An NMR metabolomics approach for the diagnosis of leptomeningeal carcinomatosis

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Abbreviation: LC, Leptomeningeal carcinomatosis; CSF, cerebrospinal fluid; HR-MAS, high resolution magic angle spinning; OPLS-DA, orthogonal projections to latent structure-discriminant analysis;
Abstract

Leptomeningeal carcinomatosis (LC) is the third most common metastatic complication of the central nervous system. However, the current modalities to reliably diagnose this condition are not satisfactory. Here we report a preclinical proof of concept for a metabolomics-based diagnostic strategy, using a rat LC model incorporating glioma cells that stably expressed green fluorescent protein. Cytological diagnoses gave 66.7% sensitivity for the 7-day LC group and 0% for the 3-day LC group. MR imaging could not diagnose LC at these stages. In contrast, NMR-based metabolomics on cerebrospinal fluid (CSF) detected marked differences between the normal and LC groups. Predictions based on the multivariate model provided sensitivity, specificity and overall accuracy of 88-89% in both groups for LC diagnosis. Further statistical analyses identified lactate, acetate, and creatine as specific for the 7-day LC group, with glucose a specific marker of the normal group.

Overall, we demonstrated that the metabolomics approach provided both earlier and more accurate diagnostic results than cytology and MR imaging in current use.
Introduction

Leptomeningeal carcinomatosis (LC) is a disorder caused by the seeding of the leptomeninges, the pia, arachnoid and cerebrospinal fluid (CSF) within the subarachnoid space, by malignant cells. This disorder is the third most common metastatic complication of the central nervous system (CNS) and increasingly common in cancer patients (1, 2). Although LC most often presents in patients with widely disseminated and progressive systemic cancer (>70%), it can present after a disease-free interval (20%) and even be the first manifestation of cancer (5–10%), occasionally occurring even in the absence of other evidence of systemic disease (3). For patients diagnosed with LC, the median survival time of untreated patients is known to be 4 - 6 weeks, which can extend to 3 - 6 months with treatment (4, 5). Treatment can also improve or stabilize a patient’s neurological status and maintain their neurological quality of life (3). Thus, an early diagnosis of LC is important in order to alert the oncologist to begin therapy prior to neurologic deterioration.

The most useful laboratory test in the diagnosis of LC has been the examination of CSF obtained from lumbar puncture (6-8). However, abnormalities in the CSF, though suggestive of LC, are not diagnostic. The presence of malignant cells in the CSF detected in a cytological examination is diagnostic of LC. However, the assignment to a particular tumor is often not possible, which is the true for most cytological analyses (6-8). Of the patients with final results that reveal positive CSF cytology, up to 45% will be cytologically negative upon initial examination (6, 7, 9). The yield increases to 80% with a second round of CSF examination, but there is little benefit for further rounds of examination (6, 7, 9). The low sensitivity of CSF cytology makes it difficult not only to diagnose leptomeningeal metastasis but also to assess the patient’s response to treatment (2). Biochemical markers, immunohistochemistry and molecular biology techniques have been applied to CSF in an attempt to find reliable biological markers of the disease. Numerous biochemical markers have been evaluated, but their use has been generally limited by poor sensitivity and specificity (3). Therefore, it is important to develop a new method that improves the
diagnostic sensitivity of LC, which can enhance the evaluation of prognosis and reduce unnecessary medical procedures.

While genomic and proteomic approaches focus on upstream genetic and protein variations, metabolomics is increasingly being used to understand the global metabolic changes that occur in response to alterations in nutrition, genetics and environment (10, 11), which can complement the information obtained from genomics and proteomics. In addition, metabolomics has been used to identify metabolite-based biomarkers for a variety of diseases conditions (see (12) for a recent review). Metabolic profiles can be investigated using high-throughput analytical tools, such as nuclear magnetic resonance (1H-NMR) spectroscopy. 1H-NMR spectroscopy of biofluids such as urine, serum, and bile can give comprehensive small molecular profiles of metabolites that are regulated by various physiological signals involved in maintaining homeostasis. Homeostasis can be disrupted by changes in a patient’s physiological condition, resulting in the perturbation of metabolite levels. Thus, monitoring these perturbations can provide not only novel information of the patient’s physiological condition but also new biomarkers for disease diagnosis.

Because the metabolite composition of CSF is directly related to the biological processes of the brain, profiling CSF metabolites can provide important information for diagnosing various brain abnormalities, such as brain injury (13), Huntington’s disease (14) and Parkinson’s disease (15). Himmelreich et al. (16) demonstrated a practical application of rapidly identifying the bacterial species in bacterial meningitis based on CSF metabolite profiles, which may help appropriate selection of antibiotics in a clinical setting. With the recent report by Wishart et al. (17) detecting approximately 70 CSF metabolites and building a public database, we expect CSF metabolomics studies to be applied to a wider variety of systems.

The purpose of our study was to develop a new NMR spectroscopic method that could be used to improve the diagnosis of LC. We first designed a rat LC model with glioma cells stably-expressing GFP and used NMR-based metabolomics to characterize CSF metabolites. As a result, we were able to obtain
earlier and significantly improved diagnostic results compared to currently-used cytological or radiological approaches.
Materials and Methods

This experiment was approved by the animal care committee at Seoul National University Hospital.

Rat LC model and sham operation

To produce LC rats, GFP-expressing F-98 cells (SI Materials and Methods and Fig. S1) were prepared in 50 μL of serum free DMEM and intradurally transplanted into the parietal area of 6-week old Fisher 344 rats (1 x 10⁶ cells / rat) (SI Materials and Methods). For the sham operation, rats (n = 4) were intradurally injected with 50 μL of serum free DMEM. In vivo MR imaging and CSF collection (SI Materials and Methods) were performed 3 (n = 8; 3-day LC group) and 7 days (n = 9; 7-day LC group) after cell implantation. Rats in the sham group also underwent in vivo MR imaging 3 days after the operation.

NMR spectroscopy data acquisition

All NMR spectra were acquired at 19 °C with a 500 MHz NMR spectrometer (Agilent, VNMRS 500 system, 11.7 T) equipped with an HR-MAS nano-probe® (Agilent, Walnut Creek, CA). A CSF sample (40 μL) was placed into the probe with inverse-detection and single Z-gradient capability. The receiver gain was adjusted carefully not to cause signal overflow. The sample was spun at 5 KHz by a stream of nitrogen gas with 54.7° (the magic angle) relative to the applied magnetic field. The spectra were acquired with a total number of complex points of 16 K, sweep width of 7961 Hz, and 1024 transients. The 90 degree pulse was calibrated with each sample on the water resonance. The water signal was saturated using weak power continuous wave during the recycle delay.

Data processing

The time-domain spectra were apodized with an exponential function (1 Hz), Fourier transformed,
phased and manually baseline-corrected. The signals were referenced to the TSP signal at 0.00 ppm and normalized against the total integration values. Water and ethanol peaks were removed to prevent artifacts in downstream analyses. To reduce the complexity of the NMR data for pattern recognition, the spectra were binned at every 0.0025 ppm interval. The normalization and binning were performed using an in-house built Perl program.

**Statistical analysis**

Statistical analysis was performed with R (from R Project for Statistical Computing) and SIMCA-P 11.0 (Umetrics, Sweden). Chenomx (Spectral database; Edmonton, Alberta, Canada) and an in-house built database were used for metabolite identification. Partial least square regression and OPLS-DA (See supplementary information S2) were performed to identify latent patterns and compare the overall metabolite profile. Class discrimination models were built until the cross-validated predictability value did not significantly increase to avoid over-fitting of the statistical model. Diagnostic performance was obtained by prediction of one left-out sample based on the distinction model constructed with the rest of the samples. The method was repeated until all the samples were left out once. An *a priori* cut-off value of 0.5 was used to evaluate the prediction results (18). Signals specific for each group were identified by performing the Wilcoxon rank sum test on all the ppm variables using the in-house written R script.
Results

CSF cytology, MR imaging, and histology

Among the 9 CSF samples obtained 7 days after F-98 cell implantation (7-day LC), 6 (66.7%) were found to have malignant cells in the CSF cytological examination as evidenced by the expression of green fluorescent protein (GFP) (Fig. 1). In contrast, no samples collected 3 days after F-98 cell implantation (3-day LC) exhibited positive CSF cytology. MR imaging revealed meningeal enhancement in all 17 rats that underwent glioma cell implantation (Fig. 2A and 2B), but it also showed the enhancement in the 4 rats from the sham group (Fig. 2C). All implanted rats (n = 17) had F-98 cells infiltrating the leptomeninges as evidenced by GFP expression, which confirmed the proper establishment of LC in both 7-day (Fig. 3A) and 3-day (Fig. 3B) groups. Still, leptomeningeal infiltration of F-98 cells was more prominent in 7-day LC rats than in 3-day LC rats, suggesting that 3-day LC group is in an earlier phase of the LC development. Overall, among the 17 histology-confirmed LC rats, 6 in the 7-day LC group could be diagnosed with cytology, and MR imaging was not useful due to the false positive effects.

HR-MAS MR spectra and multivariate analysis

We acquired the NMR data with a high resolution magic angle spinning (HR-MAS) probe because the CSF volume obtained from a rat (typically ~ 40 μL) is far smaller than required for usual 5 mm (~ 500 μL). HR-MAS MR spectra of the 26 CSF samples (9, 8 and 9 samples for normal, 3-day LC, and 7-day LC rats, respectively) from the animal model gave reasonable signals, even though the sample volumes were less than one tenth of that used for 5 mm NMR probe (Fig. 4 and Supplementary Fig. S3). Chemical shifts and scalar coupling values were used to identify a number of metabolites known to be present in CSF, such as lactate (1.35 and 4.12 ppm) and glucose (multiple signals between 3 - 4 ppm). As the intra-group variation hindered the assignment of the spectral characteristics of each group by simple visual
inspection, we applied a more holistic approach of multivariate statistical analysis to the entire NMR data set. First, partial least square (9) regression analysis was performed with all 3 groups to determine the differences with time (Fig. 5A). Our results demonstrate that spectral features move toward the right side along the first component axis as time increases. The metabolite profiles of 7-day LC rat samples were quite different from those of normal rats. In comparison, the profiles of 3-day LC rats were between the normal and 7-day LC rat samples in the direction of the first component. Still, the directional changes in the second component were opposite between the 3-day and 7-day LC rat samples, suggesting that the overall metabolite profiles may not change in a simple linear fashion with time.

To maximize the difference between a given pair and obtain quantitative information about the diagnostic performance at each time point, orthogonal projections to latent structure-discriminant analysis (OPLS-DA) modeling was performed for samples on normal vs. 3-day or 7-day LC rats. Both pairs yielded differentiation models with 1 predictive and 2 orthogonal components (Fig. 5B and 5C). The models featured 0.965 (0.963) of goodness of fit and 0.759 (0.717) of predictive values for normal vs. 3-day LC rats (normal vs. 7-day LC rats) differentiation. Both 3-day and 7-day LC rat samples were well separated from normal rat samples, suggesting that relatively early metabolic changes at day 3 may be sufficient to distinguish these samples from those of normal rats.

**Diagnostic predictions**

Good statistical characteristics of a differentiation model do not necessarily translate into good practical performance. Therefore, we performed a diagnostic prediction test with the established differentiation models. We left out one CSF sample at a time, constructed a new model with the remaining samples, and predicted the disease status of the left-out sample. The procedure was repeated until all of the CSF samples were tested once, with a diagnosis based on *a priori* cut-off value of 0.5. The test was performed on both 3-day (Fig. 6A) and 7-day LC samples (Fig. 6B) against normal rat samples. The
prediction results showed that the models correctly predicted 16 samples out of a total of 18 for both groups. One identical normal sample (from case 3) was mispredicted as LC samples, and 1 LC sample from each of the 3-day and 7-day LC groups was mispredicted as normal. Thus, our metabolomics model showed sensitivities of 89 (7-day LC group) or 88% (3-day LC group), a specificity of 89%, and an overall accuracy of 89% in the prediction of LC. These results suggest that this method could be useful for both relatively early (day 3) and full-stage (day 7) LC diagnosis.

Analysis of marker signals

To identify the metabolites that are characteristic of LC, we performed a Wilcoxon-rank sum test on all ppm variables (Fig. 7A). Although we could not identify all of the significant signals (blue dots) due to the relatively low signal-to-noise ratio and overlaps, we were able to identify glucose (3.40, 3.48, 3.73, and 3.83 ppm) as being specific for the normal group and lactate (1.35 and 4.12 ppm), acetate (1.93 ppm), and creatine (3.05 and 3.93 ppm) for the 7-day LC group by database analysis and comparisons with standard compounds (Fig. 7B; See supplementary information S4). We also tried correlating the NMR spectral markers with the thickness of the LC infiltration in the 7 day-LC animals. We built a partial least square regression model between the levels of the four markers as independent variables and the thickness as a dependent variable. The regression result gave good correlation with R² value of 0.95 between the actual values and regression–predicted values (Supplementary Fig. S5). The number and intensities of significant signals were smaller for the analysis of normal vs. 3-day LC rat samples, and reliable identification of metabolites was difficult for 3-day LC rat samples (Fig. 7A lower panel). Therefore, the distinction between normal and 3-day LC rat samples should be due to the sum of small and unassignable signals rather than several conspicuous metabolite signals. Additionally, the pattern of the significant signals of 3-day and 7-day LC rat samples appeared to be different.
Discussion

Metabolomics is a global assessment of endogenous metabolites within a biological system. This assessment is gaining increased attention for its role in diagnosing cancer (19), refining preoperative differential diagnosis, detecting tumor progression and monitoring responses to treatment (20). Magnetic resonance spectroscopy (MRS) is a method that detects metabolites within cells, tissues or biofluids and has been used in medical fields along with MR imaging. Notably, proton MRS has been used for the detection of brain, breast and prostate cancers, which led to the discovery of choline, lactate and amino acid peaks as biomarkers for cancer. Analogously, in vitro NMR spectroscopy of fine-needle aspiration biopsy samples of primary tumors exhibited 93% accuracy in differentiating between benign and malignant samples by the measurement of choline peaks (21, 22). With these successful applications of NMR-based metabolomics to the medical field, we hypothesized that LC could also be diagnosed using the different metabolite profiles in CSF measured by in vitro NMR spectroscopy. In the present study, we used a rat LC model using F-98 glioma cells expressing GFP to enhance the detectability of cancer cells in cytology and histopathology.

We applied the NMR spectroscopic method using an HR-MAS probe for the diagnosis of LC in a rat model. The approach showed a sensitivity and specificity of 89% and an overall accuracy of 89% for the 7-day LC model. In the 7-day LC group, we observed increases in lactate, creatine and acetate levels and decreases in glucose levels compared to those of the normal group (P < 0.006 for all). Although we could not reliably identify marker signals in the model of an earlier stage (3-day LC rats), we were able to obtain similar diagnostic performance at day 3. This good performance constitutes the most significant results of our study because they were significantly better than those obtained using other currently available modalities on the same animals. For example, cytology, which is often used to confirm the presence of LC, failed to detect any glioma cells on day 3 and detected cancer cells in significantly less samples (66.7%) at a later stage (day 7). In addition, although in vivo MR imaging detected meningeal
enhancement in LC model rats in both early and later stage groups (3-day and 7-day LC rats), enhancement in the sham operation group was also detected. Therefore, radiological enhancement is non-specific and in vivo MR imaging has very low specificity, which may limit their use.

In many tumor tissues, more than 50% of cellular adenosine triphosphate (ATP) can be generated from glycolysis, even in the presence of oxygen, which led to the term aerobic glycolysis (or “Warburg Effect”) (23). By using aerobic glycolysis, even in the presence of oxygen and functional mitochondria, tumor cells divert a large portion of pyruvate to extra-mitochondrial breakdown to lactate, the typical product of anaerobic glycolysis (24). In vivo MRS of the brain showed increased aerobic glycolysis in gliomas, which was measured as increases in lactate levels and correlate to the grade of gliomas (25). Thus, we believe that the observed increase in lactate levels and decrease in glucose levels can be explained by the aerobic glycolysis of disseminated cancer cells in CSF.

In terms of the elevated levels of acetate or creatine in the CSF of 7-day LC rats, similar observations have been made in another malignancy, which were performed using in vitro NMR spectroscopy. Recently, Imperiale et al. (26) reported the metabolic content of intact biopsy samples of adrenal neuroblastomas using HR-MAS NMR spectroscopy, which exhibited higher levels of creatine, glutamine/glutamate, acetate and glycine in neuroblastomas. They also showed that acetate and creatine are characteristic of stage IV neuroblastomas, which led to the conclusion that high levels of acetate and creatine in tumors could be used in diagnosis and staging. High acetate concentrations in tumors are known to be mainly due to the enhanced lipid synthesis in the generation of cell membranes, which reflects the high growth activity of neoplasm (27). Thus, [1-11C]-acetate PET has become a useful tool for detecting various types of malignant tumors, such as prostate cancer (28, 29), renal carcinoma (30), hepatocellular carcinoma (31), brain astrocytoma (32), and gliomas (33).

The elevation of creatine levels in the CSF of 7-day LC rats can be attributed to the changes in energy metabolism (34). A significant down-regulation of creatine kinase has been reported in some cancers,
such as oral squamous cell carcinoma, resulting in elevated levels of creatine (35). However, the creatine levels of the gliomas used in the present study are known to be lower than those of the cerebral cortex where creatine is highly produced. In addition, the total creatine concentration in gliomas tends to decrease according to the degree of malignancy (36), and the F-98 cell is derived from a high-grade glioma of a Fisher 344 rat. Thus, we believe that the elevation of creatine levels in the CSF of 7-day LC rats is due to the destruction of the cerebral cortex by seeded cancer cells in the CSF space (subarachnoid space).

Contrast-enhanced (e.g., gadolinium chelate agents) MR imaging is the technique of choice used to evaluate patients that are suspected to have LC (37). Some changes, such as cranial nerve enhancement and subarachnoid-enhancing nodules, can be considered diagnostic of LC in patients with cancer. Nonetheless, contrast-enhanced MR imaging has more than a 30% incidence of false-negative results. Thus, a normal study does not exclude the diagnosis of LC (3). In addition, other diseases, including infectious, inflammatory diseases, trauma or subdural hematomas, can lead to similar MR imaging findings (37-41). Similar to the present study, surgically induced contrast enhancement in meninges is also well known (42). Thus, the detection of malignant cells in the CSF has been pursued to confirm the diagnosis of LC. However, our study further emphasizes that contrast-enhancement MR imaging may not be sufficient for the diagnosis of LC mainly due to its low specificity.

Although we obtained good diagnostic performance in both early (day 3) and late (day 7) stage LC models, we could not reliably identify the marker metabolites in the early stage LC group. Most of the contributing signals for 3-day LC rats were of low intensity or overlapped. Therefore, the distinction at the early stage may have been a result of the sum of small contributions. Still, the overall profiles of the significant signals were different between 3-day and 7-day LC groups and changed non-linearly with time (see Fig 5A and 7). For example, glucose and lactate, important and conspicuous marker signals on day 7, did not exhibit significantly different levels on day 3. As we failed to detect glioma cells on day 3 by
cytology, a small number of cancer cells may have generated cancer-related metabolites just enough to
make the overall profile different, but not enough to allow each metabolite identification. Future
experiments with human samples with both a larger number of samples and increased sample volumes
should enable metabolite detection with higher sensitivity.

As stated above, the diagnosis of LC can be performed by MR imaging, CSF cytology, histology and
NMR-based metabolomics. Among these modalities, histology can provide a definite answer in animal
models but cannot be performed in clinical settings. From a practical perspective, NMR-based
metabolomics showed the best performance among the aforementioned clinically applicable techniques.
One theoretical advantage of NMR metabolomics over cytology is that NMR measures small molecules
(typically those with MW < 400), which are highly diffusible, exhibiting uniform distribution regardless
of the sampling positions used. In comparison, the cells observed in cytology are much less diffusible in
the subarachnoid space where the CSF is collected; therefore, these cells may reflect rather local status.
The significantly higher sensitivity of NMR method at day 7 (89%) over cytology (66.7%) may be due to
these factors.

For a diagnostic measure, another important aspect is specificity. Although we cannot assess the
specificity of LC diagnosis against all possible brain diseases, we may address the specificity against LC
models using cancer cells from different tissues. It is expected that the distinction between related LC
models based on different primary cancer cells should be more difficult than that between LC and other
brain pathologies. In addition, it is clinically important to determine the tissue origin of a particular LC
case. Therefore, we established another LC model using mammary adenocarcinoma cells (13762 MAT
B III), measured its CSF metabolite profiles, and compared the results from the original model using
brain cancer cells. For the specificity, we were able to observe a distinct group of significant marker
signals (choline, glutamate, creatine, and lactate) of the 7-day LC developed with the breast cancer cells
from those with the brain cancer cells (acetate, glucose, creatine, and lactate) (Supplementary Fig. S6).
These results suggest that different metabolite profiles can be detected depending on the particular types of metastasizing cancer cells. We admit that this comparison of LC models is not a full resolution of the specificity concerns. Still, the results are encouraging in that we obtained differential metabolic profiles even for different subtypes of one brain pathology (LC). In actual human cases, additional information is available. Differentiation with meningitis, for example, can be also helped by clinical information such as medical history (e.g. presence of the underlying cancer or not), clinical picture (e.g. fever or not) and CSF findings (e.g. protein level). Therefore, we believe that the metabolomics approach is quite promising in human cases, and that our results warrant its human application. The diagnostic specificity can be ultimately addressed using patients with diverse brain pathological conditions.

For humans, NMR techniques require lumbar puncture for CSF collection, which is somewhat invasive, whereas MR imaging is non-invasive. CSF sampling is still routinely performed as MR imaging suffers from low specificity as described above. In addition, we only needed 0.04 ml of CSF, which can be readily provided from routine CSF samples (~ 10 ml) for cytology examinations. Thus, NMR samples can be obtained almost “for free” in terms of the reduced amount of labor of clinicians and suffering by patients. In addition, the NMR metabolomics approach does not require sample pre-treatment and the data can be obtained in less than 5 minutes. As with other analytical approaches, there are also some difficulties in NMR metabolomics, including low sensitivity, resonance overlap, and possible processing biases. These may be addressed with better hardware and software in the near future with the availability of cryogenic microprobes, higher field magnets, and signal deconvolution algorithms. Particularly for the sensitivity of quantifying low level metabolites, the NMR metabolomics is expected to perform much better for actual human samples which have the ultimate relevance to the diagnosis. That is due to the larger volume of attainable CSF volume (see above) which enables the use of standard 5 mm probes for enhanced sensitivity and/or concentration of the samples. Overall, we expect to see more examples of NMR metabolomics applied to diagnostic research with CSF as recently shown with pneumococcal or...
cryptococcal meningitis (16).

In conclusion, we have demonstrated that the diagnosis of LC is possible using a metabolomics based approach in a rat model. This approach was superior to the currently used CSF cytology and MR imaging procedures. It also enabled detection of LC at an early stage when CSF cytology failed. To the best of our knowledge, this study is the first report of a metabolomic diagnostic approach for LC, and it may become a useful clinical protocol that can augment or replace current diagnostic methods, if proven in human samples.
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Figure Legends

Fig. 1. Fluorescence-assisted cytological analysis of glioma cells in the pellets of centrifuged CSF of a 7-day LC rat, in which glioma cells in CSF expressed green fluorescence as shown under the enhanced GFP filter. However, no samples collected from 3-day LC rats exhibited positive CSF cytology, and therefore are not shown here.

Fig. 2. Contrast-enhanced TIWI MR imaging of rat brain. A normal rat (A) shows no meningeal enhancement, whereas meningeal enhancement (arrows) was observed in both rats in LC model rats (B) and sham operations (C). Thus, meningeal enhancement was not a specific finding for the LC. CC, cerebral cortex; CL, cerebellum; OB, olfactory bulb; T, thalamus.

Fig. 3. Histopathologic examination showed the F-98 cells that expressed GFP infiltrating the leptomeninges. Leptomeningeal infiltration (indicated by arrows) of F-98 cells was more prominent in 7-day (A) than in 3-day LC rats (B). Histopathology from the brains of 7-day (A) and 3-day (B) LC rats present the advanced and early LC status, respectively.

Fig. 4. Representative HR-MAS spectra of CSF. NMR spectra of CSF samples from rat models from normal (upper), 3-day LC (middle) and 7-day LC (lower) groups. Specific metabolite peaks (marked in the top spectrum) were assigned with Chenomx (Spectral database; Edmonton, Alberta, Canada). tCreatine means total creatine including creatine and phosphocreatine. All NMR spectra were acquired with a nano-probe® installed in a 500 MHz NMR spectrometer (Agilent, VNMRS 500 system).

Fig. 5. Multivariate analysis of the spectral data. PLS regression of the entire set of samples (A), where black, red and blue represent normal, 3-day LC and 7-day LC groups, respectively. Mean values of the
modeled scores are represented by square, triangle, and circle symbols. Vertical and horizontal error bars represent standard deviations. Binary discrimination with OPLS-DA between normal and 3-day LC samples (B), where black and red represent the normal and 3-day LC group, respectively. Binary discrimination with OPLS-DA between normal and 7-day LC rat samples (C), where black and blue represent normal and 7-day LC groups, respectively. Both OPLS-DA models were obtained with 1 predictive and 2 orthogonal components.

Fig. 6. Diagnostic performance of the differentiation model. To create a diagnostic prediction model, one sample was excluded from the full data set and a new OPLS-DA model was established with the rest of the data. The excluded sample was predicted based on a priori cutoff value of 0.5 (dashed line). These steps were repeated until all the samples were predicted once. Predictions of the 3-day (A) and 7-day LC samples (B); Black, Normal; Red, 3-day LC; Blue, 7-day LC. The Y values of the filled symbols are derived from the OPLS-DA discrimination model using the entire dataset. Open symbols represent mis-predicted samples, and the Y values correspond to those obtained from the prediction model.

Fig. 7. Marker metabolites for the LC group. (A) The Wilkoxon rank sum tests of 7-day LC group (upper) and 3-day LC group (lower) were performed with an in-house written R script. The X and Y axes represent the ppm variable axis and the log of 1-p values, respectively. Values with p values < 0.01 are shown as blue dots. (B) The actual levels of the LC marker metabolites (7 day-LC group) are shown using the normalized average spectra of the corresponding peaks. Blue, normal; Green, 3-day LC; Red, 7-day LC. The p-values for normal vs. the 7-day LC group are also shown.
Fig 3

A

B

Legend:
- GFP: Green Fluorescent Protein
- DAPI: 4',6-Diamidino-2-phenylindole (DNA stain)
- Merge: Combined images

Scale bars: 50 μm (A) and 100 μm (B)
Fig 4

Normal

3 Day-LC

7 Day-LC

1H (ppm)
Fig 6

A

B
Fig 7

A

B

Acetate

p < 8 × 10^{-4}

Mean Intensity

ppm

500

1000

1500

2000

2.0

1.9

tCreatine

p < 0.004

Mean Intensity

ppm

500

1000

1500

2000

4.0

3.9

Lactate

p < 0.004

Mean Intensity

ppm

500

1000

1500

2000

4.3

4.2

4.1

4.0

Glucose

p < 8 × 10^{-4}

Mean Intensity

ppm

3.6

3.5

3.4

3.3
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