Metabolomics of Vitreous Humour from Retinoblastoma Patients

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Summary

Retinoblastoma (Rb) is the most common malignant tumor of the eye in children. Inactivation of both copies of the RB1 gene in retina is known to be the cause of cancer. Here, we present metabolomic studies on vitreous humor samples to identify differential metabolites in Rb patients that can provide a direct or indirect link to the pathways found in cancerous tissue. 9 patient and 2 controls samples were used. The extracted samples were subjected to LC/QTOF-MS and GC/QTOF-MS analysis. More than 1000 features were identified using these two techniques . Wide variety of compounds ranging from amino acids, carbohydrates, nucleobases and lipids were identified. Among lipids, Phosphatidyl cholines (PC), ether linked phosphatidyl ethanolamines (PE), ceramides, sphingomyelins and sphinganines were identified. Lipids, especially PCs and ether linked PEs were found to be up regulated in patient samples. Many of the ether lipids found to be 5 folds more in patient samples. Carnitines and free fatty acids were also up regulated in patient samples. As the biosynthesis of ether lipids starts in peroxisomes, this study suggests an altered peroxisomal metabolism in these patients.

Introduction

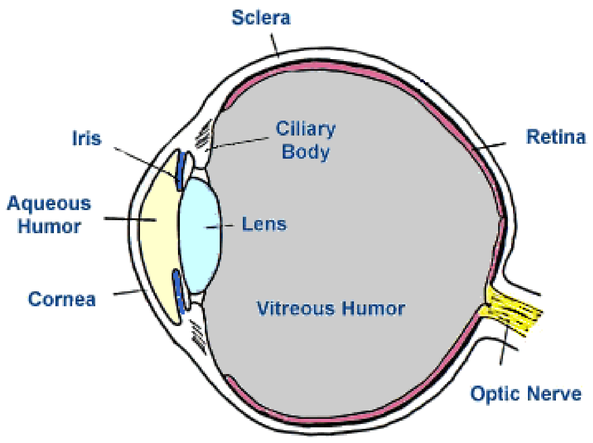

Figure 1. The location of vitreous humour.

This study illustrates a metabolomics approach to study molecular events leading to progression of retinoblastoma. Retinoblastoma is a pediatric ocular cancer affecting children usually less than five years of age. It is a complex disease predisposed primarily by mutations in the RB1 gene. From a cohort of 9 patients undergoing enucleation of the affected eyes, we obtained tumor, aqueous humor, vitreous humor and tear samples. We obtained retina, aqueous humor and vitreous humor from enucleated eyes of 2 deceased pediatric controls, whose cause of death is not due to any eye related disease. The results show overlap of key cellular pathways which can be mechanistically linked to disease progression. The study provide new biological insights that are made accessible by combining data from different biological and biochemical domains with a comprehensive integrated method. The information is useful not only to correlate expression markers with disease mechanism but also to better predict appropriate chemotherapy regimens and identify new mechanisms to treat even advanced stages of retinoblastoma.

Experimental

Method

The samples from 9 patient and 2 controls were extracted using methanol: ethanol (1:1 v/v). The extracted samples were subjected to LC/QTOF-MS and GC/QTOF-MS analysis. For LC-QTOF analysis, data was acquired using electrospray ionization in positive and negative ion modes using modified polar reverse phase C18 column, and HILIC column. Molecular features were searched against METLIN database and confirmed by METLIN library using data dependent MS/MS acquisition. For GC-QTOF analysis, data was acquired using EI source on a DB-5ms column. The results were searched against Fiehn RTL library. Gene expression microarray studies were performed using SurePrint G3 Human GE 8X60K V2 Microarray while miRNA studies were performed using Agilent SurePrint G3 Human v16 miRNA 8X60K Microarray kit. The metabolomics and gene expression results were combined and analyzed using pathway architect module of the GeneSpring 13.1 MPP. The metabolomics workflow in shown in Figure 2.

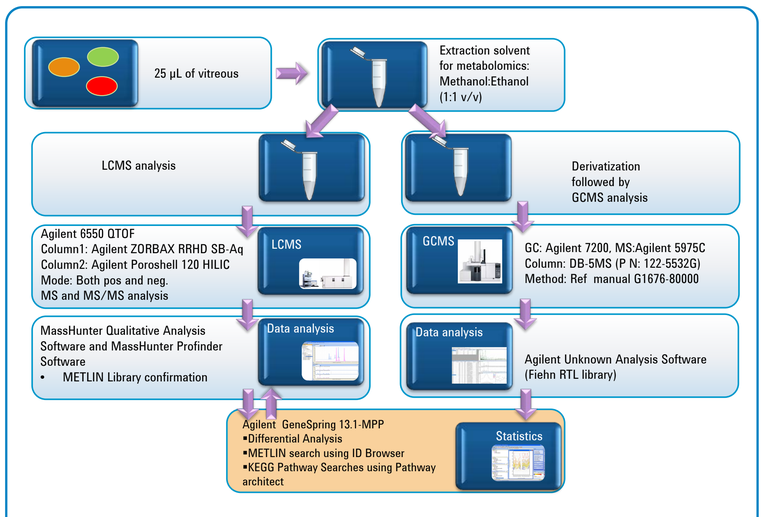

Figure 2. Metabolomics workflow for retinoblastoma study

Results and Discussion

Sample-Sample correlation

A pair wise analysis between samples (9 patient and 2 controls) within vitreous humor (C18 Pos) metabolomics experiments is shown in figure 3. The 9 patient samples are classified based on clinical and pathological risk as high risk (H), low risk (L) and no risk (N). The correlation analysis followed by clustering showed the relationship between the three groups of 9 patients. The results showed that high risk group patients correlate positively with each other marked by red color. Most of the other samples showed no (yellow) correlation or negative (blue) correlation with controls.

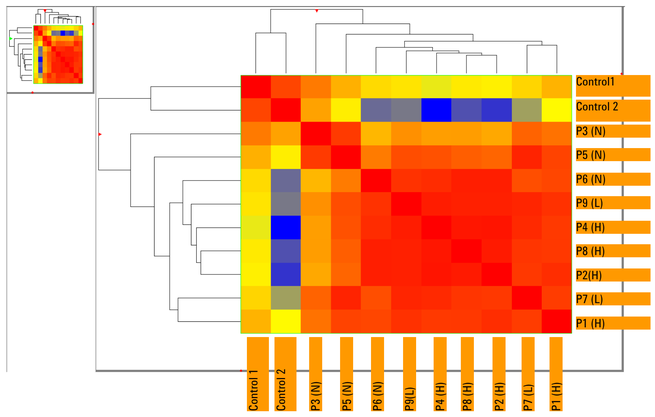

Figure 3. A pair wise analysis between samples (9 patients and 2 controls) from LCMS analysis.

Statistical analysis

Statistical analysis on vitreous humour samples reveal 350 differential metabolites as shown in the volcano plot (Figure 4) and hierarchical clustering between samples is shown in Figure 5.

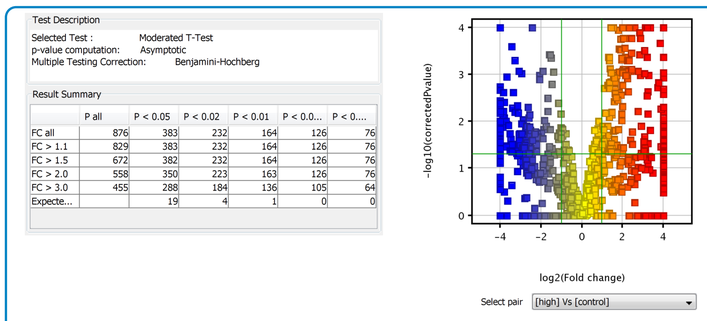

Figure 4. Volcano plot showing statistical analysis and fold change.

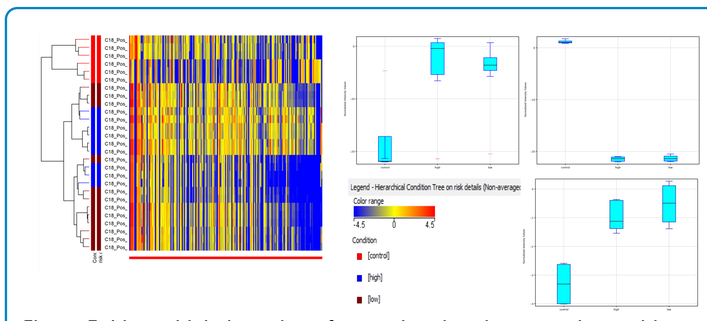

Figure 5. hierarchial clustering of control and patient samples and box and whiskers plots of Guanine (A), PC (16:0/16:1) (B) and Hydroxy Lauric acid (C) identified among control, high risk and low risk groups

Database and Library search

The accurate mass database search for LC/Q-TOF and GC/Q-TOF data resulted in the detection of about 1000 and 200 compounds respectively. Differential compounds are identified by database search. Compounds are further confirmed by matching with the spectra of authentic compounds from MSMS spectral library. As an example, comparison of acquired and library spectra of Guanine were shown in figure 6. SimLipid was used for the identification of lipids using MS/MS data along with accurate mass information to get unambiguous hits.

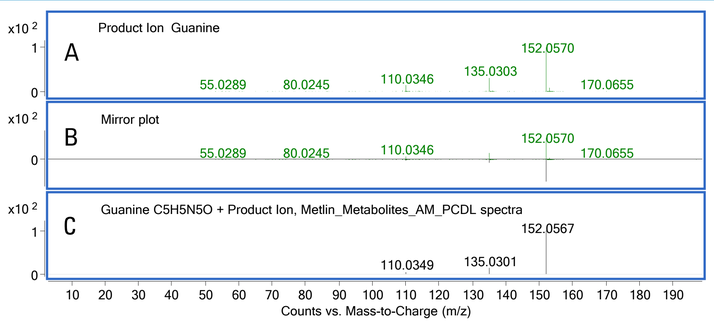

Figure 6. LC/MS/MS results of guanine showing MS/MS spectra (A), mirror plot (B) and library spectra from PCDL (C)

Table 1. Lipid identification using SimLipid software

ID24#_PC(16:0/16:1(9Z))@754.5355_1_14.160

Rank	Lipid ID	Chemical Composition	Experimental m/z	Theoretical m/z	Delta Mass(ppm)	Score
1	LMGP01011479	[C40H78NO8P+Na] 1+	754.5355	754.5363	0.9953	0.1924
2	LMGP01010490	[C40H78NO8P+Na] 1+	754.5355	754.5363	0.9953	0.0565
2	LMGP01010492	[C40H78NO8P+Na] 1+	754.5355	754.5363	0.9953	0.0565
2	LMGP01010882	[C40H78NO8P+Na] 1+	754.5355	754.5363	0.9953	0.0565
2	LMGP01011329	[C40H78NO8P+Na] 1+	754.5355	754.5363	0.9953	0.0565
2	LMGP01011352	[C40H78NO8P+Na] 1+	754.5355	754.5363	0.9953	0.0565
2	LMGP01011389	[C40H78NO8P+Na] 1+	754.5355	754.5363	0.9953	0.0565
2	LMGP01011413	[C40H78NO8P+Na] 1+	754.5355	754.5363	0.9953	0.0565
2	LMGP01011439	[C40H78NO8P+Na] 1+	754.5355	754.5363	0.9953	0.0565
2	LMGP01011500	[C40H78NO8P+Na] 1+	754.5355	754.5363	0.9953	0.0565
2	LMGP01011525	[C40H78NO8P+Na] 1+	754.5355	754.5363	0.9953	0.0565
2	LMGP01011582	[C40H78NO8P+Na] 1+	754.5355	754.5363	0.9953	0.0565
2	LMGP01011757	[C40H78NO8P+Na] 1+	754.5355	754.5363	0.9953	0.0565
2	LMGP01011805	[C40H78NO8P+Na] 1+	754.5355	754.5363	0.9953	0.0565

ID78#_SM(d18:1/16:0)@703.5741_1_15.131

Rank	Lipid ID	Chemical Composition	Experimental m/z	Theoretical m/z	Delta Mass(ppm)	Score
1	LMSP03010003	[C39H79N2O6P+H] 1+	703.5741	703.5754	1.8532	0.3163
2	LMSP03010042	[C39H79N2O6P+H] 1+	703.5741	703.5754	1.8532	0.3163
3	LMSP03010043	[C39H79N2O6P+H] 1+	703.5741	703.5754	1.8532	0.285

Results and Discussion

GCMS data analysis

GCMS acquisition was performed using Fiehn RTL method using Agilent 7200 Q-TOF mass spectrometer. The data analysis results using Agilent Unknown analysis software is shown in Figure 7. A selected list of GCMS metabolites include galactosamine, 3-hydroxy-3-methylglutaric acid, glucose, sorbose, pantothenic acid, trehalose, glutamic acid and 3- (4-hydroxyphenyl)lactic acid.

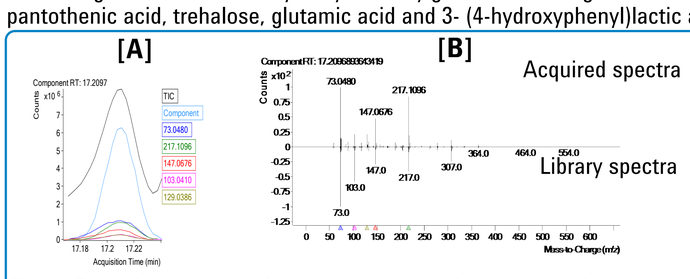

Figure 7. Unknown analysis results showing the extracted ion chromatogram (A) and the header-to-tail plot of L-Sorbose (B).

Pathway Analysis

Pathway analysis was carried out using the Pathway Analysis Module in GeneSpring 13.1. The differentially expressed entity list (p ≤0.05 and fold change ≥2.0) was selected for pathway analysis. Curated pathways from the KEGG were used for pathway analysis.

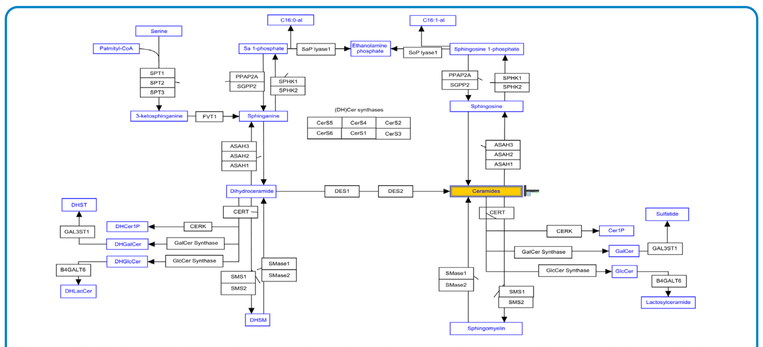

Figure 8. Spingolipid pathway showing the metabolism of ceramides which was significantly up regulated.

Genomics and metabolomics data were co-visualized in the pathway context using the Multi-Omics Analysis tool of GeneSpring 13.1, which enabled simultaneous viewing of the differential entities from both gene expression and metabolomics. Table 2 shows the list of predominant pathways as revealed by combined analysis of LCMS of vitreous humor and gene expression.

Table 2. Predominant pathways revealed by combined LCMS and gene expression multi-omic analysis in vitreous humor

Pathways	
ABC transporters	Glycolysis / Gluconeogenesis
AMPK signaling pathway	Glyoxylate and dicarboxylate metabolism
Alanine, aspartate and glutamate metabolism	HIF-1 signaling pathway
Arachidonic acid metabolism	Inositol phosphate metabolism
Arginine and proline metabolism	Insulin secretion
Bile secretion	Metabolism of xenobiotics by cytochrome P450
Cysteine and methionine metabolism	Nicotinate and nicotinamide metabolism
Fatty acid biosynthesis	Pantothenate and CoA biosynthesis
Fatty acid elongation	Pentose phosphate pathway
Fructose and mannose metabolism	Phenylalanine metabolism
Galactose metabolism	Phosphatidylinositol signaling system
Glycerolipid metabolism	Purine metabolism
Serotonergic synapse	Tyrosine metabolism
Type II diabetes mellitus	Valine, leucine and isoleucine biosynthesis

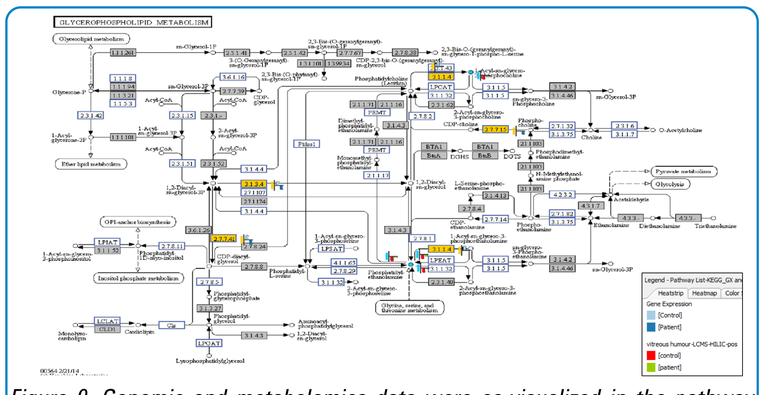

Figure 9. Genomic and metabolomics data were co-visualized in the pathway context using the Multi-Omics Analysis tool of GeneSpring 13.1 Combined analysis of transcriptomics and metabolomics data shows glycerophospholipid pathway to be significantly affected.

Conclusions

- Vitreous humour being in closest proximity to the retinoblastoma tissue showed characteristics exo-metabolites from the cancer tissue.
- Different classes of metabolites were confirmed using LCMS and GCMS techniques.
- Accurate mass LCMS libraries along with SimLipid software facilitated analysis of various class of metabolites including lipids.
- Pathway search of differential metabolites using GeneSpring software yielded key biological pathways which were also reflected in the genomics study (data not shown).
- Up regulation of phosphatidyl cholines ,free fatty acids and lipid transporters like carnitine Indicates an altered lipid metabolism in the patients.
- Although retrieval of vitreous humour would require evasive procedures, a more detailed study using other biological fluids such as tears are underway.