

Identification of Phosphorylated Proteins Regulated by SDF2L1 in Nasopharyngeal Carcinoma Cells

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Evolutionary Bioinformatics
Volume 18: 1–6
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DOI: 10.1177/11769343221095862



ABSTRACT: SDF2L1 is a new type of endoplasmic reticulum stress inducible protein, which is related to poor prognosis of various cancer, we initially studied the low expression level of SDF2L1 in NPC, but the molecular mechanism of SDF2L1 in NPC needs further elucidation. To identify phosphorylated proteins regulated by SDF2L1 in nasopharyngeal carcinoma (NPC), Label-free Quantitative (LFQ) Proteomics and 2D-LC-MS/MS analysis were performed on high metastatic NPC 5-8F cells with overexpression of SDF2L1 and empty segment. Western blotting was applied to validate the differentially expressed phosphorylated proteins (DEPPs). As a result, 331 DEPPs were identified by proteomics, and PARVA phosphorylation (ser8) was validated. The present results suggested that PARVA phosphorylation may be a new promising biomarker for predicting NPC and play a key role in the occurrence and development of NPC.

KEYWORDS: SDF2L1, nasopharyngeal carcinoma, Label-free Quantitative Proteomics, 2D-LC-MS/MS

RECEIVED: December 3, 2021. **ACCEPTED:** April 4, 2022.

TYPE: Bioinformatics Resources for Understanding the Epitranscriptome and General Omics – Original Research

FUNDING: The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This study was supported by grants from the National Natural Science Foundation of China (no. 81460414) and Natural Science Foundation of Guangxi (no. 2018GXNSFAA281055).

DECLARATION OF CONFLICTING INTERESTS: The author declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Introduction

Nasopharyngeal carcinoma (NPC) is an epithelial carcinoma arising from the nasopharyngeal mucosal lining.¹ According to the International Agency for Research on Cancer, 133 354 new cases of NPC were reported in 2020. Compared to other cancer types, NPC is uncommon, with a very unique geographical distribution, whereby more than 70% of new cases are reported in East and Southeast Asia.² Unfortunately, it is almost impossible to make a diagnosis of NPC in the early stage, as its concealed location. Consequently, the 5-year survival rate of NPC patients less than 30%.³ In the late stage of tumor, various new treatment strategies, such as intensity modulated radiotherapy (IMRT), magnetic resonance imaging (MRI) and concurrent chemoradiotherapy, have been applied in clinic. Moreover, the MR-based radiomic analysis has been utilized in predicting progression-free survival of advanced NPC patients, local recurrence in non-metastatic T4 NPC, distant metastasis before initial treatment, and response to induction chemotherapy in patients with NPC.⁴ Though the symptoms of patients with NPC can be alleviated, the disease cannot be completely cured so that patients still need to endure the adverse consequences of treatment.⁵⁻⁷ Unlike standard radiotherapy and chemotherapy, molecular targeted therapy is a new method to inhibit tumor growth, metastasis and infiltration by interfering with specific molecules.⁸ Therefore, we urgently need to further understand the molecular mechanism of NPC and find new biomarkers for early diagnosis and treatment strategies. Recently, genomics and phosphorylated proteomics research based on macro perspective may provide practicable schemes.

Stromal cell derived factor 2 like 1 (SDF2L1), located at 22q11.21, was first isolated from mice and humans in 2001.⁹ SDF2L1 is a new type of endoplasmic reticulum stress inducible protein, and its C-terminal has the endoplasmic reticulum reservation motif (HDEL), belonging to the pmt/rt protein family.¹⁰ So far, the specific functions of SDF2L1 are still unknown. It was found that in the pancreas β cells and in the mouse model, SDF2L1 combined with endoplasmic reticulum related degradation mechanism (ERAD) jointly blocked the degradation of proinsulin, suggesting that SDF2L1 may participate in the process of glucose and lipid metabolism in the body.¹¹ Meanwhile, SDF2L1 regulates liver glucose and lipid metabolism through endoplasmic reticulum stress response, suggesting that SDF2L1 may play an important role in the occurrence and development of diabetes.¹² In addition, Hanafusa et al found that ERdj3/DNAJB11 and SDF2L1 complexes are more likely to have active chaperone function, which can prevent the aggregation of misfolded proteins without transferring the substrate to immunoglobulin heavy chain binding proteins, thus maintaining endoplasmic reticulum homeostasis.¹³ Up to now, few studies on SDF2L1 reported in cancer. Previous studies have shown that low level SDF2L1 is related to poor prognosis of colorectal cancer, breast cancer and ovarian cancer, and SDF2L1 can be used as an independent prognostic survival marker for these cancers.¹⁴⁻¹⁶ Herein, we speculated that SDF2L1 gene is likely to be an important potential tumor suppressor gene, and its down-regulation or deletion may be related to the progress of tumor. As far as we know, there is no report on the application of sdf2l1 in NPC.



Our group has been committed to the study of the role of SDF2L1 in NPC. In the early stage, our team initially studied the low expression level of SDF2L1 in NPC, and found that SDF2L1 is closely involved the proliferation, invasion and migration of tumor cells.¹⁷ However, the molecular mechanism of SDF2L1 in NPC remains unclear and needs further elucidation.

Protein phosphorylation, as the most common modification method after protein translation, is the most basic and universal way to regulate and control protein activity and function, and is also the most important mechanism.^{18,19} Simultaneously, protein phosphorylation plays an important role in the process of cell signal transduction.²⁰ Phosphorylation proteomics has been paid attention to the identification of biomarkers related to NPC and the elucidation of pathogenesis.²¹⁻²³

In this study, we used label free phosphorylated proteins quantitative technique and tandem mass spectrometry to detect the DEPPs in NPC cells before and after the change of SDF2L1 expression, and validated PARVA phosphorylation (ser8) in NPC cells. This study paved the way for further exploring the role of PARVA phosphorylation (ser8) in NPC.

Materials and Methods

Cell lines and tissues

Nasopharyngeal carcinoma cell lines (HK1, HONE1, 5-8F) and Nasopharyngeal epithelial cell line (NP69) were gained from Department of Otolaryngology, Guangxi Medical University. Our research group successfully constructed a cell line stably expressing SDF2L1 and empty fragment on the basis of 5-8F cell lines (high metastasis), namely group A and group B, respectively. All cells were cultured in a complete medium containing 89% DMEM basic medium, 9% fetal bovine serum, and 1% penicillin streptomycin mixture in a humidified incubator with 5% CO₂ at 37°C.

Protein extraction, digestion, and enriching with CAE-Ti-IMAC reagents

Cells were split in lysis buffer (8M UREA, 100mM Tris-HCl, 0.1mM phenyl methylsulfonyl fluoride, 0.1mM Phosphatase inhibitor cocktail) at 4°C for 40minutes, and then centrifuged at 12000× g for 15 minutes at 4°C. The supernatant was collected, and then the proteins concentration was determined by BCA Quantification kit (meilunbio, China). Trypsin digestion was performed according to the manufacturer's protocol (Wallis, China). Briefly, 100 μg proteins of each sample was reduced and alkylated, then digested overnight at 37°C with trypsin (mass spectrometry grade; Thermo Scientific, USA), and then proteins concentration was determined by Nano-drop. And then Phospho-peptides enriched with CAE-Ti-IMAC Reagents (Bailingwei, China) as follows: The protease hydrolysate and loading buffer (80% ACN/6% TFA aqueous solution) were mixed according to the volume ratio of 1:1. Next, the CAE-Ti-IMAC material

chelated with Ti⁴⁺: protease hydrolysate=20:1-30:1 (m/m). Then the wash buffer 1, 2 and elution buffer were added into the mixture in turn, shocked for 30 minutes, centrifuged at 12000× g for 5 minutes. Finally, the supernatant was collected for mass spectrometry.

LC-MS/MS analysis

The mixed peptides were dephlegmated by Easy-nano-LC 1200 chromatographic system (Thermo Scientific, USA). Briefly, the polypeptides were dissolved into liquid A (water with 0.1% formic acid, containing IRT standard peptide), and then separated on the analytical column at a flow rate of 600NL/minute. The next separation steps as follows: From 0 to 15 minutes, liquid B (containing 0.1% formic acid and 80% acetonitrile) increased linearly from 7% to 15%; From 15 to 85 minutes, liquid B increased linearly from 15% to 30%; From 85 to 110minutes, liquid B increased linearly from 30% to 50%, then rose to 95% within 2 minutes and maintained for 120minutes. The mass spectrometry data were collected using thermo Qexactive HF mass spectrometer (Thermo Scientific, USA). The specific parameter settings are as follows: the spray voltage of the ion source is set to 2.1 kV. The scanning range of the first level is 300 to 1400M/Z, the resolution is 60 K (@ m/Z 200), the AGC target is 3e6, and the maximum is 80 ms; The secondary resolution is 15 K (@ m/Z 200), isolation window is 1.6 Th, AGC target is 5e4, maximum IT is 40 ms, loop count is 20, colligation energy is 30, charge state is 2 to 6, and dynamic exclusion time is 30seconds.

Qualitative and quantitative analysis of proteins

The LC-MS/MS original files were imported into maxquant software (version No. 1.3.0.5) for database search. The Andromeda search engine was applied for LFQ non-standard quantitative analysis. The inverse database based on the UniProt database is used to calculate the false discovery rate (FDR) of each peptide and proteins. Maxquant software integrates the LFQ algorithm by extracting the isotopic peak of each peptide in each analysis. Maxquant platform calculates the protein ratio by using the median value of the common peptide ratio in all analyses. This represents a fairly approximate estimate of the protein ratio. The differential multiple greater than 1.5 times or less than 0.67 times and the *P* value less than .05 were regarded as differential proteins. The main parameters are showed in Supplemental Table 1.

Western blotting analysis

HK1, HONE1, 5-8F, and NP69 cell line were used for western blotting analysis. For western blotting or immunostaining, primary antibodies against the following proteins were used: PARVA phosphorylation (ser8) (1:500; Abcam) and GAPDH (1:1000; Abcam). Cells were washed 3 times with PBS and then lysed in cold RIPA buffer supplemented with phenyl

methylsulfonyl fluoride and Phosphatase inhibitor cocktail (meilunbio, China) on the ice. Cell lysate was centrifuged at $12000\times g$ for 15 minutes, boiled in $4\times$ loading buffer for 10 minutes, separated on an 8% gradient SDS-PAGE gel and blotted onto a PVDF membrane. The membrane was blocked with high efficiency sealing solution or 0.1% Tween-20 in TBS containing 5% non-fat milk and probed with the indicated primary antibody overnight at 4°C , followed by incubation with goat anti-rabbit antibody (1:5000; LI-COR) for 1 hour at room temperature. The signal was visualized via IgG Cy3-labeled anti-fluorescence (Cell Signaling Technology (CST)) by Odyssey scanner (LI-COR, USA) and quantitated by densitometry using Image Quant image analysis system (Storm Optical Scanner, Molecular Dynamics, Sunnyvale, CA).

Statistical analysis

Data were expressed as means \pm SD. Statistical analyses were performed by Statistical Package for Social Science (SPSS for Windows, Version 26.01, USA). Student's *t* test was used to assess the statistical significance. $P < .05$ was considered statistically significant.

Bioinformatics analysis

The GO annotation and KEGG signaling pathway enrichment of differentially expressed phosphorylated proteins (DEPPs) were performed using "cluster Profiler" package in R language. The set threshold is $P < .05$ and $\text{FDR} < 0.05$. A protein-protein interaction network (PPI) of the DEPPs was constructed according to the interaction score ≥ 0.4 by Search Tool for the Retrieval of Interacting Genes/Proteins (STRING; <https://www.string-db.org/>) and visualized by Cytoscape (version:3.7.2).

Results

Identification of DEPPs between 5-8F-SDF2L1 and 5-8F-NC (control) cell using LFQ method and 2D-LC-MS/MS

A total of 3121 phosphorylation sites, 2558 phosphorylation peptides and 1390 phosphorylation proteins were identified by LQF protein quantification and 2D-LC-MS/MS. The detailed parameters information as followed: the overall identified protein molecular weight distribution, peptide length distribution, map matching error distribution, peptide charge distribution, map matching score distribution, map matching delta score distribution, phosphorylated amino acid distribution, peptide phosphorylation number distribution and protein phosphorylation number distribution (Supplemental Figure S-1).

To identify the DEPPs regulated by SDF2L1 in NPC cells, phosphorylated proteins expressional profiles between the 2 cell lines were compared. The phosphorylated proteins which met the following criteria were spontaneously considered as DEPPs: (1) Map false positive (PSM FDR) < 0.01 ; (2)

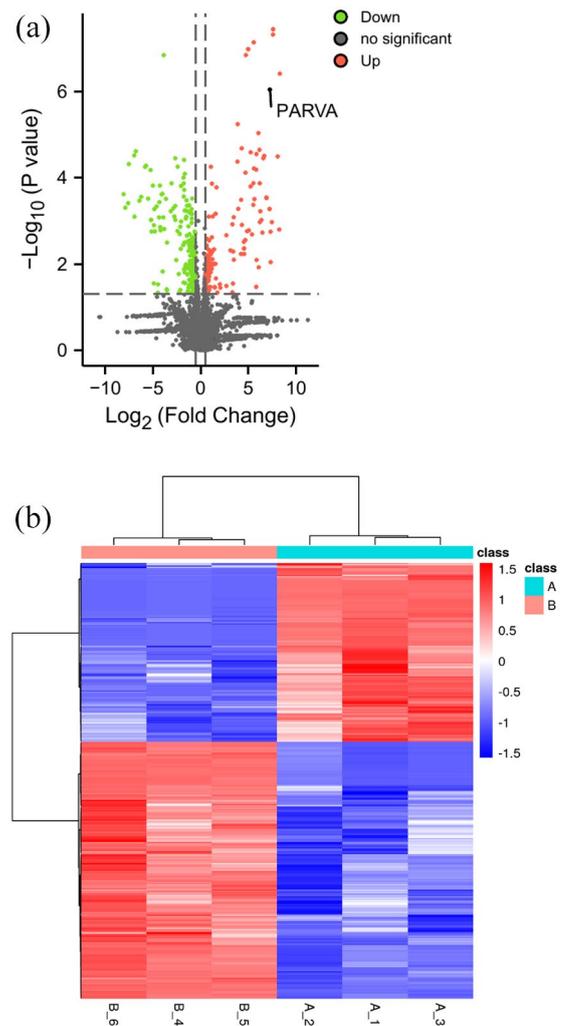


Figure 1. The volcano plot and Heat map of differential phosphorylated proteins. (a) The volcano plot of differential phosphorylated proteins; (b) The Heat map of differential phosphorylated proteins.

Phosphorylation probability > 0.75 ; (3) Score for modified peptides > 40 ; (4) Delta score for modified peptides > 6 ; (5) Phosphorylated proteins showed mean ratio fold changes > 1.5 or < 0.67 and $P < .05$. Finally, compared with 5-8F-NC cells, 331 DEPPs were quantified in 5-8F-SDF2L1 cells. The detailed information of these DEPPs were shown in Supplemental Table S-1. The Volcano map and heat map of DEPPs were shown in Figure 1.

Validation of DEPPs identified by quantitative proteomics

Based on the Uniport and PubMed research, PARVA phosphorylation (ser8) identified by 2D-LC-MS/MS analysis was selected from 331 DEPPs for verification.

In order to elucidate the effect of SDF2L1 on PARVA phosphorylation in NPC, western blotting was performed to detect the expression between 5-8F-SDF2L1 and 5-8F-NC cells. As shown in Figure 2A, the change of the level in protein consistent with the finding in MS analysis. In addition,

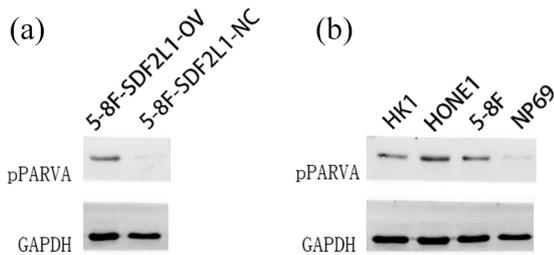


Figure 2. The western blotting of PARVA Phosphorylation in NPC cells. (a) The expression of PARVA Phosphorylation in 5-8F-SDF2L1-OV cells and 5-8F-SDF2L1-NC cells; (b) The expression of PARVA Phosphorylation in nasopharyngeal carcinoma cells (HK1, HONE1 and 5-8F) and normal nasopharyngeal epithelial cells (NP69).

to determine the PARVA phosphorylation level in NPCs, we compared the expression of PARVA phosphorylation (ser8) between NPC cells (HK1, HONE1 and 5-8F) and Normal nasopharyngeal epithelial cells (NP69). The result showed that the expression of this phosphorylated proteins was significantly higher in NPC cells than that in normal cells (Figure 2B).

GO, KEGG pathways analysis and PPI of the DEPPs

To get insight into the biological function of the DEPPs in NPC, functional enrichment analysis including biological process, cellular component molecular functions, and signaling pathways were performed using “cluster profiler” package in R language.

In the biological process category, DEPPs are mainly involved in the transport of mRNA, RNA and nucleic acid, RNA splicing and localization and the regulation of the ribonucleoprotein complex. The cellular component category indicated that the DEPPs are mainly associated with nucleus, focal adhesion, cell-substrate junction, chromosomal region and kinetochore. The molecular function category showed that the DEPPs are related to binding, transcription activity and catalysis. The top 30 GO terms with the most significant enrichment were shown in Figure 3. KEGG pathway enrichment analysis was conducted on the basis of 331 DEPPs. As a result, the differentially expressed phosphorylated proteins were mainly involved in nearly 200 signaling pathways. The pathways closely related to tumor were MAPK, PI3K-AKT, Ras, cGMP-PKG, HIF-1, AMPK, mTOR, FOXO and VEGF pathways are shown in Table 1.

Discussion

In order to obtain the characteristics of phosphorylated proteins expression profile in NPC cells before and after the change of SDF2L1 expression, we successfully constructed 5-8F^{SDF2L1-OV} and 5-8F^{SDF2L1-NC} cells based on lentiviral vector technology. After LFQ and 2D-LC-MS/MS analysis, a total of 331 DEPPs were identified, including 136 up-regulated DEPPs and 195 down-regulated DEPPs. Next, PARVA

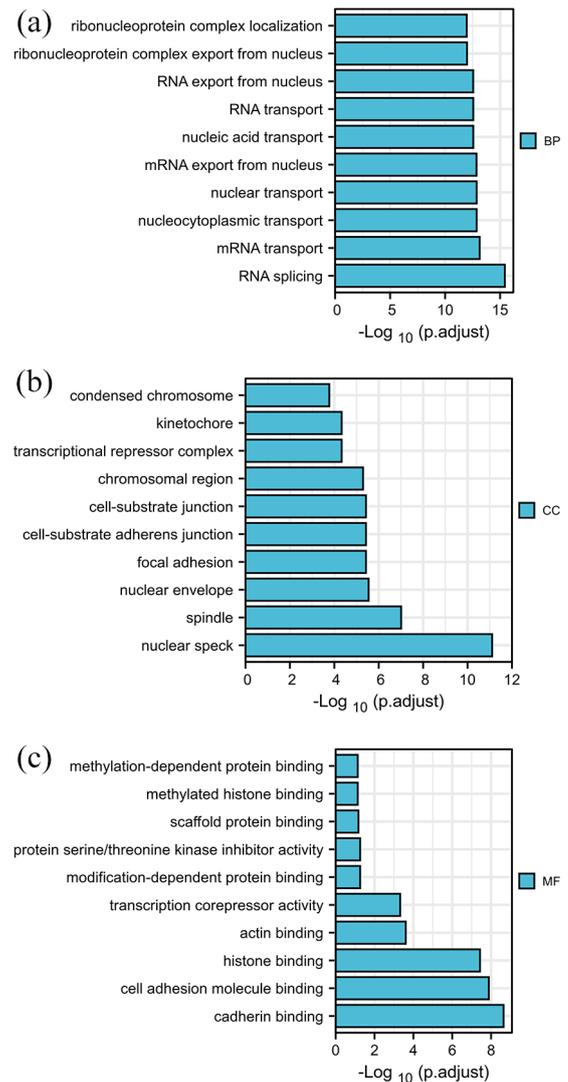


Figure 3. The 30 top GO terms of differential phosphorylated proteins. (a) The 10 top biological process of differential phosphorylated proteins; (b) The 10 top cellular components of differential phosphorylated proteins; (c) The 10 top molecular function of differential phosphorylated proteins.

phosphorylation (Ser8), one of the DEPPs, was selected to validation and further explore function in NPC, suggesting that this phosphorylated protein is a potential biomarker for warning NPC.

PARVA, also known as Actopaxin/ α -parvin, an essential component of focal adhesions, a member of mammalian parvin protein family, involved in the linkage of integrins with several serine phosphorylation sites at the amino terminal, regulation of actin cytoskeleton dynamics and cancer cell morphology, migration, invasion, and survival signals.²⁴⁻²⁷ PARVA Phosphorylation was increased in a variety of cancer cells with multiple functions. Briefly, PARVA increased phosphorylation in osteosarcoma cells and invasive breast cancer cell lines. Phosphorylated PARVA regulates matrix degradation through Rho GTPase signaling pathway, thereby promoting the proliferation and invasion of tumor cells.^{28,29} In human cervical cancer,

Table 1. Differential expression of phosphorylated proteins in tumor associated KEGG pathway.

PROTEIN ID	GENE ID	MAP ID	DESCRIPTION
Q99956/P21333/O75369/P04792/P16949/P36507	DUSP9/FLNA/FLNB/HSPB1/STMN1/MAP2K2	hsa04010	MAPK signaling pathway
Q14643/Q14573/P36507/P21796	ITPR1/ITPR3/MAP2K2/VDAC1	hsa04022	cGMP-PKG signaling pathway
P07900/P16144/P36507/Q8N122	HSP90AA1/ITGB4/MAP2K2/RPTOR	hsa04151	PI3K-Akt signaling pathway
Q14643/Q14573/P36507	ITPR1/ITPR3/MAP2K2	hsa04912	GnRH signaling pathway
Q86T10/Q96EB6/Q8N122	TBC1D1/SIRT1/RPTOR	hsa04152	AMPK signaling pathway
Q14643/Q14573/P21796	ITPR1/ITPR3/VDAC1	hsa04020	Calcium signaling pathway
O95835/P46937	LATS1/YAP1	hsa04390	Hippo signaling pathway
P00558/P36507	PGK1/MAP2K2	hsa04066	HIF-1 signaling pathway
P04792/P36507	HSPB1/MAP2K2	hsa04370	VEGF signaling pathway
P36507/Q96EB6	MAP2K2/SIRT1	hsa04068	FoxO signaling pathway
P36507/Q8N122	MAP2K2/RPTOR	hsa04150	mTOR signaling pathway
Q7Z569	MAP2K2/BRAP	hsa04014	Ras signaling pathway
P36507	MAP2K2	hsa04662	B cell receptor signaling pathway
P36507	MAP2K2	hsa04012	ErbB signaling pathway
P36507	MAP2K2	hsa04660	T cell receptor signaling pathway
P36507	MAP2K2	hsa04024	cAMP signaling pathway
Q9NYZ3	GTSE1	hsa04115	p53 signaling pathway

cyclin (B1/cdc2) dependent on PARVA phosphorylation regulating actin cytoskeleton reorganization during cell division.³⁰ Meanwhile, PARVA phosphorylation may regulate the association between PARVA and TESK1, thus affecting the growth and proliferation of cancer cells.³¹

Our LFQ results showed that PARVA phosphorylation expression levels increased in NPC, supporting that PARVA phosphorylation is a protein. To our best knowledge, it is first report on PARVA phosphorylation's role in NPC.

To deeply understand the biological functions of the differentially expressed phosphorylated proteins in NPC advancement, GO annotation, KEGG pathways enrichments analysis and PPI were conducted on 331 differential phosphorylated proteins. GO annotation analysis indicated that the DEPPs mainly involved in 30 aspects, including biological activity of mRNA and RNA, cell cycle, depolymerization of protein complexes, molecular transduction activity, cell adhesion, and cytoskeleton movement. KEGG pathway analysis revealed that the DEPPs were closely related to tumor signaling pathways, such as MAPK, PI3K-AKT, Ras, cGMP-PKG, HIF-1, AMPK, mTOR, FOXO, and VEGF.

In summary, we identified a total of 331 DEPPs in NPC cells loading SDF2L1 by LFQ method combined with 2D-LC-MS/MS and suggested that PARVA phosphorylation may be serve as a novel potential biomarker for predicting NPC. These findings reported here could have potential

clinical value in early warning NPC and also provide valuable information for further study of molecular mechanisms that govern NPC metastasis. We plan to operate the PARVA phosphorylation in vitro and in vivo to further explore the role of PARVA phosphorylation in the development of NPC.

Author Contributions

Chengchang Luo, Zunni Zhang, and Qisheng Su designed the study. Chengchang Luo and Zunni Zhang gathered the data and performed the data analysis. Chengchang Luo and Qisheng Su wrote the manuscript and helped with the validation. Wuning Mo helped with the revision. All authors contributed equally to the article and approved the submitted version.

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Supplemental Material

Supplemental material for this article is available online.

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