Clinical and microbiological characteristics of *Cryptococcus gattii* isolated from 7 hospitals in China

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Abstract

Background: Infection, even outbreak, caused by Cryptococcus gattii (C. gattii) has been reported in Canada and the United States, but there were sparsely-reported cases of C. gattii in China. Our interest in occurrence, clinical manifestation, laboratory identification and molecular characterization of Chinese C. gattii strains leads us to this research.

Methods: A total of 254 clinical isolates primarily identified as Cryptococcus neoformans (C. neoformans) were collected. VITEK 2 compact, canavanine glycine bromothymol blue (CGB) agar and Bruker Biotyper matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) were used for strain identification. Multi-locus sequence typing (MLST) was performed for genotyping. Antifungal susceptibility test was carried out with commercial kits of both ATB fungus 3 and Yeast one. Clinical information of patients was reviewed retrospectively. Label-free proteome technique was used to quantitatively analyze the differential proteins of C. gattii.

Results: Out of 254 clinical isolates, we identified eight strains as C. gattii. MLST showed genotype VGI accounted for the most (6 / 8), the other two strains were genotype VGII，VGIIa and VGIIb respectively，with 3 specific spectra of molecular weight about 4342, 8686, 9611 Dalton by MALDI-TOF MS. The minimal inhibitory concentrations (MICs) of Fluconazole with Yeast one was 2～4 times higher than that with ATB fungus 3. Higher MICs of antifungal agents were exhibited against VGII strains than against VGI strains. C. gattii genotype VGI and VGII possessed 418 and 774 specific proteins respectively. Comparative proteome analysis showed that 329 and 180 proteins were highly expressed in C. gattii VGI and VGII. The enrichment of differentially expressed proteins was directed to Golgi complex.

Conclusions: Infection by C. gattii in China might have been underestimated because of initial mis-identification. Genotype VGI was predominant but VGII was more resistant to antifungal agents. There was significant difference in protein expression profile between VGI and VGII C. gattii.

Background

Cryptococcus gattii (*C. gattii*), one of the most common pathogenic cryptococcal species, primarily infects immunocompetent hosts, in contrast to *Cryptococcus neoformans* (*C. neoformans*), which mainly infects immunocompromised individuals [1, 2]．Previous studies revealed that *C. gattii* infection was restricted geographically to tropical and subtropical regions [1-3]. However, outbreaks have been recorded in temperate regions, such as British Columbian of Canada and Pacific Northwest of the United States [4, 5]. In China, majority of people reside in temperate and subtropical regions with climates suitable for fungal growth and spread. The incidence of cryptococcal infection increased significantly in recent years, but unfortunately, little investigation has been carried out on this organism [6]. The objectives of the present study were to determine the frequency of *C. gattii* in 254 clinical isolates initially identified as *C. neoformans* from 7 hospitals in China and to investigate clinical and microbiological characterization. The antifungal susceptibility and molecular features were also studied.

Results And Discussion

1. Identification and antifungal susceptibility profile of *C. gattii* isolates

C. neoformans and *C. gattii* are two important fungal pathogens for humans and animals. Both of them are round with capsule and positive urease test. These routine laboratory identification methods are unable to distinguish the two species [7]. In this study, we analyzed 254 strains primarily identified as *C. neoformans*, 8 (3.1%) showed pigmentation on CGB agar and were then identified as *C. gattii* by Bruker MALDI-TOF MS with software 3.0 with scores from 2.043 to 2.275，showed in Table1．. However, VITEK 2 compact could not differentiate *C.gattii* from *C. neoformans*. Although there were several reports about infection of *C. gattii* from China [8-10], the number of *C. gattii* infections may have been underestimated because most laboratories utilize ink staining and biochemical methods for routine identification of cryptococcal species (such as VITEK 2 compact). There might be more cryptococcal cases of *C. gattii* if appropriate identification methods had been used. CGB agar was recommended to identify *C. gattii* from *C. neoformans* due to its convenience and low cost [7], but it took longer time to get the result. Molecular methods would be faster and more accurate for differentiation of the two pathogenic Cryptococci species. However, they are not available in all clinical laboratories, where an accurate identificaction of species should be made in order to install the proper treatment. In this study, eight strains of *C. gattii* were correctly and timely identified by Bruker MALDI-TOF MS with software 3.0. Additionally, as showed in Fig.1, *C. gattii* VGII possessed three specific spectra with molecular weight of about 4342, 8686, 9611 Dalton compared with *C. gattii* VGI, therefore, we suggest that Bruker MALDI-TOF MS with software 3.0 should be used not only to identify *C. gattii* but also to differentiate *C. gattii* VGII from *C. gattii* VGI (showed in Fig.2). Of course, eight isolates was not

enough and more strains should be collected and studied to observe the spectrum difference between *C. gattii* VGII and other genotypes of *C. gattii*.

The susceptibility of eight *C. gattii* strains to six kinds of antifungal agents were investigated with two kinds of commercial kits. As showed in Table1, it was remarkable that the results were in good agreement by two kits, except that MICs of Fluconazole were 2–4 times higher by Yeast one than by ATB fungus 3. All the *C.gattii* isolates were susceptible to 5-Flucytosine, Amphotericin B, Itraconazole, Voriconazole and Posaconazole. While only one strain was resistant to Fluconazole with MIC of 128 by Yeast one. However, interpretation of MICs of antifungal agents including Fluconazole for category of “susceptibility” or “resistance” was hampered by the lack of clinical breakpoints for *C. gattii*. Some previous studies have shown that *C. gattii* may be less susceptible to antifungal agents compared with *C. neoformans*. In a study from Taiwan, *C. gattii* was less susceptible to 5-Flucytosine and amphotericin B, and in Spain, MICs of Fluconazole, Voriconazole and Posaconazole to *C. gattii* were significantly higher [11-13].Our data indicated that antifungal agents exhibited higher MICs against isolates of genotype VGII than genotype VGI, which agreed with the data of Hagen et al and Lockhart et al. [14-17]. The correlation between susceptibility profile and genotype of *C. gattii* has rarely been studied [14], however, Clinical relevance between MIC breakpoint and epidemiological cut-off value (ECV) based on MIC distributions of wild-type strains has currently been studied on both *C. neoformans* and *C. gattii* isolates from Europe, USA, Australia, Brazil, Canada, India and South Africa. ECVs of Amphotericin B (0.5–1μg/ml), 5-Flucytosine (4 –16μg/ml), Fluconazole (8–32μg/ml) and other azoles varied similarly by molecular type for both *C. neoformans* and *C. gattii* [15, 16].

2. MLST analysis and Genotyping

As showed in Table 1, Our study indicated six strains of *C. gattii* as genotype VGI (predominant genotype) and two strains as genotype VGII （VGIIa and VGIIb respectively）, Some gene mutation existed in unlinked gene loci such as FTR1 and RAS1 as indicated in Fig. 3. Up to now, four genotypes of *C. gattii* have been recognized, they were VGI, VGII, VGIII and VGIV. Lockhart and colleagues had reported that VGII and VGIII were the most-frequently identified isolates in America, VGIV were almost exclusively in Africa, and VGI predominated in Europe, Australia and Asia [18]. *C. gattii*, as an important pathogen, caused outbreak in British Columbia, Canada and the Pacific Northwest, the United States. But 8 strains in our study were pathogens causing sporadic infections in accordance with the strain origination and the isolation time.

3. Patient information and clinical characteristics

As showed in Table 1, the eight strains of *C. gattii* were isolated from eight patients. Two patients with isolate numbers of 2 and 6 were from Hainan and Yunnan Province, which were recognized as tropical areas. Three patients with isolate number 3 (from Chongqing city), 4 and 7 (from Fujian Province) were recognized as being from subtropical area. The other three patients with isolate numbers of 1, 5 and 8 were from Shandong Province, Inner Mongolia Autonomous Region and Henan Province, which were recognized as temperate regions. Information of patient infected with isolate number 3 was un-available, the other 7 patients were immune-competent young male adults, with age from 21 to 60 years old, which was considered as the reason for increased exposure to environmental sources and increased chance for infection[19]. Their clinical and laboratory information were summarized in Table 2. All the patients in our study had no recent travel history, the *C. gattii* infection occurred regionally and domestically. Most patients had headache and fever, three patients showed neck stiffness, positive Kernig’s sign and meningeal lesions（meningeal enhancement or lacunar infarction by brain MRI. Most of them also demonstrated irregular nodule, consolidation and mass by lung CT, which was consistent with the research made by Ngamskulrungroj who indicated even though *C.gattii* failed to cross the blood-brain barrier and cause fatal meningoencephalitis, *C. gattii* could cause fatal lung infection [20].

It was reported that host response varied based on the genotype of the organism and concomitant illnesses [21]. There was also study which revealed that *C. gattii* VGII strains were more virulent than VGI strains and VGIIa were even more virulent than VGIIb independent of their clinical or environmental origin [22]. In our study, all patients were treated by Fluconazole plus amphotericin B, most of them improved effectively without severe neurological sequelae. This might be explained by the fact that these patients infected by *C. gattii* VGI more than *C. gattii* VGII on the one hand, and on the other hand, two of them were infected with *C. gattii* VGII which showed sequence mutations in the gene location of RAS1 and FTR1 compared with reference strains of R265 and R272 respectively. Whether the mutations were relevant to virulence decrease, further work needs to be done.

4. Proteomic analysis.

In this study, the proteome expression profiles of two strains of VGII and four strains of VGI *C. gattii* were created. A total of 3628 proteins were identified, of which 418 were VGI specific proteins 774 were VGII specific proteins, and 2436 were common proteins for both VGI and VGII. Comparative proteome analysis showed that 329 proteins were highly expressed in *C. gattii* VGI and 180 proteins were highly

expressed in *C. gattii* VGII as shown in Fig. 4. A cluster analysis of the differentially expressed proteins was shown in Fig.5, which clearly demonstrated that the protein expression profile of VGII *C. gattii* isolates was significantly different from that of VGI *C.gattii* isolates. The information of GO (Gene ontology) term and KEGG (Kyoto Encyclopedia of Genes and Genomes) metabolic pathway analysis exhibited that the enrichment of differential expressed proteins between VGII and VGI was mainly directed to Golgi apparatus, Golgi membrane and Golgi vesicle as shown in Table 3. The most significant enriched metabolic pathways were oxidative phosphorylation, ribosome and metabolic pathway (shown in Fig. 6)

GO is a very important method and tool in the field of biological information. By establishing a set of controlled words with dynamic form, the attributes of genes and gene products in organisms can be described comprehensively. KEGG is the main public database with which the most important biochemical metabolic pathways and signal transduction pathways can be determined. Our results suggested that the differential protein of *C. gattii* VGII and VGI was mainly located on the organelles associated with the Golgi body, that meant energy metabolism *of C. gattii* might be involved in the difference of pathogenesis mechanism for different genotype of *C. gattii*. So it was suggested that study on the secretory protein and the secretory vesicles of the two types of *C. gattii* would be beneficial to the understanding of the pathogenesis of *C. gattii*, which provided another cutting angle for further study.

Conclusions

Our results demonstrated that *C. gattii* Genotype VGI was predominant in hospitals of China, but VGII was more resistant to antifungal agents. *C. gattii* VGII isolates possessed obvious protein peaks with molecular weight of approximate 4342, 8686, 9611 Dalton. The full protein spectrum data of *C.gattii* were provided for the first time, a total of 3628 proteins were identified, 418 and 774 specific proteins were found from *C. gattii* genotype VGI and VGII respectively. The enrichment of differentially expressed proteins between VGI and VGII was mainly directed to Golgi complex. It will lay a foundation for better understanding and further research on the pathogenic mechanism for different genotype of *C. gattii*. The limitation of this study was only 8 strains were available for analysis.

Methods

1. Collection and initial identification of isolates

A total of 254 clinical isolates initially identified as *C. neoformans* by biochemical methods were collected from seven hospitals in China, they were all stored at −80°C. All the isolates were sub-cultured onto Sabouraud dextrose agar medium at 25 °C for 48–72 h and identified initially by VITEK 2 Compact (bioMérieux SA, France). Each isolate was also inoculated on canavanine-glycine-bromthymol blue (CGB) agar[23] at 25°C for 24–120h, The isolates which showed blue pigmentation on CGB agar were then subjected to further identification.

2. Identification by MALDI-TOF MS

Isolates were re-identified and analyzed by Bruker Biotyper MALDI-TOF MS (Bruker, Daltonik, Bremen, Germany). Briefly, one single colony was smeared directly on the MALDI-TOF MS analysis plate and formic acid was added. After 3–5 minutes, matrix was added and the plate was put into the MALDI-TOF MS instrument with MALDI Biotyper software 3.0 (Bruker Daltonik GmbH) for species identification and dendrogram analysis. Two strains of *C.neoformans* were included as outgroup control.

3. Analysis by multilocus sequence typing (MLST)

Genomic DNA of each isolate identified as *C. gattii* was then extracted using the TianGen® TIANamp Yeast DNA Kit (Tiangen Biotech Beijing CO., LTD, China) complying with the manufacturer's instruction, seven genes of unlinked loci were amplified, including six housekeeping genes (CAP59, GPD1, LAC1, PLB1, SOD1, URA5) and one non-coding region gene (IGS1)[24] , bi-directional sequencing for each gene was then carried out, sequence comparison for each locus was done according to the method described by ISHAM Cryptococcal Working Group. Sequences were uploaded to the MLST Database for the *C.neoformans/C.gattii* species complex (http://mlst.mycologylab.org) one by one. A sequence type (ST) number and seven allele type (AT) numbers were given to each isolate. New AT and ST number will be assigned for new sequence. To differentiate VGII subtypes, twelve unlinked genes (SXI1α, TEF1, FTR1, CBP1, ICL1, HOG1, TOR1, STE7, TRR1, FHB1, RAS1, PAK1) were also amplified and sequenced according to the method of Fraser et al[25], they were analyzed and compared with Vancouver Island strains of R265 and R272.

4. Antifungal susceptibility test of *C. gattii* isolates

Antifungal susceptibility test was carried out by both ATB fungus 3 (bioMérieux SA, France) and Yeast one (Trek Diagnostic Systems Ltd, UK) following their instructions. *Candida krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019 were used for quality control.

5. Clinical information of patients

Under authorization of Medical Record Department, Clinical information of patients infected by *C. gattii* was reviewed retrospectively, including age, sex, underlying diseases, symptoms, imaging findings, laboratory examinations and antifungal therapy.

6. Analysis of differential proteins

According to the regional distribution and the integrity of clinical data of the strains, six strains (4 of VGI and 2 of VGII) of *C. gattii* were selected for proteome analysis. All the isolates were sub-cultured onto Sabouraud dextrose agar medium, incubated at 25°C for 48–72 h, the proteins were extracted from the strains, The protein concentration was 0.5μg/μl, after being separated by SDS-PAGE electrophoresis (One-dimensional), the proteins were hydrolyzed and analyzed. Original mass spectrum files of 6 strains were imported into Maxquatt software (version 1.3.0.5) for analysis. The database source was for *C.gattii*. in Uniprot、http://www.geneontology.org/、. Plus or minus 15ppm of polypeptide molecular weight, or missing 2 cutting sites were set as Maxquatt parameters, false discovery rate (FDR) for polypeptide identification was set as 0.01. A standard of $p<0.05$ and 2-fold expression were considered as different proteins. The enrichment and pathway analysis was carried out by using the GO plot package of R language and KEGG online tool (https://david.ncifcrf.gov/, http://www.kegg.jp/kegg/pathway.html). The bubble diagram was drawn according to the R language ggplot2 package.

Abbreviations

AMB Amphotericin B

AT allele type

CGB canavanine glycine bromothymol blue

C. gattii Cryptococcus gattii

C. neoformans Cryptococcus neoformans

ECVs epidemiological MIC cut-off values

FCA Fluconazole

FC Fold change

FDR false discovery rate

GO Gene Ontology

ITR Itraconazole

KEGG Kyoto Encyclopedia of Genes and Genomes

MALDI-TOF MS matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

MICs minimal inhibitory concentrations

MLST multi-locus sequence typing

PSZ Posaconazole

ST sequence type

VRC Voriconazole

5-FU 5-Flucytosine

Declarations

Ethics approval and consent to participate: Not applicable

Consent for publication: All authors agreed for publication.

Availability of data and materials: The datasets used and/or analysed during the current study are available from the corresponding author and first author on reasonable request.

Competing interests: The authors declare that there are no competing interests.

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Authors' contributions：DXS designed and organized this study, revised the manuscript, LJ performed most of the experiments and wrote the manuscript；XYX collected strains, analyzed the sequence, HW, LFW and LG collected and identified strains, JRC performed and analyzed MALDI-TOF MS data. All authors read and approved the final manuscript.

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Tables

Table 1 The geographical region of the isolation, species identification, antifungal susceptibility profile, genotype and sequence type of 8 *C. gattii* strains

Isolate No.	ID by VITEK 2 Compact (Identification rate)	pigmentation on CGB agar	ID by MALDI-TOF MS (score)	geographical region of the isolation	STs	Genotype	5-FU		AMB		FCA		ITR		VRC		PSZ
							a	b	a	b	a	b	a	b	a	b	b
1	C. neoformans (93%)	blue	C. gattii (2.190)	temperate regions	20	VGII	<4	2	<0.5	1	8	32	0.25	0.25	0.25	0.25	0.25
2	C. neoformans (90%)	blue	C. gattii (2.043)	tropical areas	106	VGI	<4	0.5	<0.5	0.25	1	4	<0.125	0.06	0.06	0.06	0.06
3	C. neoformans (96%)	blue	C. gattii(2.275)	subtropical area	57	VGI	<4	0.5	<0.5	0.25	1	4	<0.125	0.12	0.06	0.12	0.12
4	C. neoformans (99%)	blue	C. gattii (2.092)	subtropical area	197	VGI	<4	1	<0.5	0.25	2	4	<0.125	0.06	<0.125	0.06	0.12
5	C. neoformans (86%)	blue	C. gattii (2.118)	temperate regions	57	VGI	<4	0.5	<0.5	0.25	2	4	<0.125	0.12	0.06	0.06	0.12
6	C. neoformans (91%)	blue	C. gattii (2.126)	tropical areas	7	VGII	<4	2	<0.5	0.5	8	128	0.25	0.25	0.25	0.25	0.5
7	C. neoformans (98%)	blue	C. gattii (2.188)	subtropical area	57	VGI	<4	0.5	<0.5	0.25	1	4	<0.125	0.06	0.06	0.06	0.12
8	C. neoformans (99%)	blue	C. gattii (2.194)	temperate regions	161	VGI	<4	1	<0.5	0.25	2	4	<0.125	0.12	0.06	0.06	0.12

Notes: a and b represented the method of ATB fungus 3 and Yeast one respectively; the abbreviations for antifungal drugs were: 5-FU: 5-Flucytosine; AMB: Amphotericin B; FCA: Fluconazole; ITR: Itraconazole; VRC: Voriconazole; PSZ: Posaconazole.

Table 2 Clinical and laboratory information for patients infected with *C. gattii*

	1	2	4	5	6	7	8
Location (Province)	Shangdong	Hainan	Fujian	Neimeng	Yunnan	Fujian	Henan
isolation period of *C. gattii*	2014.11	2015.12	2014.04	2016.06	2016.07	2015.09	2016.08
Gender/Age(years old)	Male/40	Male/33	Male/21	Male/47	Male/61	Male/33	Male/36
History(medical/contact)	No	No	No	No	No	No	No
Immunity	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Fever	+	+	+	-	+	+	+
Headache	+	+	+	+	+	+	-
Nausea and Vomiting	-	+	+	+	-	-	-
Seizure	-	-	+	-	-	-	-
Neck stiffness	+	-	+	+	-	-	-
Kernig's sign	+	+	+	-	-	-	-
Papilloedema	-	-	+	+	-	-	-
Lung CT	Irregular nodule with spicules and lobulation	Left lower lung mass	Normal	Left lung mass	Left lower lung consolidation	Multiple small nodules	Right upper lung mass
Brain MRI	Meningeal lesions	Normal	Meningeal enhancement	Left lacunar infarction	NA	Normal	Normal
Organism also found by lung biopsy	Yes	NA	NA	NA	NA	Yes	Yes
Blood culture	No growth	No growth	No growth	NA	NA	NA	No growth
Cryptococcal antigen titre in serum	1:1024	NA	NA	NA	NA	NA	1:640
CSF test:							
Pressure (mmH2O)	260	330	330	330	NA	200	normal
Glucose (mmol/L)	0.5	4.11	2.49	2.3	NA	2.22	normal
Protein (g/L)	1.57	0.71	0.35	0.73	NA	29	normal
Chloride (mmol/L)	111	144.7	117	102	NA	126.6	normal
White blood cells($\times 10^6$cells/L)	377	80	720	91	NA	368	normal
Ink staining	positive	positive	positive	positive	NA	NA	positive
Cryptococcal antigen titre	1:1024	NA	NA	NA	NA	NA	1:640
Organism cultured from	CSF	CSF	CSF	CSF/	sputum	CSF	sputum
Antifungal therapy regime	Flu+AmB	Flu+AmB	Flu+AmB	Flu+AmB	Flu+AmB	Flu+AmB	Flu+AmB
Improvement (follow-up)	alive	alive	alive	alive	alive	alive	alive
Neurological sequelae	No	No	Yes	No	No	No	No

Notes: + and – represented symptoms appeared and not appeared; NA represented data not available.

Table 3 Results of GO enrichment analysis for Cellular Component

GO ID	GO Term	Gene Ratio	Bg Ratio	P value
GO:0005794	Golgi apparatus	40	51	3.26E-07
GO:0000139	Golgi membrane	26	32	1.37E-05
GO:0005798	Golgi-associated vesicle	12	16	0.011314374
GO:0031982	vesicle	17	26	0.021147847
GO:0030662	coated vesicle membrane	14	21	0.028770779
GO:0044433	cytoplasmic vesicle part	15	23	0.030922717
GO:0034708	methyltransferase complex	4	4	0.036668632
GO:0030120	vesicle coat	12	18	0.042486706
GO:0098796	membrane protein complex	50	95	0.046997979
GO:0030135	coated vesicle	14	22	0.047857677
GO:0031988	membrane-bounded vesicle	15	24	0.049777137

Figures

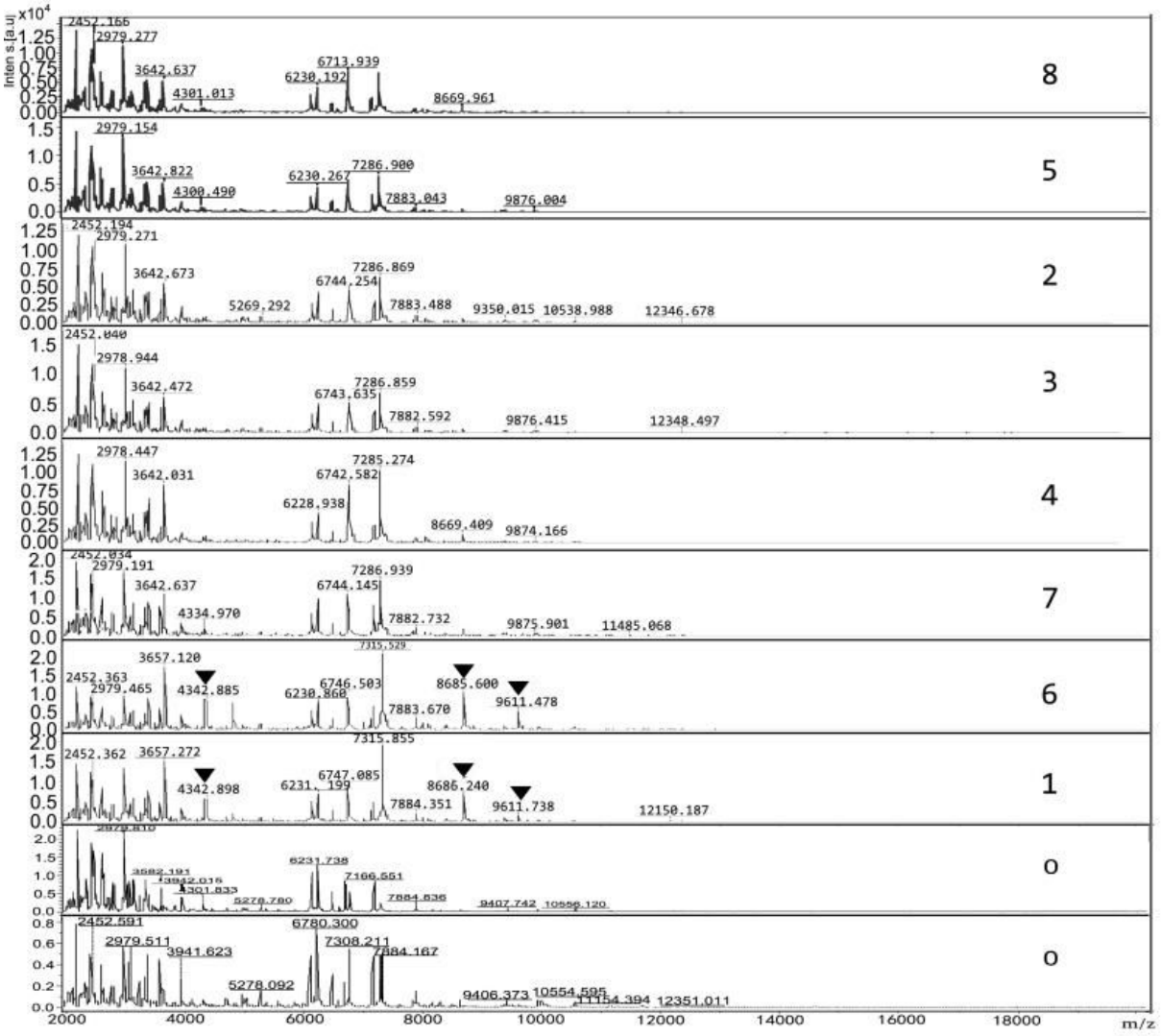

Figure 1

Mass spectra of 8 strains of C. gattii and 2 strains of C.neoformans by MALDI-TOF MS. Notes: Three specific spectra of C. gattii VGII with molecular weight of about 4342, 8686, 9611 dalton were indicated as arrows; 0 represented C.neoformans.

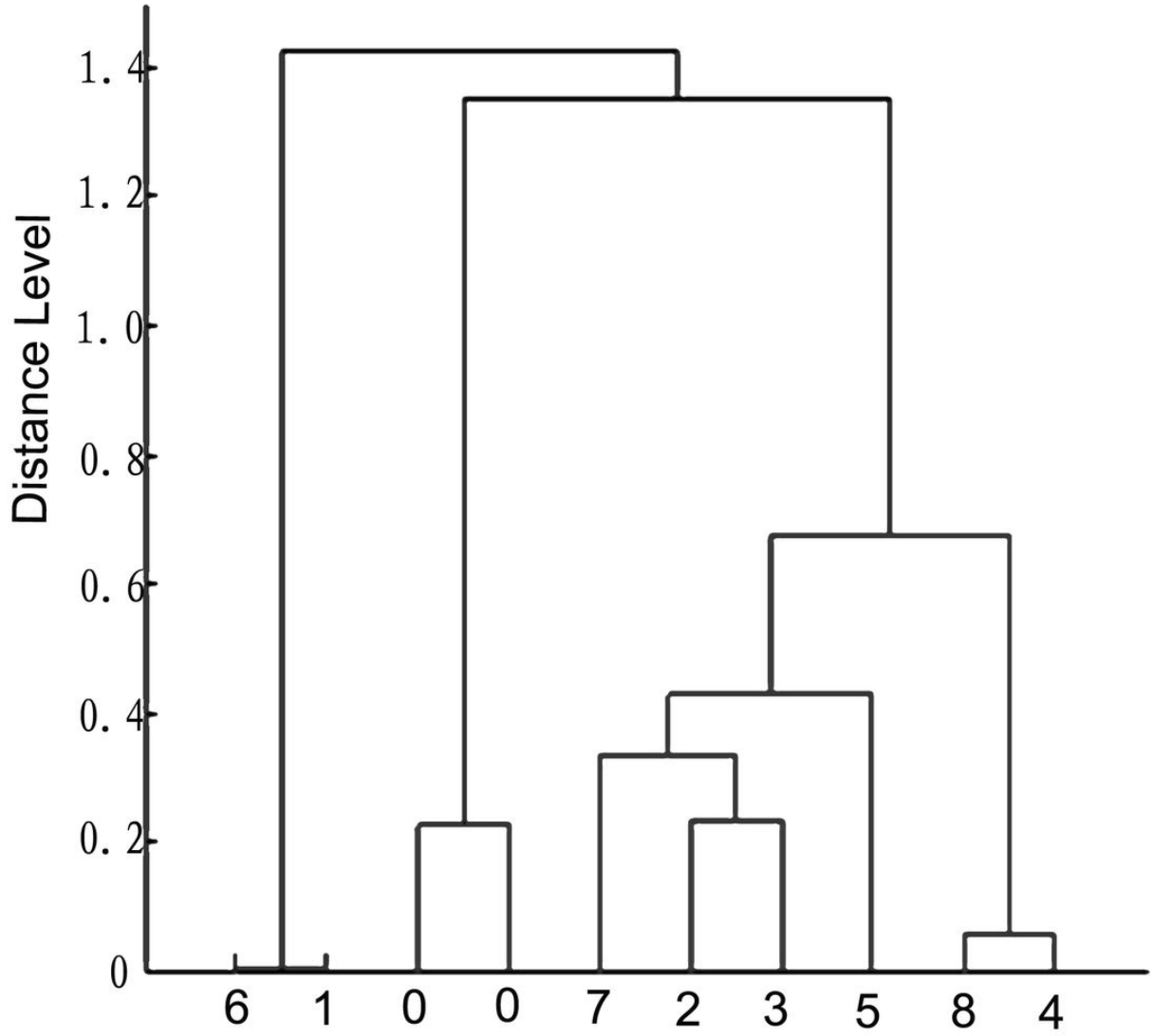

Figure 2

The dendrogram of 8 strains of C. gattii and 2 strains of C.neoformans

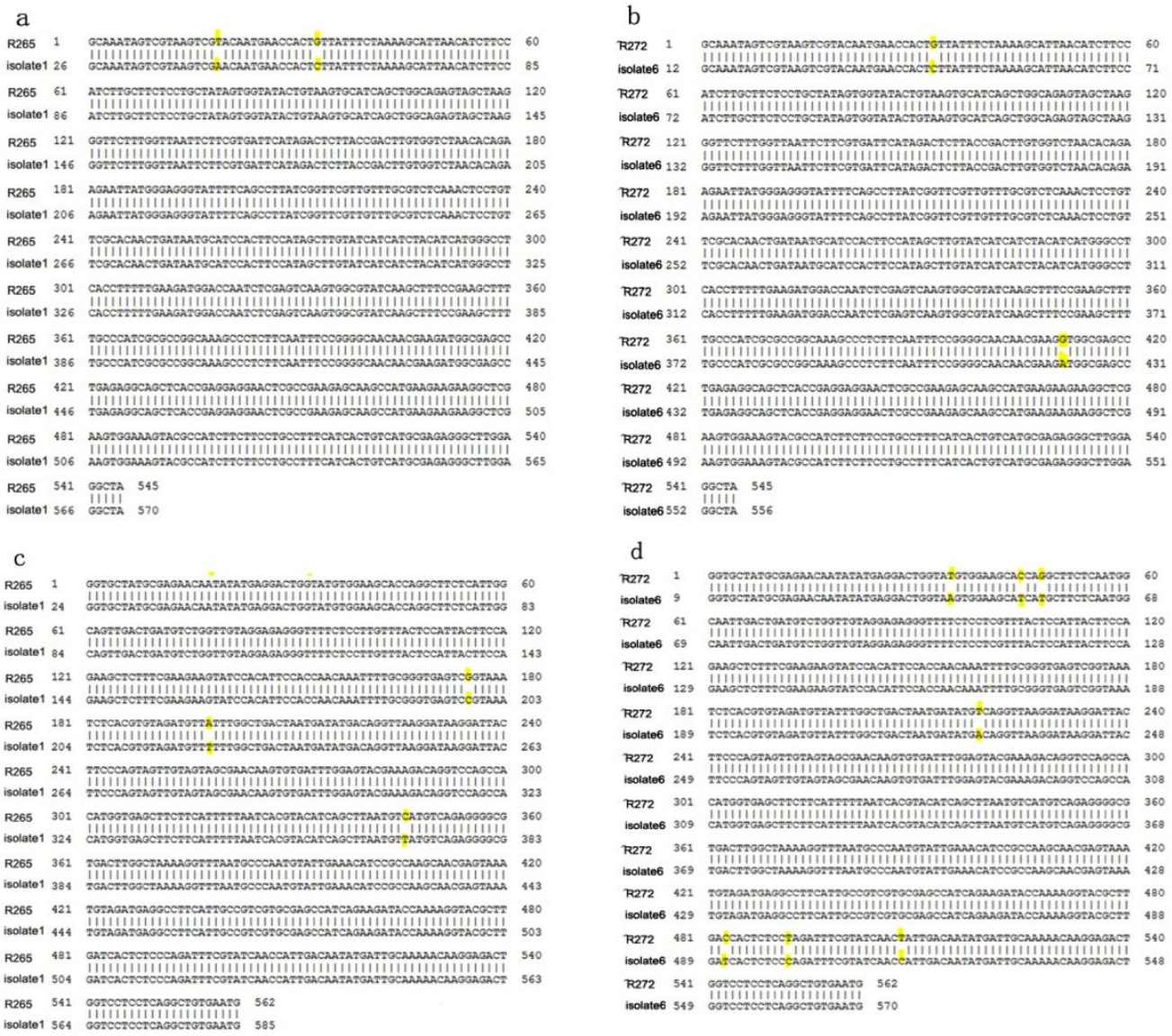

Figure 3

Gene mutations of 2 VGII strains (isolate number 1 and 6) in unlinked gene loci FTR1 and RAS1 compared with reference strains R265 and R272

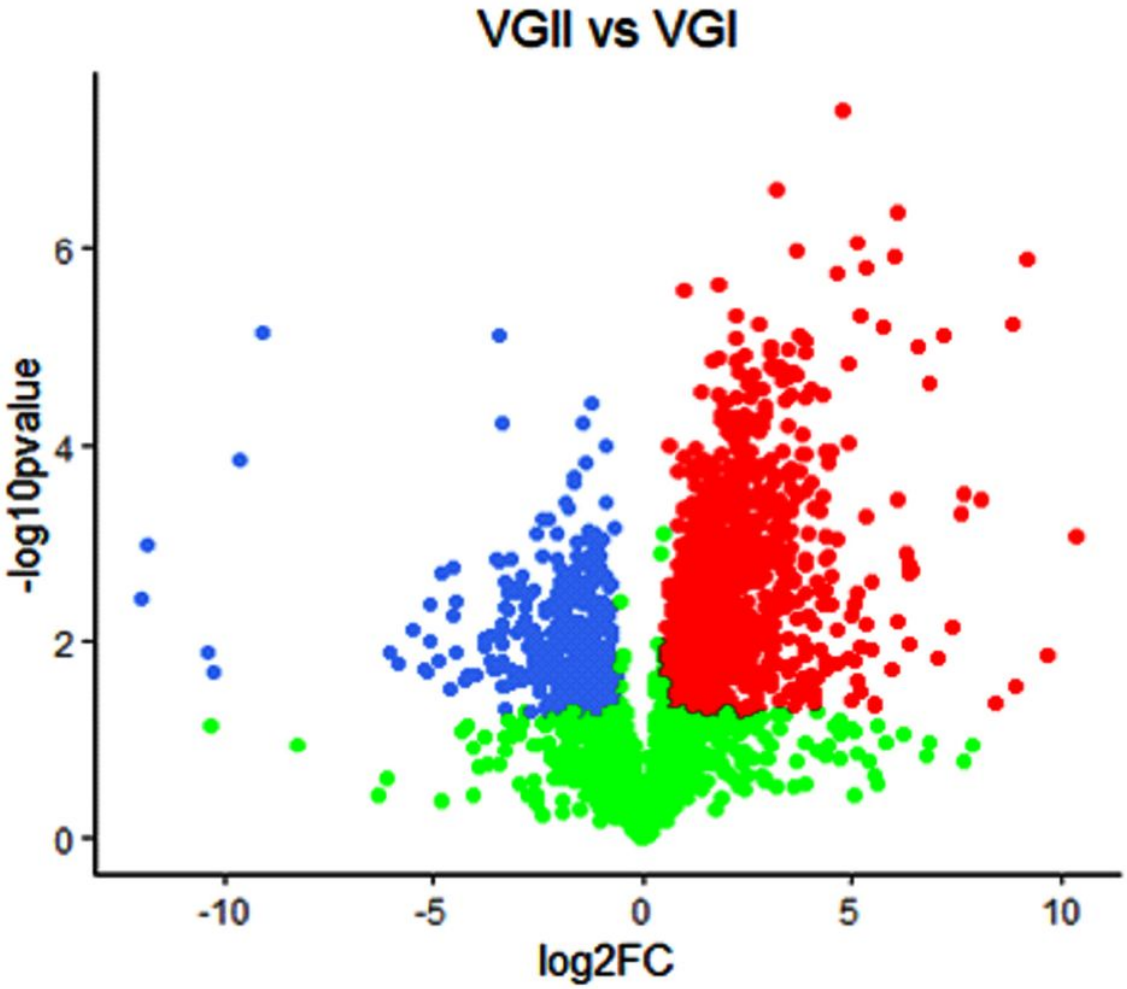

Figure 4

Volcanic map of identified proteins Notes: The red and blue dot represented the profile for 329 and 180 highly expressed proteins of C.gattii VGI and VGII ($p < 0.05$); the green dot represented the proteins without significant difference.

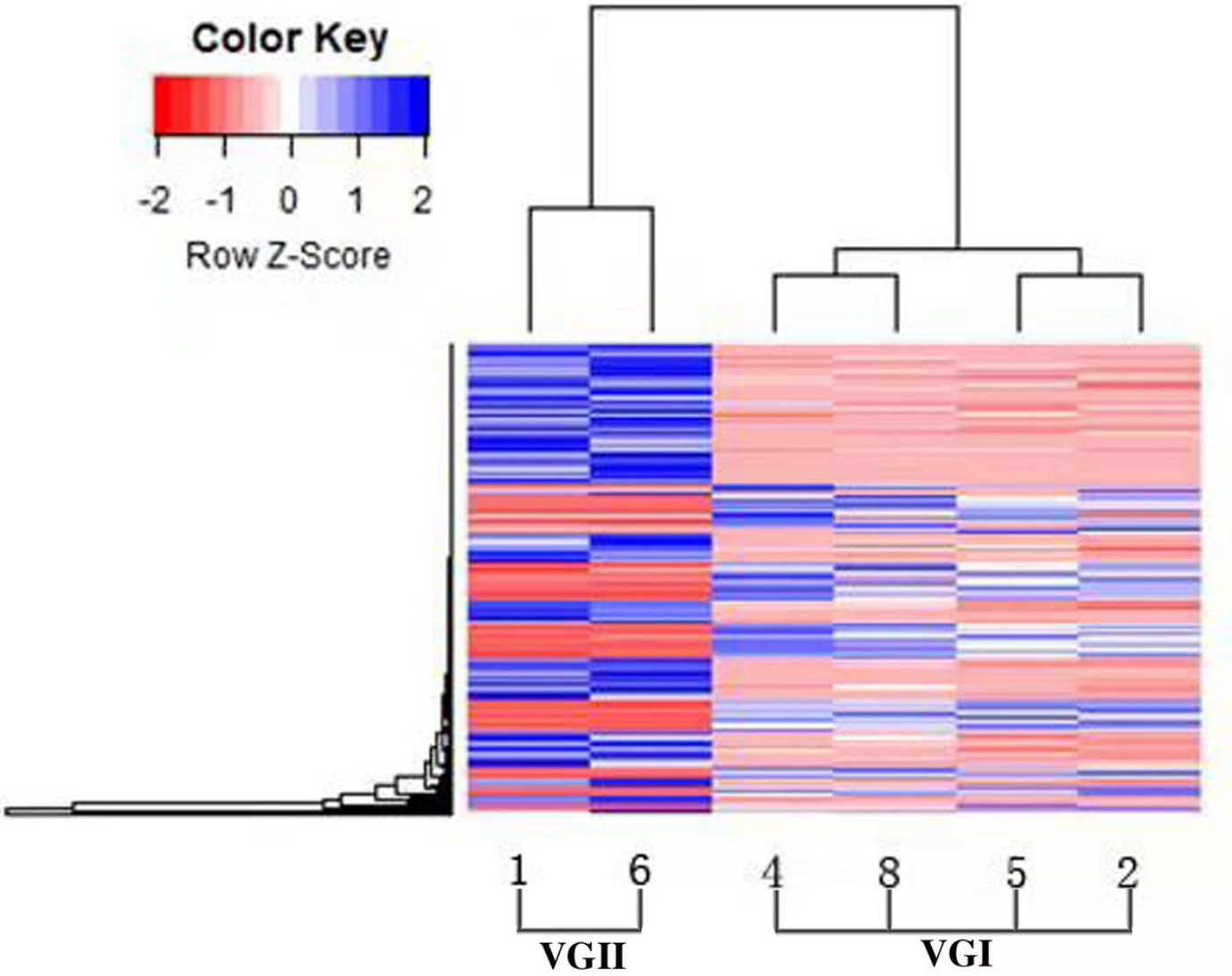

Figure 5

Clustering of differential expression proteins Notes: Blue and red represented high and low expression proteins respectively

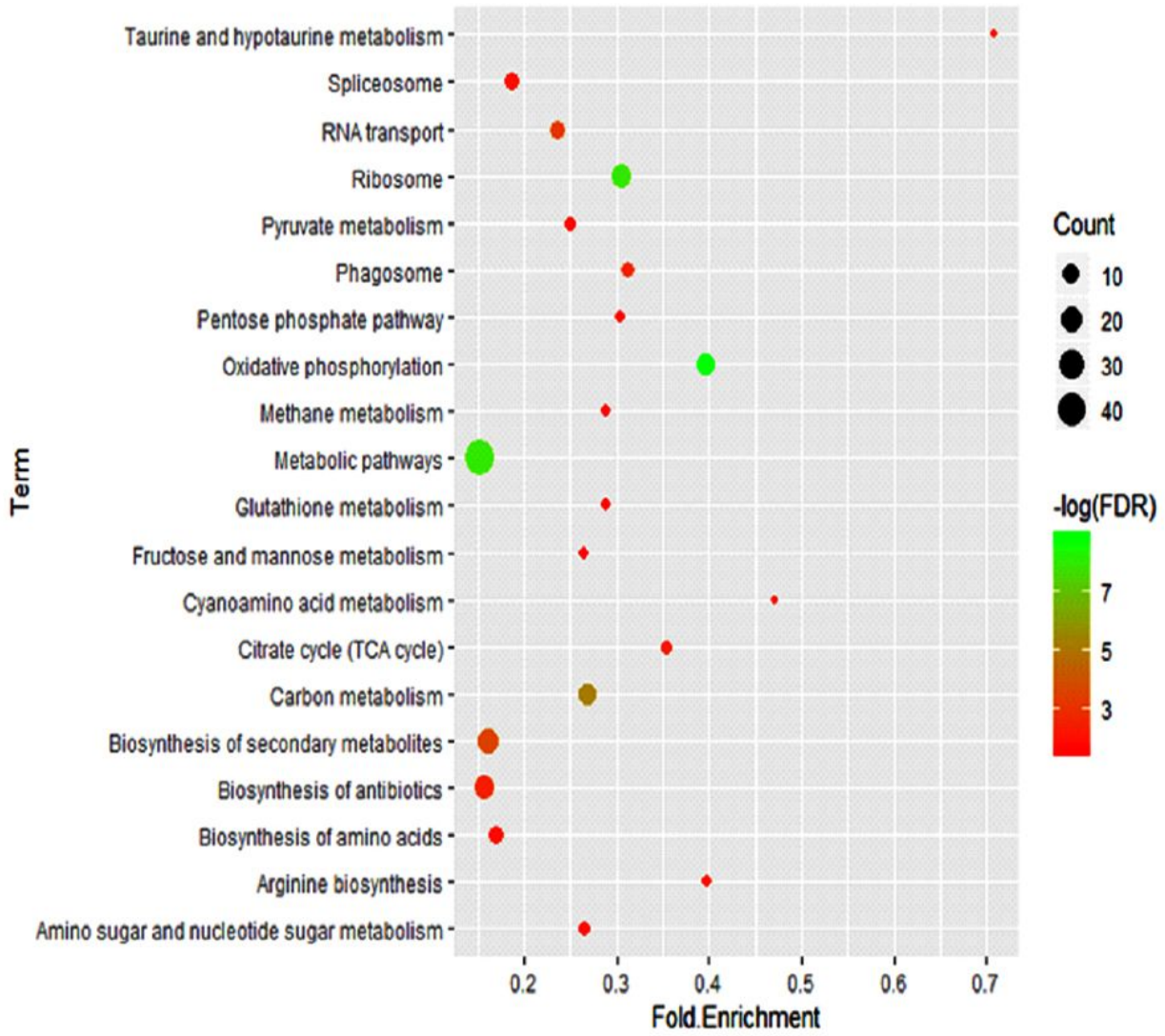

Figure 6

Enrichment Analysis of differential proteins by KEGG metabolic pathway Notes: The circle was used to represent the enrichment of each KEGG term, the diameter of the circle represented the number of differentially expressed genes commented to each KEGG term species, the color of the circle represented the significance of the corresponding KEGG term (p value corrected by FDR). Bottom transverse coordinate represented “Rich factor”, the larger the value, the higher the proportion of the number of differentially expressed genes to the total number of corresponding KEGG term gene.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryTables.docx