 6.34(t), 7.69(s)	0.03 $\pm$ 0.01	0.02 $\pm$ 0.01	0.02 $\pm$ 0.01	0.01 $\pm$ 0.01
19	Glutamate	2.05(m), 2.14(m), 2.34(m), 2.37(m), 3.76(dd)	9.46 $\pm$ 0.91	13.72 $\pm$ 1.31	5.97 $\pm$ 0.57	8.67 $\pm$ 0.83
20	Glutamine	2.1(m), 2.15(m), 2.44(m), 2.47(m), 3.77(t), 6.83(s),	5.38 $\pm$ 1.92	4.57 $\pm$ 0.76	3.40 $\pm$ 1.21	2.89 $\pm$ 0.48
21	Methionine	2.11(m), 2.14(s), 2.2(m), 2.65(t), 3.86(dd) 2.16(m), 2.18(m), 2.52(m),	2.01 $\pm$ 0.71	1.44 $\pm$ 0.28	1.27 $\pm$ 0.45	0.91 $\pm$ 0.18
22	Glutathione	2.57(m), 2.95(dd), 2.98(dd), 3.74(d), 3.77(m), 4.58(m) 2.3(m), 2.41(m), 4.2(m),	2.50 $\pm$ 0.55	1.65 $\pm$ 0.23	1.58 $\pm$ 0.35	1.04 $\pm$ 0.15
23	dCTP	4.58(m), 4.73(m), 6.13(d), 6.31(t), 7.86(d)	0.15 $\pm$ 0.04	0.06 $\pm$ 0.01	0.09 $\pm$ 0.03	0.04 $\pm$ 0.01
24	Malate	2.36(dd), 2.67(dd), 4.3(m)	2.47 $\pm$ 0.29	1.57 $\pm$ 0.15	1.56 $\pm$ 0.18	0.99 $\pm$ 0.09

Key	Metabolites	$\delta^1\text{H}$ (ppm) and multiplicity	Concentration (μmg) Mean $\pm$ SD		Estimates of absolute concentrations in tissue (mM) Mean $\pm$ SD	
			Tumor	Control	Tumor	Control
25	2-Oxoglutarate	2.44(t), 2.99(t)	0.71 $\pm$ 0.37	0.38 $\pm$ 0.07	0.45 $\pm$ 0.23	0.24 $\pm$ 0.04
26	Isocitrate	2.49(m), 2.57(m), 2.99(m), 4.06(d)	3.51 $\pm$ 1.10	4.44 $\pm$ 0.79	2.22 $\pm$ 0.69	2.80 $\pm$ 0.50
27	2'-Deoxyguanosine	2.5(m), 2.78(m), 3.75(dd), 3.81(dd), 4.12(m), 4.61(m), 6.31(t), 7.98(s)	0.04 $\pm$ 0.01	0.01 $\pm$	0.03 $\pm$ 0.01 0.005	0.01 $\pm$ 0.003
28	Citrate	2.54(d), 2.68(d)	0.57 $\pm$ 0.13	0.42 $\pm$ 0.07	0.36 $\pm$ 0.08	0.27 $\pm$ 0.04
29	Aspartate	2.67(dd), 2.82(dd), 3.9(dd)	6.89 $\pm$ 0.80	9.75 $\pm$ 1.16	4.35 $\pm$ 0.51	6.16 $\pm$ 0.73
30	Asparagine	2.85(dd), 2.95(dd), 4(dd)	3.53 $\pm$ 1.09	1.77 $\pm$ 0.35	2.23 $\pm$ 0.69	1.12 $\pm$ 0.22
31	Trimethylamine	2.88(s)	0.03 $\pm$ 0.02	0.01 $\pm$ 0.01	0.02 $\pm$ 0.01	0.01 $\pm$ 0.01
32	Tyramine	2.92(t), 3.23(t), 6.9(m), 7.2(m)	0.22 $\pm$ 0.13	0.16 $\pm$ 0.04	0.14 $\pm$ 0.08	0.10 $\pm$ 0.03
33	Histamine	3(t), 3.29(t), 6.8(s), 7.97(s)	0.22 $\pm$ 0.02	0.12 $\pm$ 0.02	0.14 $\pm$ 0.01	0.08 $\pm$ 0.01
34	Creatine phosphate	3.01(s), 3.94(s)	0.60 $\pm$ 0.29	0.30 $\pm$ 0.16	0.38 $\pm$ 0.18	0.19 $\pm$ 0.10
35	Creatinine	3.04(s), 3.98(s)	0.40 $\pm$ 0.17	0.56 $\pm$ 0.08	0.25 $\pm$ 0.11	0.35 $\pm$ 0.05
36	Creatine	3.04(s), 3.91(s)	0.32 $\pm$ 0.10	0.43 $\pm$ 0.10	0.20 $\pm$ 0.06	0.27 $\pm$ 0.06
37	Tyrosine	3.06(dd), 3.2(dd), 3.93(dd), 6.9(m), 7.2(m)	2.51 $\pm$ 1.20	1.69 $\pm$ 0.41	1.59 $\pm$ 0.76	1.07 $\pm$ 0.26
38	Phenylalanine	3.11(dd), 3.27(dd), 3.99(dd), 7.33(m), 7.38(m), 7.43(m)	2.19 $\pm$ 0.90	1.28 $\pm$ 0.22	1.38 $\pm$ 0.57	0.81 $\pm$ 0.14
39	Histidine	3.14(dd), 3.24(dd), 3.98(dd), 7.06(s), 7.79(s)	0.75 $\pm$ 0.39	0.40 $\pm$ 0.10	0.47 $\pm$ 0.25	0.25 $\pm$ 0.06
40	Ethanolamine	3.14(m), 3.82(m)	1.44 $\pm$ 0.75	1.15 $\pm$ 0.26	0.91 $\pm$ 0.47	0.73 $\pm$ 0.16
41	Choline	3.21(s), 3.52(m), 4.07(m)	1.98 $\pm$ 1.27	1.19 $\pm$ 0.28	1.25 $\pm$ 0.80	0.75 $\pm$ 0.18
42	O-Phosphocholine O-Phosphoethanolamine	3.21(s), 3.57(m), 4.17(m)	0.74 $\pm$ 0.31	0.62 $\pm$ 0.24	0.47 $\pm$ 0.20	0.39 $\pm$ 0.15
43		3.22(m), 3.98(m)	4.77 $\pm$ 0.60	9.18 $\pm$ 0.89	3.01 $\pm$ 0.38	5.80 $\pm$ 0.56
44	sn-Glycero-3-phosphocholine	3.23(s), 3.6(dd), 3.67(m), 3.68(dd), 3.86(m), 3.92(m), 3.95(m), 4.32(m)	4.47 $\pm$ 3.84	4.60 $\pm$ 0.49	2.82 $\pm$ 2.43	2.91 $\pm$ 0.31
45	n-Methylhistidine	3.22(dd), 3.3(dd), 3.73(s), 3.95(dd), 6.8(s), 8(s)	0.16 $\pm$ 0.05	0.07 $\pm$ 0.03	0.10 $\pm$ 0.03	0.04 $\pm$ 0.02
46	Trimethylamine N-oxide	3.23(s)	0.67 $\pm$ 0.47	0.52 $\pm$ 0.35	0.42 $\pm$ 0.30	0.33 $\pm$ 0.22
47	Glucose	3.23(m), 3.4(m), 3.5(m), 3.53(dd), 3.7(dd), 3.72(dd), 3.78(m), 3.83(m), 3.84(m), 3.94(dd), 4.65(d), 5.23(d)	5.20 $\pm$ 0.77	4.86 $\pm$ 1.16	3.28 $\pm$ 0.49	3.07 $\pm$ 0.73
48	Taurine	3.26(t), 3.43(t)	28.11 $\pm$ 2.94	38.87 $\pm$ 3.91	17.75 $\pm$ 1.86	24.55 $\pm$ 2.47
49	Betaine	3.27(s), 3.91(s)	0.52 $\pm$ 0.25	0.32 $\pm$ 0.10	0.33 $\pm$ 0.16	0.20 $\pm$ 0.06
50	myo-Inositol	3.28(t), 3.53(dd), 3.62(t), 4.06(m) 3.3(dd), 3.48(dd), 4.05(dd),	3.78 $\pm$ 2.02	3.96 $\pm$ 0.65	2.39 $\pm$ 1.28	2.50 $\pm$ 0.41
51	Tryptophan	7.2(dd), 7.29(dd), 7.32(s), 7.54(d), 7.74(d),	0.40 $\pm$ 0.19	0.26 $\pm$ 0.04	0.25 $\pm$ 0.12	0.16 $\pm$ 0.03
52	Methanol	3.36(s) 3.51(dd), 3.58(m), 3.78(dd),				
53	UDP-glucuronate	4.13(d), 4.17(m), 4.23(m),	0.10 $\pm$ 0.07	0.09 $\pm$ 0.07	0.06 $\pm$ 0.04	0.06 $\pm$ 0.04

Key	Metabolites	$\delta^1\text{H}$ (ppm) and multiplicity	Concentration (μmg) Mean $\pm$ SD		Estimates of absolute concentrations in tissue (mM) Mean $\pm$ SD	
			Tumor	Control	Tumor	Control
		4.27(m), 4.36(m), 5.61(dd), 5.97(d), 5.99(d), 7.97(d)				
54	Glycine	3.56(s)	12.08 $\pm$ 3.01	6.81 $\pm$ 1.66	7.63 $\pm$ 1.90	4.30 $\pm$ 1.05
55	Glycerol	3.56(dd), 3.65(dd), 3.78(m) 3.72(dd), 3.75(dd), 3.81(m), 3.91(dd), 4(d), 4.16(m),	0.91 $\pm$ 0.63	1.18 $\pm$ 0.43	0.57 $\pm$ 0.40	0.75 $\pm$ 0.27
56	UDP-galactose	4.19(m), 4.24(m), 4.28(m), 4.37(m), 5.63(dd), 5.98(m), 7.95(d) 3.8(dd), 3.89(dd), 4.13(m),	0.11 $\pm$ 0.05	0.10 $\pm$ 0.08	0.07 $\pm$ 0.03	0.06 $\pm$ 0.05
57	Uridine	4.23(t), 4.36(t), 5.9(d), 5.92(d), 7.88(d), 3.81(dd), 3.92(dd), 4.12(m),	0.97 $\pm$ 0.15	0.90 $\pm$ 0.12	0.61 $\pm$ 0.09	0.57 $\pm$ 0.08
58	Cytidine	4.19(t), 4.3(m), 5.89(d), 6.06(d), 7.81(d)	0.37 $\pm$ 0.33	0.49 $\pm$ 0.20	0.23 $\pm$ 0.21	0.31 $\pm$ 0.13
59	Adenosine	3.83(dd), 3.91(dd), 4.29(m), 4.42(dd), 4.79(dd), 6.05(d), 8.24(s), 8.34(s) 3.84(dd), 3.92(dd), 4.27(m),	0.02 $\pm$ 0.01	0.02 $\pm$ 0.01	0.01 $\pm$ 0.006	0.01 $\pm$ 0.006
60	Inosine	4.44(m), 4.76(t), 6.11(d), 8.23(s), 8.35(s)	0.50 $\pm$ 0.33	0.62 $\pm$ 0.39	0.32 $\pm$ 0.21	0.39 $\pm$ 0.25
61	Serine	3.84(dd), 3.95(dd), 3.99(dd)	7.27 $\pm$ 4.13	3.23 $\pm$ 0.81	4.59 $\pm$ 2.61	2.04 $\pm$ 0.51
62	AMP	4.01(m), 4.36(m), 4.49(m), 4.78(dd), 6.11(d), 8.24(s), 8.62(s) 4.2(m), 4.4(m), 4.57(dd),	0.04 $\pm$ 0.01	0.01 $\pm$ 0.01	0.03 $\pm$ 0.006	0.006 $\pm$ 0.006
63	ATP	4.74(dd), 6.15(d), 8.23(s), 8.38(s) 4.2(m), 4.37(m), 4.57(dd),	0.08 $\pm$ 0.02	0.16 $\pm$ 0.02	0.05 $\pm$ 0.01	0.10 $\pm$ 0.01
64	ADP	4.74(dd), 6.13(dd), 8.27(s), 8.58(s)	0.05 $\pm$ 0.03	0.06 $\pm$ 0.02	0.03 $\pm$ 0.02	0.04 $\pm$ 0.01
65	GTP	4.24(m), 4.35(m), 4.55(m), 4.74(dd), 5.94(d), 8.12(s)	0.04 $\pm$ 0.01	0.01 $\pm$ 0.003	0.03 $\pm$ 0.006	0.006 $\pm$ 0.002
66	Uracil	5.8(d), 7.53(d)	0.89 $\pm$ 0.52	0.37 $\pm$ 0.11	0.56 $\pm$ 0.33	0.23 $\pm$ 0.07
67	Fumarate	6.52(s)	0.11 $\pm$ 0.02	0.08 $\pm$ 0.01	0.07 $\pm$ 0.01	0.05 $\pm$ 0.006
68	Benzoate	7.48(m), 7.55(m), 7.86(m)	0.04 $\pm$ 0.02	0.05 $\pm$ 0.01	0.03 $\pm$ 0.01	0.03 $\pm$ 0.006
69	Niacinamide	7.6(dd), 8.26(m), 8.71(m), 8.94(d)	0.30 $\pm$ 0.02	0.39 $\pm$ 0.02	0.19 $\pm$ 0.01	0.25 $\pm$ 0.01
70	Xanthine	7.91(s)	0.46 $\pm$ 0.11	0.08 $\pm$ 0.04	0.29 $\pm$ 0.07	0.05 $\pm$ 0.03
71	Hypoxanthine	8.19(s), 8.21(s)	1.46 $\pm$ 0.36	1.46 $\pm$ 0.26	0.92 $\pm$ 0.23	0.92 $\pm$ 0.16
72	Oxypurinol	8.19(s)	8.92 $\pm$ 0.67	8.97 $\pm$ 1.74	5.63 $\pm$ 0.42	5.67 $\pm$ 1.10
73	Formate	8.46(s)	0.61 $\pm$ 0.28	0.84 $\pm$ 0.28	0.39 $\pm$ 0.18	0.53 $\pm$ 0.18

* s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; dd, doublet of doublet

Abbreviations: dTTP, Deoxythymidine triphosphate; dCTP, Deoxycytidine triphosphate; UDP-glucuronate, Uridine diphosphate glucuronate; UDP-galactose, Uridine diphosphate galactose; AMP, Adenosine monophosphate; ATP, Adenosine triphosphate; ADP, Adenosine diphosphate; GTP, Guanosine-5'-triphosphate

Table 2

Melanoma induced metabolic changes in spleen tissue extracts

Key	Metabolites	Correlation coefficients
14	Alanine	0.817
16	Thymidine	0.832
19	Glutamate	−0.911
23	dCTP	0.874
24	Malate	0.897
27	2'-Deoxyguanosine	0.854
29	Aspartate	−0.870
33	Histamine	0.936
43	O-Phosphoethanolamine	−0.967
48	Taurine	−0.892
63	ATP	−0.936
65	GTP	0.924
69	Niacinamide	−0.925
70	Xanthine	0.931