

Identification of CALM as the potential serum biomarker for predicting the recurrence of nasopharyngeal carcinoma using a mass spectrometry-based comparative proteomic approach

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Abstract. To date, there are no serum biomarkers available for the prediction of recurrent nasopharyngeal carcinoma (rNPC). The diagnosis of rNPC mostly depends on imaging and biopsy of diseased tissue; however, both of these methods work mostly if the target tumor is at an advanced stage. Therefore, the identification of recurrent biomarkers is urgently required. In the present study, we used tandem mass tag (TMT) labeling and high performance liquid chromatography (HPLC) fractionation followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) to identify differentially expressed proteins. Serum was collected from 40 patients with NPC [recurrence (n=20) and no recurrence (n=20)]. Compared to non-recurrent NPC (nrNPC), we found 59 proteins to be significantly dysregulated in rNPC; most of these have been previously reported to play a role in carcinogenesis. The dysregulation of calmodulin (CALM) was confirmed in 74 new patients [recurrence (n=32) and no recurrence (n=42)] by ELISA. Moreover, we performed a preliminary pathway analysis which revealed that oxidative phosphorylation was altered in the patients with rNPC compared to those with nrNPC. Taken together, these data identify a potential diagnostic biomarker for rNPC and elucidate the potential molecular mechanisms that are dysregulated and contribute to the pathogenesis of rNPC.

Introduction

Nasopharyngeal carcinoma (NPC) is one of the most common types of cancer affecting populations in Southern

China and Southeast Asia. It has led to a very serious health concern in these areas (1,2). As far as we know, due to its location-specific characteristics and sensitivity to radiation, radiotherapy (RT) is the main treatment for NPC (3). With the appearance of intensity-modulated radiation therapy (IMRT), dose escalation, and the use of concurrent chemotherapy, the 5-year survival rate for patients with early-stage NPC can reach approximately 90%, although for patients with advanced NPC, the rate is 70% (4). However, approximately 20% of patients with NPC suffer from local recurrence even after the above-mentioned effective therapies (1). Currently, the detection of recurrent NPC (rNPC) mainly depends on the imaging or biopsy examination of diseased tissue; both of these methods work mostly if the target tumor is at an advanced stage (5). Furthermore, it is difficult to obtain tissue from recurrent disease for biopsy, and the difference between the side of recurrence and the tissue which changed by RT in imaging is difficult to distinguish. Therefore, the identification of serum biomarkers is warranted. In the first place, compared to tissue biopsy, the collection of serum is a less harmful method and serum is more easily obtained. Moreover, tumor biomarkers are molecules produced specifically or in excess of normal tissue by the pre-malignant cell, tumor cell or recurrent cancer cell, which may accumulate and are released into the circulation. These can help in the diagnosis, monitoring and prognosis of the treatment response (6). It is known that alpha-fetoprotein (AFP), cancer antigen (CA)-125, prostate-specific antigen (PSA) are used as serum biomarkers for the diagnosis, monitoring and the prognosis of liver, ovarian and prostate cancer, respectively. Serum biomarkers may also be used to identify recurrent cancers. At present, carcinoembryonic antigen (CEA) is a recurrent-related biomarker for colorectal tumors (7).

In addition, previous studies have reported some early predictive biomarkers for NPC, such as heat shock protein 27 (HSP27) (8), cathepsin D and keratin 8 (9), alpha-2 macroglobulin (A2M or AMG) and complement factor B (CFB) (10), as well as Galectin-1 (11). To date, some biomarkers for recurrent disease have also been reported, such as serum amyloid A (SAA) (12) and Epstein-Barr virus (EBV)

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DNA (13); however, these markers for rNPC still lack high predictive value in clinical practice. Therefore, there is an urgent need for the identification of novel putative biomarkers.

Furthermore, a preliminary pathway analysis is necessary, which can provide an approach with which to reveal the potential molecular mechanisms of recurrence, and which has been widely used in other diseases (14,15).

In the present study, in an aim to identify new biomarkers for rNPC, and in order to analyze serum-expressed proteins, we performed tandem mass tag (TMT) labeling and high performance liquid chromatography (HPLC) fractionation followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). We considered that this would be an effective tool for screening proteomic biomarkers.

Patients and methods

Ethics. The present study was approved by the Joint Ethics Committee of the Guangxi Medical University Health Authority. All patients provided written informed consent prior to obtaining any samples. All human serum and clinical information was obtained from the Affiliated Cancer Hospital of Guangxi Medical University. According to the criteria established by the Chinese staging system of NPC in 2008 and the UICC/AJCC staging system in 2010; each patient with NPC was diagnosed and confirmed by a pathological examination.

Patient information. We selected 114 patients with NPC with histologically proven squamous cell carcinoma who were treated by IMRT or conventional radiotherapy (con-RT) (a total prescribed dose of ≥ 70 Gy), using a modified linear accelerator at the Affiliated Cancer Hospital of Guangxi Medical University, China, from January 2010 to June 2015. In total we recruited 52 patients with rNPC and 62 with non-recurrent NPC (nrNPC). The patients with rNPC were defined as those with histologically proven recurrence, and with no metastasis proven by imaging following radical cure RT 3 months later (with or without chemotherapy). Patients with nrNPC were those in which no proven recurrence or metastasis following radical cure RT (with or without chemotherapy) was found. The basic clinical parameters of the included patients are shown in Table I.

Serum separation. A total of 3 ml venous blood was collected from each subject. Samples were placed standing for 30 min and then centrifuged at 3,000 rpm for 10 min. The supernatant was separated and stored in different tubes at -80°C . A part of the serum was utilized for the discovery of dysregulated peptide, whereas the other part was prepared for the validation of target proteins.

Materials and reagents. Two pairs (termed studies; 10 rNPC and 10 nrNPC/study) patient sera were randomly selected and dissolved in a refrigerator at $2-8^{\circ}\text{C}$. Subsequently, for reducing individual heterogeneity (16), an equal amount of 10 NPC samples from both the rNPC and nrNPC groups were mixed to generate 2 sample pools. Of the 20 Discovery samples (20 rNPC and 20 nrNPC) study1 randomly included 10 rNPC and 10 nrNPC samples; the remaining 10 rNPC and 10 nrNPC samples, were included as study2.

Table I. Basic clinical parameters between patients with recurrent NPC, and those with non-recurrent NPC.

A, Discovery samples			
Variables	nrNPC	rNPC	P-value
Sex (no.)			
Male	15	16	>0.05
Female	5	4	>0.05
Clinical stage (no.)			
Low (i+ii)	5	2	
High (iii+iv)	15	18	>0.05
Median age (years)	42	48.5	>0.05

B, Validation samples

Variables	nrNPC	rNPC	P-value
Sex (no.)			
Male	35	29	>0.05
Female	7	3	>0.05
Clinical stage (no.)			
Low (i+ii)	8	5	
High (iii+iv)	34	27	>0.05
Median age (years)	45	46	>0.05

In all the parameters, no significant differences were observed between the patients with recurrent nasopharyngeal carcinoma (rNPC) and those with non-recurrent nasopharyngeal carcinoma (nrNPC).

High-abundance protein depletion. To enhance the accuracy of TMT analysis, high-abundance proteins were removed from the pooled samples using the ProteMiner (Protein Enrichment Large-Capacity kit, cat. no. 163-3006; Bio-Rad Laboratories, Inc., Hercules, CA, USA). The concentrated proteins were immunodepleted one more time. Finally, the protein was precipitated with cold 15% TCA for 2 h at -20°C . Following centrifugation at $20,000 \times g$ at 4°C for 10 min, the supernatant was discarded. The remaining precipitate was washed with cold acetone 3 times. The protein was redissolved in buffer (8 M urea, 100 mM TEAB, pH 8.0), and the protein concentration was determined with 2-D Quant kit (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA). SDS-PAGE was used to evaluate the efficiency of high-abundance protein depletion in the two pooled samples (Fig. 1).

Trypsin digestion. For digestion, the protein solution was reduced with 10 mM DTT for 1 h at 37°C and alkylated with 20 mM IAA (both from Sigma, St. Louis, MO, USA) for 45 min at room temperature in the dark. For trypsin digestion, the protein sample was diluted by the addition of 100 mM TEAB to the urea concentration to <2 M. Finally, trypsin was added at 1:50 trypsin-to-protein mass ratio for the first digestion overnight and 1:100 trypsin-to-protein mass ratio for a second 4 h digestion. Approximately 100 μg protein for each sample was digested with trypsin for the following experiments.

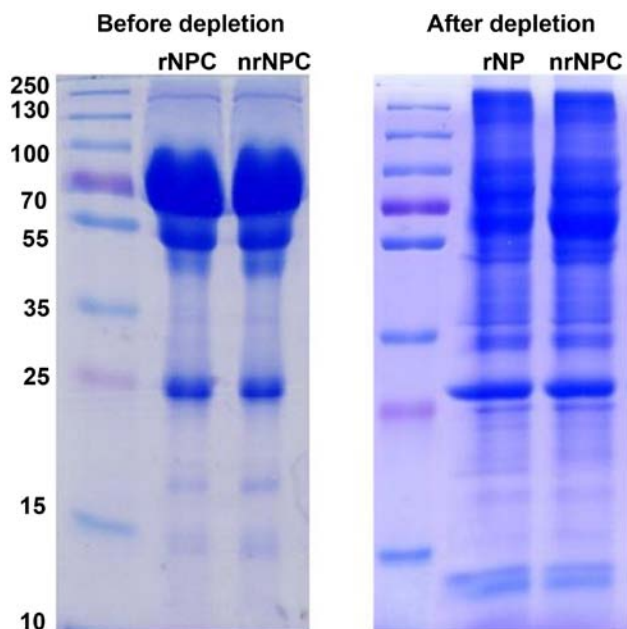


Figure 1. From SDS-PAGE, it is evident that high abundant protein was depleted well after depletion.

TMT labeling. Following trypsin digestion, for TMT labeling, peptide was desalted by a Strata-X C18 SPE column (Phenomenex, Inc., Torrance, CA, USA) and vacuum-dried. The peptide was reconstituted in 0.5 M TEAB and processed according to the manufacturer's instructions provided with the 6-plex TMT kit (Thermo Fisher Scientific, Waltham, MA, USA). Briefly, one unit of TMT reagent (defined as the amount of reagent required to label 100 μ g of protein) was thawed and reconstituted in 24 μ l ACN (Thermo Fisher Scientific, Inc.). The peptide mixtures were then incubated for 2 h at room temperature and pooled, desalted and dried by vacuum centrifugation.

HPLC fractionation. The sample was then fractionated into fractions by high pH reverse-phase HPLC using an Agilent 300Extend-C18 column (5 μ m particles, 4.6 mm ID, 250 mm length; Agilent Technologies, Santa Clara, CA, USA). Briefly, the peptides were first separated with a gradient of 2-60% acetonitrile in 10 mM ammonium bicarbonate pH 10.0 over 80 min into 80 fractions. The peptides were then combined into 18 fractions and dried by vacuum centrifugation.

LC-MS/MS analysis. The peptides were dissolved in 0.1% FA (Fluka, Buchs St. Gallen, Switzerland), directly loaded onto a reversed-phase pre-column (Acclaim PepMap 100; Thermo Fisher Scientific, Inc.). Peptide separation was performed using a reversed-phase analytical column (Acclaim PepMap RSLC; Thermo Fisher Scientific, Inc.). The gradient was comprised of an increase from 7 to 20% solvent B (0.1% FA in 98% ACN) over 24 min, 20-35% in 8 min, 35-80% for 5 min and holding at 80% for the last 3 min, all at a constant flow rate of 300 nl/min on an EASY-nLC 1000 UPLC system (Thermo Fisher Scientific, Inc.). The resulting peptides were analyzed by Q Exactive™ Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher Scientific, Inc.).

The peptides were subjected to NSI source followed by tandem mass spectrometry (MS/MS) on a Q Exactive™ mass spectrometer (Thermo Fisher Scientific, Inc.) coupled online to the UPLC. Intact peptides were detected in the Orbitrap at a resolution of 70,000. Peptides were selected for MS/MS using NCE setting as 27, 30, 33; ion fragments were detected in the Orbitrap at a resolution of 17,500. A data-dependent procedure that alternated between one MS scan followed by 20 MS/MS scans was applied for the top 20 precursor ions above a threshold ion count of 1.0E4 in the MS survey scan with 30.0 sec dynamic exclusion. The electrospray voltage applied was 2.0 kV. Automatic gain control was used to prevent overfilling of the ion trap; 5E4 ions were accumulated for generation of MS/MS spectra. For MS scans, the m/z scan range was 350-1,800. Fixed first mass was set as 100 m/z.

Database search. The resulting MS/MS data were processed using Mascot search engine (v.2.3.0). Tandem mass spectra were searched against the Swiss-Prot Human Database (20,203 sequences). Trypsin/P was specified as cleavage enzyme allowing up to 2 missing cleavages. Mass error was set to 10 ppm for precursor ions and 0.02 Da for fragment ions. Carbamidomethyl on Cys, TMT-6 plex (N-term) and TMT-6plex (K) were specified as fixed modification and oxidation on Met was specified as variable modifications. FDR was adjusted to <1% and peptide ion score was set >20.

QC validation of MS data. Firstly, we checked the mass error of all the identified peptides. The distribution of mass error was near zero and most of them were <0.02 Da, which meant that the mass accuracy of the MS data fit the requirement. Secondly, the length of most peptides was distributed between 8 and 16, which are in agreement with the property of tryptic peptides, which meant that sample preparation reached the standard (Fig. 2).

Bioinformatics methods

Gene Ontology (GO) analysis. All proteins identified in the serum samples were assigned a gene symbol using the UniProt-GOA database (<http://www.ebi.ac.uk/GOA/>). If some identified proteins were not annotated by UniProt-GOA database, the InterProScan software would be used to annotated protein's GO functional based on protein sequence alignment method. Protein classification was performed by GO annotation based on biological process, cellular component and molecular function. The differentially expressed proteins were further analyzed for pathway enrichment analysis by using Kyoto Encyclopedia of Genes and Genomes (KEGG) database. At the same time, we performed protein functional enrichment and performed a functional enrichment-based clustering for protein groups.

Selection of rNPC biomarkers. Dysregulated proteins were selected for further verification if they met one or more requirements: i) it should be a 'secreted' protein (14); ii) was shown to be involved on the KEGG database; and iii) the fold change of the protein was ≥ 1.2 or ≤ 0.83 .

ELISA validation. Human ELISA kits were used for calmodulin (CALM; TSZ Bioscience, North Brunswick, NJ, USA), and experimental steps were performed as recommended by

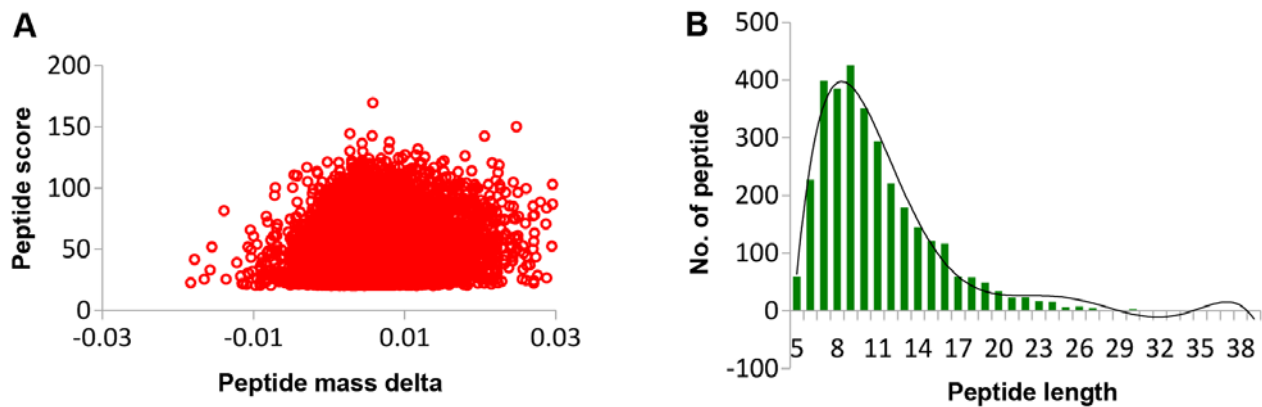


Figure 2. QC validation of MS data. (A) Mass error distribution of all identified peptides; (B) peptide length distribution. MS, mass spectrometry.

the manufacturer. Serum samples were analyzed for a total of 74 patients with NPC (32 rNPC + 42 nrNPC).

Statistical analysis. All statistical analyses were performed using SPSS 21.0 software and GraphPad Prism 5.0 software. The differences between the two states of NPC were analyzed using a Mann-Whitney U test. In addition, the target protein was evaluated for its capacity to distinguish between rNPC and nrNPC by making their ROC curves based on the ELISA scores. A value of $P < 0.05$ was considered to indicate a statistically significant difference.

Results

A schematic of the work flow is shown in Fig. 3. Using TMT labeling and HPLC fractionation followed by high-resolution LC-MS/MS analysis, we compared the 2 pooled NPC samples from the 10 patients with rNPC and the 10 patients with nrNPC (controls) in biological repeats. We identified 635 protein groups from pooling human serum with a 99% confidence score and a 1% local FDR in each of the 2 analyzed groups, among which 413 proteins were quantified proteins, all were considered to be dysregulated if TMT ratios were ≥ 1.2 or ≤ 0.83 in $\geq 50\%$ in rNPC relative to nrNPC; finally, dysregulated proteins were obtained in each group. The dysregulated proteins are shown in Table II and Fig. 4.

Functional classification of differentially quantified proteins. According to the GO annotation information of identified proteins, we calculated the number of differentially expressed proteins in each GO term of level 2. The upregulated proteins were main involved in the biological process of single-organism process (26/29), cellular process (25/29), response to stimulus 20/29 etc, and the cellular component main included cell (23/29), organelle (21/29), macromolecular complex (13/29) etc. Molecular function mainly included binding (15/29), catalytic activity (11/29) etc. The details are shown in Table III.

The downregulated proteins were main involved in single-organism process (40/54), biological regulation (39/54), response to stimulus (35/54) etc, the cellular component main included extracellular region (37/54), cell (34/54),

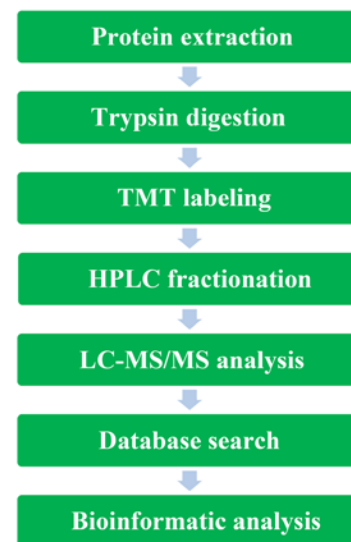


Figure 3. The aim of this study was to use an integrated approach involving TMT labeling, HPLC fractionation and mass spectrometry-based quantitative proteomics to quantify dynamic changes of the whole proteome of human serum. The general technical route is indicated. TMT, tandem mass tags; HPLC, high performance liquid chromatography.

membrane (20/54) etc. Molecular function main included binding (15/29), catalytic activity (11/29) etc. The details are shown in Table III.

GO-based enrichment analysis. We then we performed a GO-based enrichment analysis, molecular function included guanyl ribonucleotide binding, GTP binding etc, and most proteins were formed nucleosome, DNA bending complex, microtubule cytoskeleton etc. In addition, numerous proteins were involved in response to calcium, regulated cell cycle biological process etc. The details are shown in Fig. 5.

KEGG pathway enrichment. KEGG pathway enrichment included oxidative phosphorylation. According to KEGG pathway analysis, CALM was enriched in a significant pathway, and the enrichment ratio of CALM was the highest; moreover, the fold change of CALM was 2.11 (Fig. 6). Thus, we selected CALM to perform a further confirmed test.

Table II. Differentially expressed and dysregulated proteins in nasopharyngeal carcinoma.

A, Summary of differentially quantified proteins (>1.2, or <0.83)

Name	Upregulated (>1.2)	Downregulated (<0.83)
Y1vsN1	29	54
Y2vsN2	29	65
YvsN	28	47
N1vsN2	0	1
Y1vsY2	0	1

B, Top 5 of upregulated proteins (Y1:rNPC, N1:rNPC)

Protein accession no.	Gene name	Protein description	MW (Da)	pI	AASC (%)	MP	Score	Y1/N1 ratio	Y1/N1 P-value
P02741	CRP	C-reactive protein	28,631	5.45	23.7	7	781	3.862	3.249E-12
P13637	ATP1A3	Sodium/potassium-transporting ATPase subunit alpha-3	126,394	5.22	2.9	2	98	2.356	0
P02686	MBP	Myelin basic protein	37,681	9.79	7.2	2	78	2.26	0.030696
P62158	CALM2	Calmodulin	18,889	4.09	16.1	3	40	2.11	0.019282
P60174	TPI1	Triosephosphate isomerase	35,869	5.65	7	2	49	2.045	0.127868

C, Top 5 of downregulated proteins

Protein accession no.	Gene name	Protein description	MW (Da)	pI	AASC (%)	MP	Score	Y1/N1 ratio	Y1/N1 P-value
P25705	ATP5A1	ATP synthase subunit alpha, mitochondrial	66,932	9.16	12.1	6	200	0.280	8.69E-06
Q99798	ACO2	Aconitate hydratase, mitochondrial	98,946	7.36	2.2	2	64	0.329	0.036558
P06576	ATP5B	ATP synthase subunit beta, mitochondrial	62,025	5.26	12.9	5	170	0.339	0.003399
P09972	ALDOC	Fructose-bisphosphate aldolase C	44,872	6.41	10.2	2	129	0.347	0.2142
P11678	EPX	Eosinophil peroxidase	86,771	10.31	7.6	4	99	0.408	0.038348
P61160	ACTR2	Actin-related protein 2	50,976	6.3	4.6	2	91	0.501	0.148856

D, All dysregulated proteins

Protein accession no.	Protein description	Y/N ratio	Regulated type
P02741	C-reactive protein	3.862	Up
Q9BXR6	Complement factor H-related protein 5	1.433	Up
P62158	Calmodulin	2.11	Up
P67936	Tropomyosin alpha-4 chain	1.464	Up
O43852	Calumenin	1.361	Up
P04211	Ig lamda chain V region 4A	1.517	Up
P02743	Serum amyloid P-component	1.295	Up
P20742	Pregnancy zone protein	1.208	Up
Q15485	Ficolin-2	1.502	Up
P0DJ19	Serum amyloid A-2 protein	2.036	Up
P02671	Fibrinogen α chain	1.217	Up
P01598	Ig kappa chain V-I region EU	1.507	Up
Q9HBI1	Beta-parvin	1.638	Up
Q9Y490	Talin-1	1.521	Up
Q13201	Multimerin-1	1.238	Up
Q08830	Fibrinogen-like protein 1	1.361	Up

Table II. Continued.

Protein accession no.	Protein description	Y/N ratio	Regulated type
P02686	Myelin basic protein	2.26	Up
P35542	Serum amyloid A-4 protein	1.494	Up
P05155	Plasma protease C1 inhibitor	0.763	Down
P80748	Ig lamda chain V-III region LOI	0.772	Down
P05534	HLA class I histocompatibility antigen, A-24 α chain	0.545	Down
O75882	Attractin	0.616	Down
P36955	Pigment epithelium-derived factor	0.721	Down
P06314	Ig kappa chain V-IV region B17	0.759	Down
Q5QNW6	Histone H2B type 2-F	0.693	Down
P01597	Ig kappa chain V-I region DEE	0.747	Down
P04217	Alpha-1B-glycoprotein	0.729	Down
Q16610	Extracellular matrix protein 1	0.675	Down
P06312	Ig kappa chain V-IV region (fragment)	0.746	Down
P25705	ATP synthase subunit alpha, mitochondrial	0.28	Down
P25311	Zinc-alpha-2-glycoprotein	0.678	Down
P23083	Ig heavy chain V-I region V35	0.687	Down
P01009	Alpha-1-antitrypsin	0.78	Down
P01767	Ig heavy chain V-III region BUT	0.671	Down
P08571	Monocyte differentiation antigen CD14	0.58	Down
P06576	ATP synthase subunit β , mitochondrial	0.339	Down
P01042	Kininogen-1	0.817	Down
P01859	Ig gamma-2 chain C region	0.765	Down
P01860	Ig gamma-3 chain C region	0.766	Down
P32119	Peroxiredoxin-2	0.642	Down
Q99798	Aconitate hydratase, mitochondrial	0.329	Down
P01034	Cystatin-C	0.769	Down
P01593	Ig κ chain V-I region AG	0.535	Down
P01834	Ig κ chain C region	0.789	Down
P01615	Ig κ chain V-II region FR	0.785	Down
P01781	Ig heavy chain V-III region GAL	0.77	Down
P49747	Cartilage oligomeric matrix protein	0.659	Down
B9A064	Immunoglobulin lamda-like polypeptide 5	0.778	Down
P01611	Ig kappa chain V-I region Wes	0.755	Down
P00488	Coagulation factor XIII A chain	0.764	Down
P68871	Hemoglobin subunit beta	0.566	Down
P02790	Hemopexin	0.606	Down
P00738	Haptoglobin	0.818	Down
P06396	Gelsolin	0.711	Down
P62805	Histone H4	0.766	Down
P01625	Ig kappa chain V-IV region Len	0.755	Down
P02763	Alpha-1-acid glycoprotein 1	0.757	Down
P01768	Ig heavy chain V-III region CAM	0.64	Down
P05546	Heparin cofactor 2	0.754	Down

Y represents recurrent nasopharyngeal carcinoma (rNPC); N represents non-recurrent nasopharyngeal carcinoma (nrNPC).

Clustering analysis. KEGG pathway enrichment-based clustering demonstrated that the dysregulated proteins mainly involved oxidative phosphorylation etc; the details are shown in Fig. 7. Functional enrichment-based clustering revealed that the dysregulated proteins were mainly involved in responding

to calcium ion, oxidative stress, transport and secretion. The details are shown in Fig. 8.

ELISA results. We performed a preliminary analysis to assess the potential value of CALM as a biomarker in serum from

Table III. The GO terms of level 2 distribution of the proteins of Y/N.

GO terms level 1	GO terms level 2	No. of protein
Upregulated proteins		
Biological process	Single-organism process	26
	Cellular process	25
	Response to stimulus	20
	Biological regulation	19
	Localization	14
	Metabolic process	13
	Signaling	12
	Multicellular organismal process	12
	Cellular component organization or biogenesis	12
	Developmental process	9
	Immune system process	7
	Locomotion	5
	Multi-organism process	4
	Other	5
Cellular component	Cell	23
	Organelle	21
	Macromolecular complex	13
	Membrane	11
	Extracellular region	10
	Membrane-enclosed lumen	8
	Cell junction	2
	Other	2
Molecular function	Binding	15
	Catalytic activity	11
	Structural molecule activity	6
	Enzyme regulator activity	3
	Electron carrier activity	1
Downregulated proteins		
Biological process	Single-organism process	40
	Biological regulation	39
	Response to stimulus	35
	Metabolic process	29
	Cellular process	27
	Multicellular organismal process	20
	Immune system process	17
	Localization	13
	Developmental process	12
	Cellular component organization or biogenesis	11
	Multi-organism process	8
	Other	16
Cellular component	Extracellular region	37
	Cell	34
	Membrane	20
	Organelle	20

Table III. Continued.

GO terms level 1	GO terms level 2	No. of protein
	Macromolecular complex	13
	Membrane-enclosed lumen	11
	Extracellular matrix	5
	Other	2

GO, Gene Ontology. Y represents recurrent nasopharyngeal carcinoma (rNPC); N represents non-recurrent nasopharyngeal carcinoma (nrNPC).

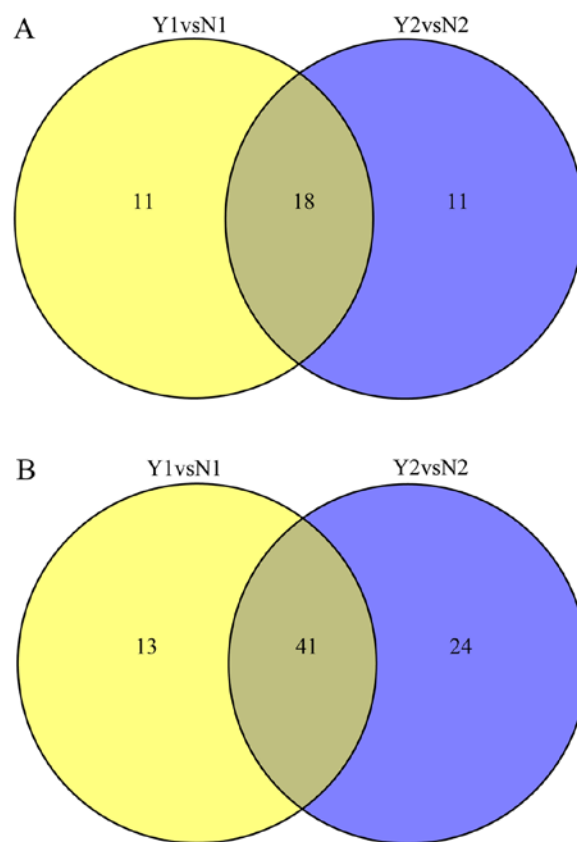


Figure 4. Venn diagrams showing the number of (A) upregulated and (B) down-regulated protein identifications and identification overlap3 of study 1 and 2. Y represents recurrent nasopharyngeal carcinoma (rNPC); N represents non-recurrent nasopharyngeal carcinoma (nrNPC).

patients with rNPC. We found a significant difference between patients with rNPC compared to those with nrNPC (mean \pm SD) (237.1 ± 307 ng/l vs. 104 ± 115.3 ng/l), respectively ($P=0.0233$, $P<0.05$; Fig. 9) in the levels of serum, which was in agreement with our MS results. Furthermore, we performed a ROC curve analysis to evaluate the predictive value of serum CALM; the area under the ROC curve was 0.6931 (95% CI: 0.5419-0.8443, $P=0.02275$, $P<0.05$). In addition, we have to point out that the total number in our sample was 74 patients (32 rNPC + 42 nrNPC), for the reason that some samples contained a low concentration which was beyond the reach of the ELISA kit; the concentration of CALM was only found

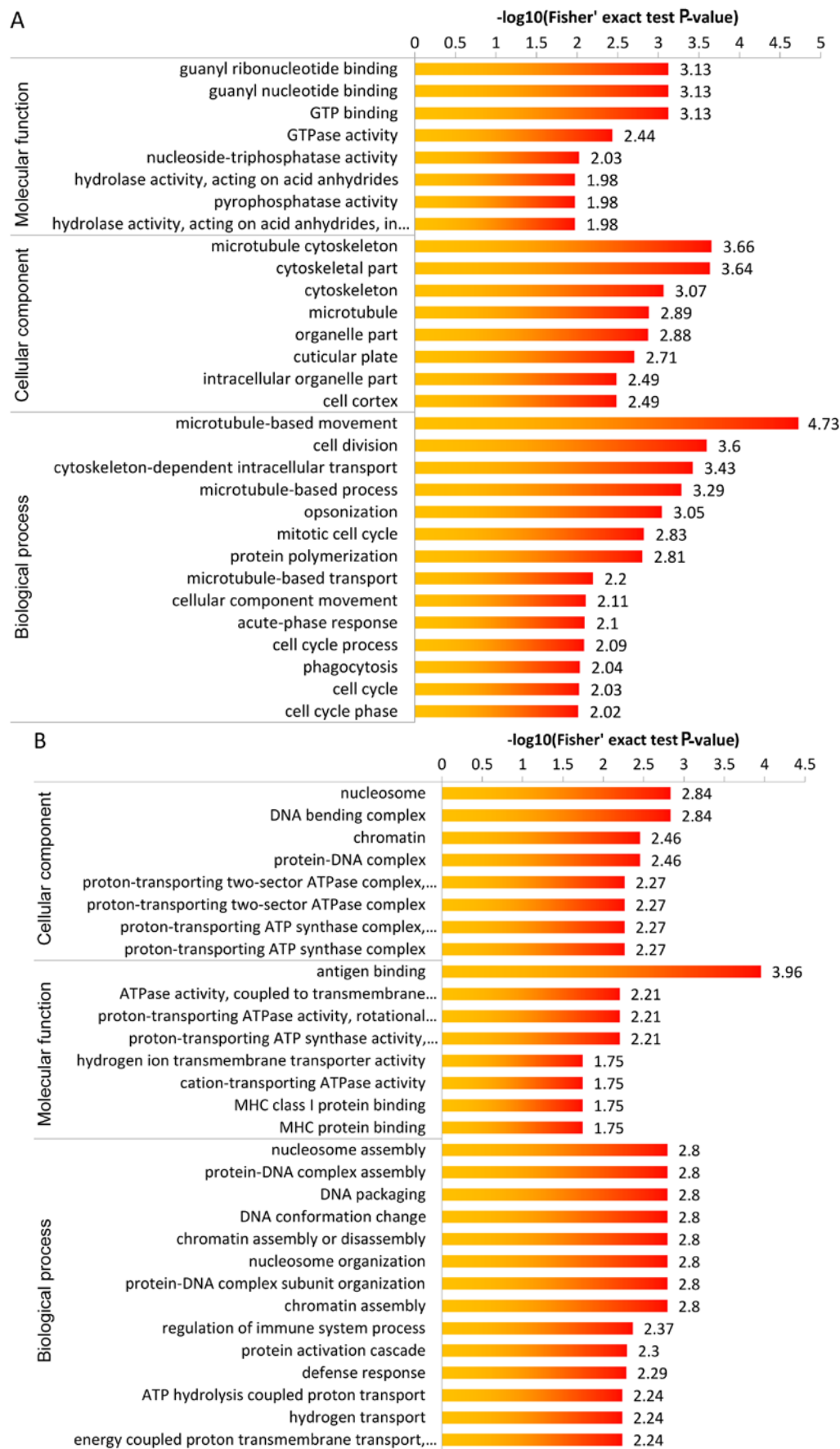


Figure 5. (A) GO-based enrichment analysis of upregulated proteins, which shows guanyl ribonucleotide binding, guanyl nucleotide binding, GTP binding were the highest enrichment in cellular component, microtubule cytoskeleton, cytoskeletal part were the highest enrichment in molecular function, microtubule-based movement was the highest enrichment in biological process. (B) GO-based enrichment analysis of downregulated proteins, which shows nucleosome and DNA bending complex were the highest enrichment in cellular component, and antigen binding was the highest enrichment in molecular function, and nucleosome assembly protein-DNA complex assembly, DNA packaging were the highest enrichment in biological process. GO, Gene Ontology.

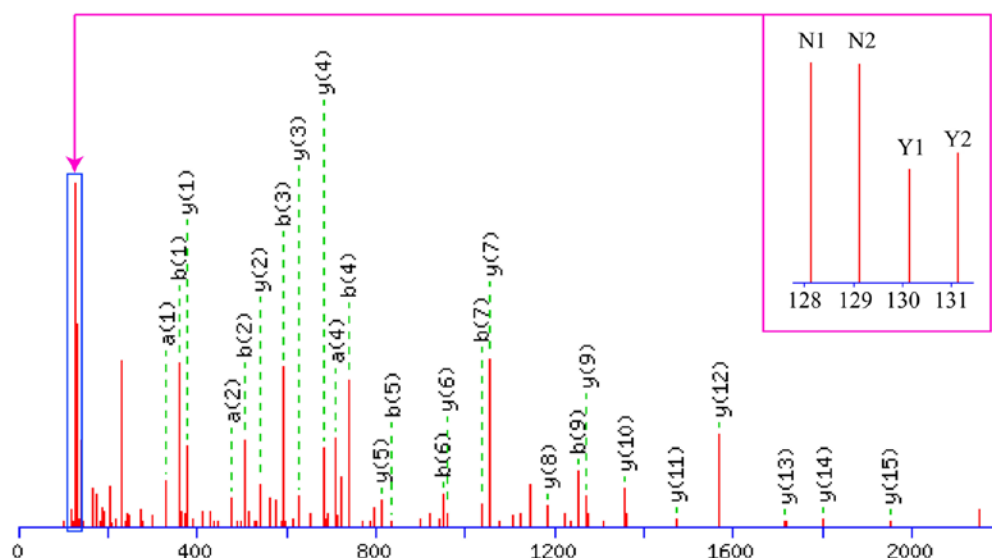


Figure 6. MS/MS spectrum of CALM. MS/MS, tandem mass spectrometry; CALM, calmodulin.

KEGG pathway

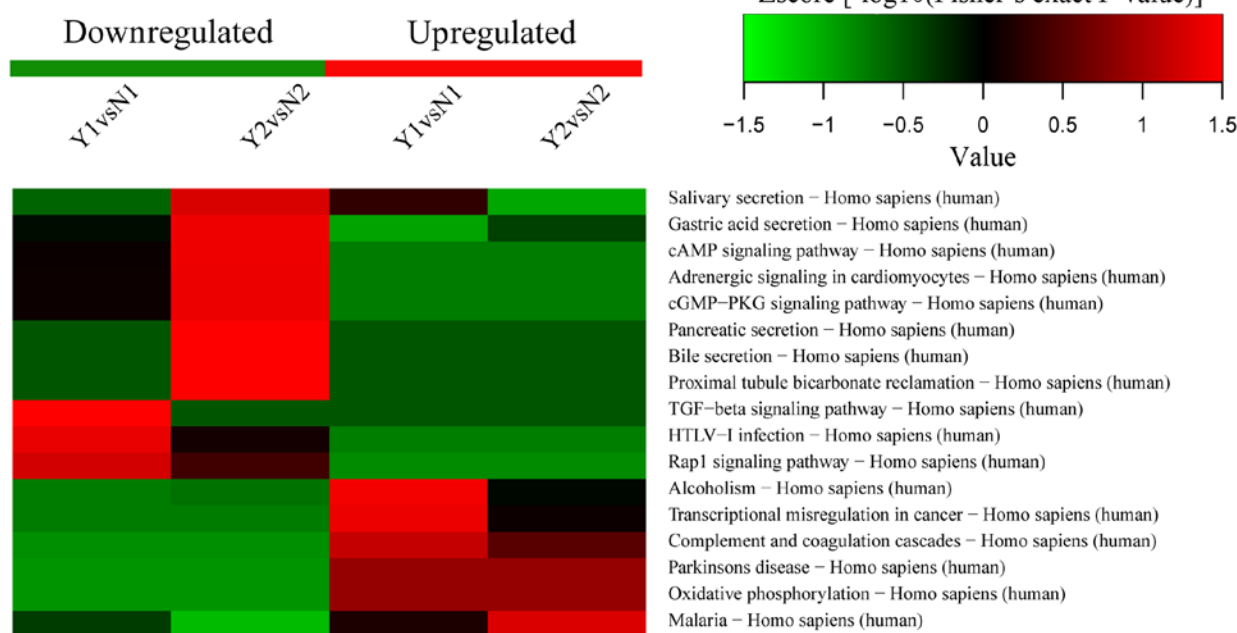


Figure 7. Heatmaps obtained from KEGG enrichment-based cluster analysis, which shown pathway of alcoholism - *Homo sapiens* (human), salivary secretion - *Homo sapiens* (human), malaria - *Homo sapiens* (human), Parkinson's disease - *Homo sapiens* (human), oxidative phosphorylation - *Homo sapiens* (human) were meaningful pathways in this process. KEGG, Kyoto Encyclopedia of Genes and Genomes. Y represents recurrent nasopharyngeal carcinoma (rNPC); N represents non-recurrent nasopharyngeal carcinoma (nrNPC).

in only 49 patients (29 rNPC + 20 nrNPC). The difference of the constituent ratio [3/29 (rNPC) vs. 22/42 (nrNPC)] was significant ($P=0.000$), which expressed that rNPC serums were more easily to be tested for CALM. These data suggest that CALM may be a promising and useful protective marker for patients with rNPC.

Discussion

A tremendous challenge in rNPC is the lack of tools which can be used for the early diagnosis of the disease. However,

imaging, such as CT, MRI or PET-CT has limited accuracy and cannot confirm the nature of the lesion. Moreover, biopsy is an invasive approach with some side-effects. This results in some patients being diagnosed at a late stage. A non-invasive blood-based test will be a revolutionary step in tumor diagnosis.

In this study, we conducted comprehensive quantitative proteomics analysis to identify a promising biomarker which can be further studied as a non-invasive test for the diagnosis of rNPC. We performed the study in biological replicates.

In two of these, we identified 94 and 83 proteins that were significantly dysregulated in rNPC compared to nrNPC,



Figure 8. (A) Heatmaps obtained from biological process enrichment-based cluster analysis, which show antigen binding, proton-transporting ATPase activity, rotational mechanism, hydrogen ion transmembrane transporter activity and MHC class I protein binding processes were the highest enrichment parts.

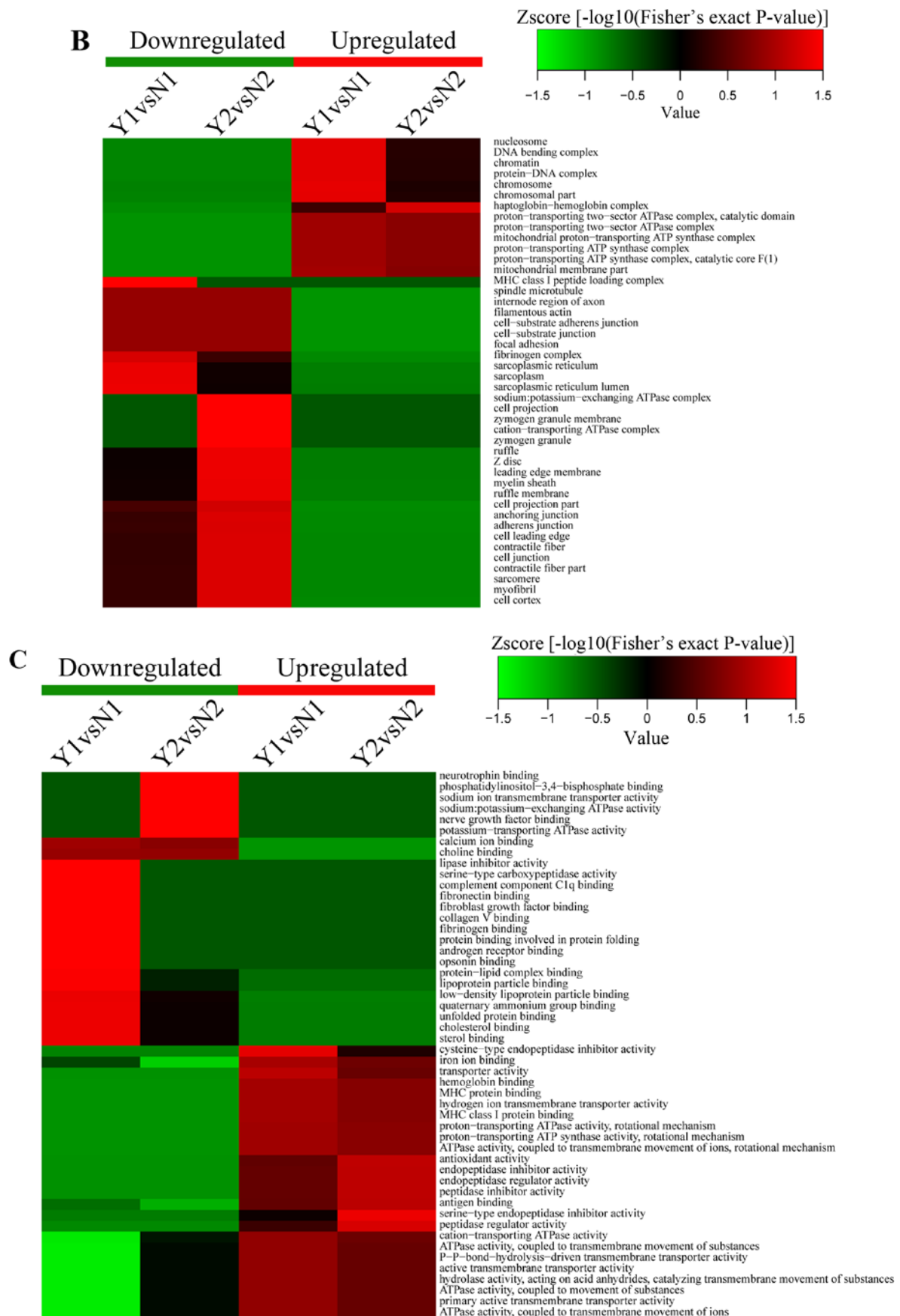


Figure 8. Continued. (B) Heatmaps obtained from cellular component enrichment-based cluster analysis, which show nucleosome, DNA bending complex, chromatin, protein-DNA complex, proton-transporting two-sector ATPase complex and ATPase activity, coupled to transmembrane movement of ions, and rotational mechanism were the meaningful enrichment parts. (C) Heatmaps obtained from molecular function enrichment-based cluster analysis, which show function of proton-transporting ATPase activity, rotational mechanism, ATPase activity, coupled to transmembrane movement of ions, rotational mechanism, heme transporter activity, protein transmembrane transporter activity and isocitrate hydro-lyase (cis-aconitate-forming) activity were the meaningful enrichment parts. Y represents recurrent nasopharyngeal carcinoma (rNPC); N represents non-recurrent nasopharyngeal carcinoma (nrNPC).

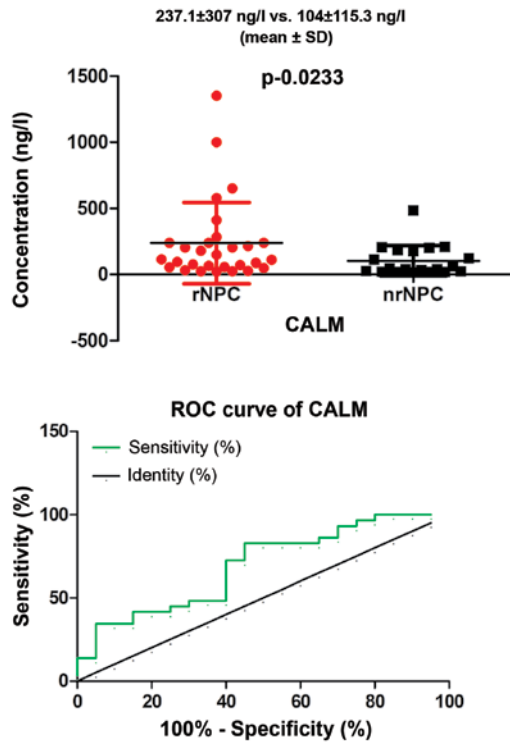


Figure 9. ROC curve of CALM proteins, the area of CALM is 0.6931, $P=0.02275$, which showed CALM got some power in clinical used. Compared to nrNPC, the concentration of CALM was higher in rNPC significantly. CALM, calmodulinin; nrNPC, no recurrence nasopharyngeal carcinoma; rNPC, recurrent nasopharyngeal carcinoma.

respectively. Intensive bioinformatics analysis was carried out to annotate these quantifiable targets, including protein annotation, functional classification, functional enrichment, functional enrichment-based cluster analysis, etc.

There are some unique aspects in the present study. The first one, was that we performed biological replicates. Moreover, the data of two trials received a low heterogeneity (Fig. 10), which showed a high confidence in LC-MS/MS analysis. In addition, we used TMT labeling which allows for the simultaneous accurate, reproducible and precise quantification of proteins across complex samples, which is considered superior to two-dimensional liquid chromatographic approach and iTRAQ (14,17). It offered a more confident expression in our study.

Furthermore, we did not find a related report about rNPC patient serum using TMT labeling, which meant that, at least to the best of our knowledge, this was the first test comparing the serum of patients with rNPC and nrNPC in a high throughput approach.

It is known that NPC is a multifactorial disease, including host genetics, chronic infection by EBV, environmental factors and others (18). Even after effective treatment, there are still 20% patients who suffer from recurrence; a well known cause of rNPC is radioresistance (19). The elucidation of the molecular mechanisms and the potential pathways involved in rNPC carcinogenesis can certainly aid in the diagnosis and prognosis.

Of 59 dysregulated proteins, most proteins have been reported to be connected with tumorigenesis. In our study, CALM was enriched in 4 pathways, the fold change was 2.11 and the enrichment ratio was the highest. In addition, CALM has been reported

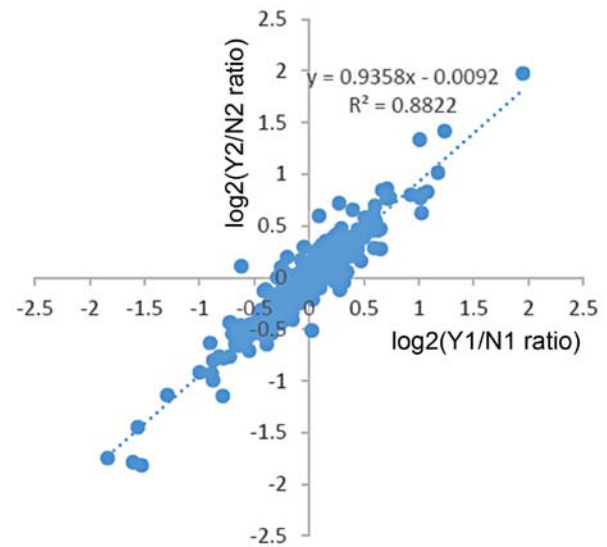


Figure 10. Reproducibility analysis of 2 repeated trials by scatter diagram. The x-axis presents the $\log_2(Y1/N1 \text{ ratio})$ value and the y-axis presents the $\log_2(Y2/N2 \text{ ratio})$ value. Y represents recurrent nasopharyngeal carcinoma (rNPC); N represents non-recurrent nasopharyngeal carcinoma (nrNPC).

to be dysregulated in other malignancies (20-23). All the above suggests that CALM plays a significant role in rNPC.

To date, it is well known that CALM is considered to be the major regulator of Ca^{2+} -dependent signaling in all eukaryotic cells, which is considered the major regulator of many physiological processes, such as cell proliferation, autophagy and apoptotic processes (24). A previous study demonstrated that compared to normal mouse liver, the concentration of CALM was high in hepatomas (20); others have demonstrated that CALM level was significantly higher in human primary lung cancer (25), and in human T lymphocytes (23) than in normal subjects. A positive correlation between the degree of tumor malignancy with the level of cellular CALM has been published (20,21). Furthermore, there are multiple CALM inhibitors that have been used to inhibit tumor cell growth in different species either *in vivo* or in culture, such as breast adenocarcinomas (21), lung carcinomas (26), colon carcinomas (27), leukemia cells (28) and resistant pancreatic cancer (29). According to our results, the CALM level was significantly higher in rNPC serum than in nrNPC serum, which suggests that the upregulation of CALM can be a potential biomarker. Even though the association between NPC or rNPC and CALM has not yet been reported, our data suggest that it plays an important role in rNPC; however, this needs to be further confirmed in a larger cohort of patients.

Interestingly, CALM is involved in the Rap1 signaling pathway, cAMP signaling pathway and cGMP-PKG signaling pathway processes, even though the enrichment ratios of the above pathways were not high ($P>0.05$), which may still demonstrate some connective associations. Several studies have also demonstrated that under hypoxic conditions, the Ca^{2+} /CALM complex increases, and it lead to an improvement in the expression of vascular endothelial growth factor and the transcriptional activity of hypoxia-inducible factor 1. CALM antagonists can inhibit the above process (30-32). As we all know, hypoxia is a reason for the radioresistance of NPC, which suggests that

CALM may be closely associated with rNPC. Further studies are warranted for elucidate to provide more insight into this.

There are limits to the present study. Firstly, we used pooled samples for TMT profiling, which is sensitive to outliers. Thus, it will possible that this may have lead to false positives. This is the reason why we undertook a validation using individual samples. Secondly, we stored the serum at -80°C, while we could not collect the sample at the same time, this may lead to the loss of some vital proteins. Finally, as mentioned above, further verifications are required with a large sample size. If the serum volume of each sample was large enough, a PCR study should have also been carried out. This may be an approach with which to elucidate the potential mechanisms responsible for rNPC.

In conclusion, CALM may be a potential biomarker which may greatly improve the prediction and management of rNPC.

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