Chapter 7

The Use of Gel Electrophoresis and Mass Spectrometry to Identify Nitroproteins in Nervous System Tumors

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Additional information is available at the end of the chapter

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Abstract

Protein tyrosine nitration is an important molecular event in nervous system tumor such as glioma and pituitary adenomas. It is the essential step to identify the protein targets and exact modified sites of tyrosine nitration for addressing the biological roles of protein tyrosine nitration in nervous system tumors and discovering effective biomarkers to understand in-depth molecular mechanisms and determine new diagnosis strategy and novel therapeutic targets. One/two-dimensional gel electrophoresis (1DGE, 2DGE), or nitrotyrosine affinity column (NTAC), coupled with tandem mass spectrometry (MS/MS) have been successfully applied in the analysis of nitroproteins in nervous system tumors. This article address the basic concept of protein tyrosine nitration, nitroproteomics methodology based on gel electrophoresis/immunoaffinity enrichment and tandem mass spectrometry, and the current status of nitroprotein study in nervous system tumors. The established nitroproteomics approach is easily translated to study other diseases.

Keywords: two-dimensional gel electrophoresis, one-dimensional gel electrophoresis, tandem mass spectrometry, tyrosine nitration, nitroprotein, nitroproteomics, nervous system tumors

1. Introduction

The incidence of central nervous system (CNS) has been increasing over the last 30 years, and unfortunately, become younger and younger [1]. More remarkable, patients (20–40%) with systemic cancer will occur metastatic disease to the CNS [2]. Primary malignant brain tumors and other metastatic disease to the CNS threaten human health. Astrocytoma is a type of primary malignant brain tumor that range from 20 to 40% of glioma, which is the most common

primary brain tumor in adults [3]. Here, we put research emphasis on two kinds of primary intracranial tumor, astrocytomas and pituitary adenomas. Astrocytomas cells possess the characteristic of high invasion to cause difficulty in therapy and high mortality (median survival = 9–12 months) [4]. A pituitary adenoma is quite common, and accounts for 10% of all primary intracranial neoplasias [5]. Even only a minority of pituitary adenomas (0.1–0.2%) develops into metastatic cancer, pituitary adenomas arise clinical problems: (a) Compress the adjacent brain organs appear symptoms including headache and visual failure. (b) An inappropriate hormone secretion led to hormone syndromes, including hypopituitarism, acromegaly, hyperprolactinemia and Cushing's syndrome [6]. The molecular mechanisms of those diseases remain unclear, and the traditional treatment models contain surgical excision, radiotherapy, and medical therapies [7]. Discovering in-depth molecular mechanisms, new diagnosis strategy, novel therapeutic targets are urgent.

Protein tyrosine nitration is an important posttranslational modification (PTM) in nervous system tumors that is related with multiple abnormal pathophysiological processes [8]. The process of protein tyrosine nitration is formed from 3-nitrotyrosine group to position 3 of the tyrosine residue phenolic ring [9], which alters the electron density and pKa value (from ~10 for tyrosine into ~7.1 for 3-nitrotyrosine) of the tyrosine phenolic ring [10]. Such changes affect biochemical characteristics of the tyrosine residue that will change interaction between enzyme-substrate, antigen–antibody or receptor-ligand, when interacting regions were in nitration. Reactive nitrogen species (RNS) act as an important mediated material for protein nitration, and our studies [11] consistent with others [12, 13] have indicated that nitric oxide and protein nitration may play important roles in the nervous system tumors. Previous studies reported that (1) the inflammatory reaction is involved in nervous system tumors [14]; (2) NO and RNS are important inflammatory mediators [15]; and (3) increased production of NO, peroxinitrite and superoxide, occurs in nervous system tumors [16]; (4) higher levels of nitrotyrosine are observed in nervous system tumors than normal tissues with biochemical approaches and immunohistochemical, and only protein nitrotubulin and protein nitro-p53 have been determined in human nervous system tumors [17]. Furthermore, the amino acid analog 3-nitrotyrosine due to functional and morphological injury of mouse-neuroblastoma cell lines and rat-glioma cell lines [18]. These studies demonstrated the importance of protein tyrosine nitration in the pathogenesis of nervous system tumors. Illustrating the functions of nitroproteins might reveal in-depth molecular mechanisms and biological function of tyrosine nitration in human nervous system tumors. Literature-based review and comprehensive annotation of proteins on the SwissProt website were used to expound the nitroprotein domains/motifs, location of nitrotyrosine sites and possible signaling pathways relevant to nervous system tumors. Nitroproteins took part in multiple biological processes in the development of tumors as follows: (a) tumor cell migration and invasion [19]; (b) cell proliferation and apoptosis [20]; (c) chemotherapy resistance [21]; (d) signal transduction [22]; (e) phenotypic dedifferentiation [23]; (f) microtubule dynamic stabilization [24]; (g) tumor recurrence; (h) others such as immunoreaction and post-transcriptional regulation. Moreover, the discovery of tyrosine nitration being a reversible reaction [25] and having a competition between phosphorylation motif [26], led us to speculate that dynamic process of protein nitration might also be regulated and controlled. However, no definite target of intervention is found for tyrosine nitration in human nervous system tumors. It takes long time to study tumor-related nitroproteins and to illustrate molecular mechanisms in tumor formation.

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Nitroproteomics methods were based on sample enrichment and mass spectrometry analysis. Modern nitroproteomics applies protein-separation-enrichment techniques such as gel methods and non-gel methods, including immunoprecipitation [11], anti-nitrotyrosine antibody-based enzyme-linked immunosorbent assay (ELISA) [27], and one/two-dimensional gel electrophoresis (1DGE/2DGE)-based Western blot analyses [9]. 1DGE/2DGE-based Western blots analyses can separate and preferentially enrich endogenous nitroproteins and also preliminarily determine the quantitative information of nitrotyrosine. Studies showed that the same protein was detected at multiple gel-spots on 2D electrophoresis gels, and single 2D electrophoresis gel-spot usually contains several proteins [28]. Therefore, 2D electrophoresis gel has advantages in protein component visualization, detection of protein species that are mainly derived from alternative splicing or PTMs [9]. Protein isoforms or variants present dynamic biological processes in vivo, and different protein species associated with different conditions and pathophysiological status [29]. We adopted 1DGE/2DGE-based Western blots analyses method: the scanned images of the silver-stained 2D electrophoresis gels and the visualization of Western blot membranes were input to a PDQuest system (Bio-Rad, version 7.1, Hercules, CA) to composite image that contained the Gaussian spots [30]. MS/MS is the mainstream technique to identify protein species and PTMs products with verification of amino acid sequence, splicing sites, and modification site [31]. However, it is also pointed out that the challenges faced by low abundance of tyrosine nitration and the elusive mass spectrometry result of a nitro group are existed [32]. For example, matrix-assisted laser desorption ionization (MALDI) is quite different from electrospray ionization (ESI)-MS when study the MS behaviors of a nitropeptide [33]. Photochemical decompositions of the nitro group ($–NO_2$) induced by the high-energy laser decrease the precursor-ion intensity of a nitropeptide, making an MS spectrum much more complex in process of MALDI. However, the decomposition pattern of a nitropeptide was well clarified by the photochemical decomposition pattern ([M + H]+, [M + H – 16]+, [M + H –30]+, and [M + H – 32]+). Even ESI does not produce photochemical decompositions, scanning for the characteristic immonium ion (m/z 181.06) by the precursor ion could help accurately identify a nitropeptide or nitroprotein under ESI conditions [34]. Above key factors, including low abundance of nitroproteins, preferential enrichment methods, sensitivity of MS analysis, complicated MS behaviors of a nitro group, that all determine success or failure in the identity of in vivo nitroproteins. Thus, nitropeptide was detected by a vMALDI MS/MS method [9, 11]. Accumulation of MS/MS scans was used to increase the signal-to-noise ratio (S/N), which is needed for the detection of endogenous nitroproteins. A single MS/MS scan can determine an amino acid sequence to study proteome and phosphoproteome.

Protein tyrosine nitration is an (PTM) in nervous system tumor such as astrocytoma and pituitary adenomas, and participates in multiple complex biological processes [9, 11, 35, 36]. For human astrocytoma and pituitary adenomas nitroproteomics studies, 2DGE-based nitrotyrosine Western blot analysis with MALDI-TOF were used to identify endogenous nitroproteins and nitrotyrosine sites from human pituitary control and adenoma tissues, and 2DGE-based nitro-tyrosine Western blot analysis with liquid chromatography-electrospray ionization-quadrupole ion trap (LC-ESI-Q-IT) were used to identify endogenous nitroproteins and nitrotyrosine sites from human astrocytoma brain tissues. Bioinformatics and pathway analysis were used to determine domains/motifs in a nitroprotein, location of nitrotyrosine sites, and possible signaling pathways. A total of eight nitrotyrosine-containing proteins in human pituitary control tissues [9, 36], and nine nitroproteins and three nitroprotein-interacting

proteins in a human nonfunctional pituitary adenoma tissue [11], 18 nitroproteins and their 23 nitrotyrosine sites in human astrocytoma tissues [35], were identified with 2DE-MS/MS. The nitration site was located onto the corresponding functional domain, and each nitroprotein was carried out pathway analysis to speculate the possible biological function.

2. Materials and methods

2.1. Tissues and extraction of proteins

A clinically nonfunctional human pituitary adenoma tissue was obtained during surgery. The expression of FSH, LH, GH, prolactin, TSH, and ACTH were all negative in tumor cells [11]. A normal human pituitary tissue acted as control group was obtained from the post-mortem sample (a drowning male) [9, 36]. Human astrocytoma brain tissues (including different clinical staging-I/II/III/IV) were obtained from the Department of Neurosurgery of Xiangya Hospital, China [35]. The tissues were frozen in liquid nitrogen immediately, then stored at −80°C. According to the protein extraction manuals (Pierce, Rockford, IL, USA),supernatant of the tissue lysate was extracted for further analysis.

2.2. 2DGE-based western blot detection of nitroproteins

2DGE: The precast IPG strips (pH 3–10 NL; 180 × 3 × 0.5 mm) and 18-cm IPGstrip holder was used for 2DGE first dimension—isoelectic focusing (IEF) on an IPGphor instrument (GE Heathcare) to separate protein sample. After IEF, the IPG strip was processed for 2DGE second dimension—sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), namely, the equilibrated proteins in the IPG strips were separated by molecular weight during electrophoresis on in 12% PAGE resolving gel (250 × 215 × 1.0 mm), and visualized with silver-staining [9, 36] or Coomassie brilliant blue G staining [35].

2DGE-based Western blotting: The 2DGE-separated proteins were transferred to a PVDF membrane, which were blocked by BSA, incubated with an anti-human nitrotyrosine antibody and secondary antibody, then visualized with 1-Step™ nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) (Thermo Product No. 3404).

Image analysis of a 2D gel and of 2D-Western blotting: The scanned images of the 2D gels and the Western blot membranes were input to a PDQuest analysis system (Bio-Rad, version 7.1, Hercules, CA) to synthesize image. Gaussian spots were applied to all subsequent spot-matching and analyses. Each spot volume was normalized to the total optical density (OD) to minimize experimental factors on a spot volume [30].

2.3. MS/MS characterization of tryptic nitropeptides from nitroproteins

LC-ESI-MS/MS: The nitrotyrosine-positive gel-spots were excised, digested, purified, eluted, air-dried, redissolved. Then, the peptide mixture was subjected to LC-ESI-quadrupole-time of flight (LC-ESI-qTOF) or LTQ-OrbiTrap Velos MS/MS analyses. The detailed operation process and parameter settings have been described [35].

MALDI-MS/MS: The immunopositive 2D gel spots were excised, digested, purified, eluted, air-dried, redissolved, and were spots onto MALDI-plate, which were subjected to MALDI-MS/MS analysis [37]. The detailed procedure has been described [9, 11, 36].

2.4. Identification of nitroproteins with MS/MS

MS/MS data were used to identify the protein and nitrotyrosine sites by searching the SwissProt and NCBInr databases with SQUEST or Mascot software, with mass modifications of +45 Da (+NO_2 – H) at Tyr, +57 Da (+NH_2COCH_2 – H) at Cys, +16 Da (oxidation) at Met. Protein domains and motifs analyses were carried out with ScanProsite software (http://us.expasy.org/tools/scanprosite). Nitrotyrosine sites within a given domain or motif were determined with MotifScan software (http://myhits.isb-sib.ch/cgi-bin/motif_scan). The functions and experimental data-based model of nitroproteins was searched on the Swiss-Prot database annotation page and the related literature resources.

2.5. Annotation of functional characteristics of the nitroproteins

Literature-based bioinformatics and comprehensive annotation of protein in the SwissProt page were used to rationalize the functional characteristics of each nitroprotein, and to provide important clues to the biological significance of each nitroprotein relevant to tumors.

3. Results and discussions

3.1. The level of protein tyrosine nitration in nervous system tumors

One1D gel-based Western blotting in combination with anti-nitrotyrosine antibody analysis revealed that the overall level of protein tyrosine nitration in astrocytoma was significantly higher than the controls (**Figure 1**).

3.2. Enrichment of endogenous nitroproteins in nervous system tumors

The challenges faced by low abundance of tyrosine nitration and the elusive mass spectrometry result of a nitro group are existed. Modern nitroproteomics applies protein-separation-enrichment techniques such as gel methods and non-gel methods, including immunoprecipitation [11, 37], anti-nitrotyrosine antibody-based enzyme-linked immunosorbent assay (ELISA) [27], and one/two-dimensional gel electrophoresis (1DGE/2DGE)-based Western blot analyses [9, 35]. 1DGE/2DGE-based Western blots analyses can separate and preferentially enrich endogenous nitroproteins and also preliminarily determine the quantitative information of nitrotyrosine. It should not be neglected that limitations of 2DGE-based method including coverage of proteome, dynamic range, sensitivity and throughput, which always were restricted by the amount of samples. Also, the non-nitrated tryptic peptides are much more than nitrated tryptic peptides after a 2DGE-separated nitroprotein was digested, which will interrupt MS/MS signal of nitrated tryptic peptides [38]. However, for the proteome study, a 2DGE gel could detect more than 1000 spots [39]. Different proteins may be contained within the same spot [40].

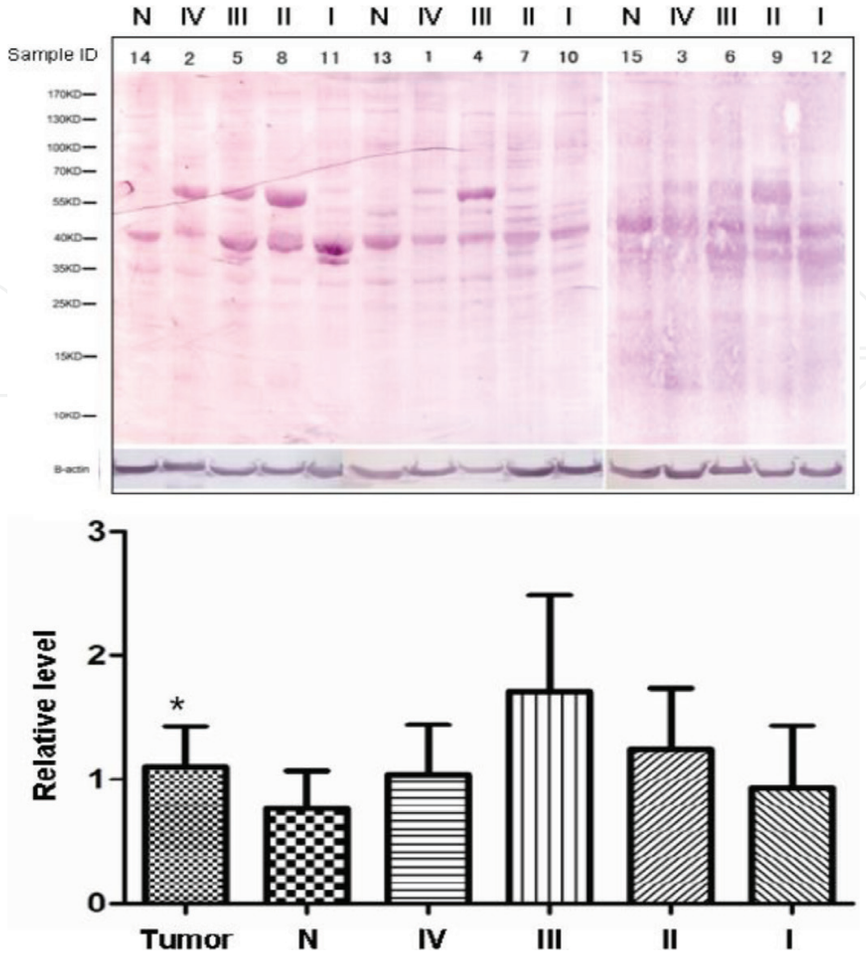

Figure 1. The nitrotyrosine immunoactivities in different grade (I, II, III, and IV) of astrocytoma tissues relative to normal controls (N), detected with nitrotyrosine immunoaffinty-based western blotting (n = 3). Tumor = sum of different grade (I, II, III, and IV) of astrocytomas. *p < 0.05. Reproduced from Peng and Zhan [35], with permission from Springer, copyright 2015.

Many proteins were detected in several analyzed spots showing 2DE-MS separate ability at the protein species level [43]. Indeed, higher sensitivity had been expected when 2DE coupled with high-sensitivity LC–MS, and will detect, identify and quantify human proteome nearly 500,000 (with an estimated resolution) protein species [28]. We adopted 2DGE-based Western blots analyses method to separate and detect nitroproteins in nervous system tumors. Our previous study obtained enrichment of endogenous nitroproteins in human pituitary [9, 36] and astrocytoma [35].

The 2DGE-based Western blot coupled with anti-nitrotyrosine antibody was used to analyze nitroproteins in human astrocytoma tissues (**Figure 2**) [17]. Nearly 1100 protein spots were detected in each Coomassie-stained 2D gel. Most proteins were distributed within a range of pI 4–8 and Mr. of 15–150 kDa. A total of 57 nitrotyrosine-immunopositive gel spots were detected, and each positive spot corresponded to a Coomassie-stained 2D gel spot. In order to show non-specific nitrotyrosine-immunopositive Western blot spots, we setup a control experiment without primary antibody to determine cross-reactivity of the secondary antibody.

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A similar 2DGE-based Western blot coupled with anti-nitrotyrosine antibody was used to analyze nitroproteins in human pituitary tissues [35, 38]. Each pituitary silver-stained 2D gel image (pI 3–10; Mr. 10–100 kDa) contained ca. 1000 protein spots, and a total of 32 nitrotyrosine immunopositive Western blot spots were detected in human pituitary tissues.

An immunoprecipitation, nitrotyrosine affinity column (NTAC)-based MS/MS approach was also used to enrich and identify nitroproteins from a pituitary adenoma tissue (**Figure 3**) [37]. Briefly, the NTAC was prepared with protein G beads cross-linked with anti-nitrotyrosine antibodies. A volume (600 μl) of protein extracts from 62 mg wet weight of a pituitary adenoma

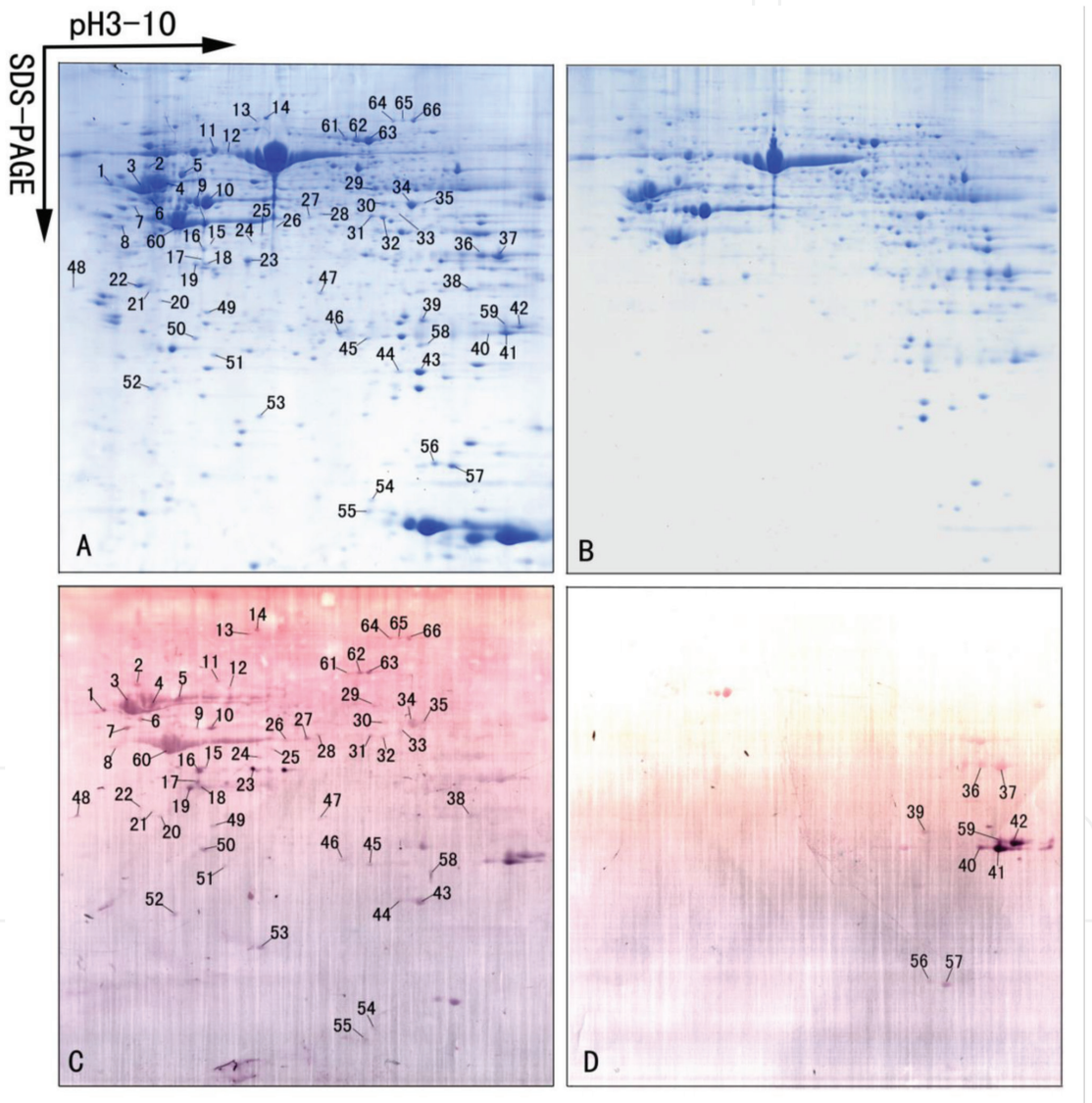

Figure 2. 2DGE-based Western blot analysis of nitroproteins in an IV-grade astrocytoma tissue (500 μg protein per 2D gel). (A) Coomassie blue-stained 2DGE image (before transfer of proteins). (B) Coomassie blue-stained 2DGE image (after transfer of proteins). (C) Western blotting image of nitroproteins (anti-nitrotyrosine antibodies + secondary antibodies). (D) Negative control of Western blotting to show the cross-reaction of the secondary antibody (only the secondary antibody; no anti-nitrotyrosine antibody). Reproduced from Peng and Zhan [35], with permission from Springer, copyright 2015.

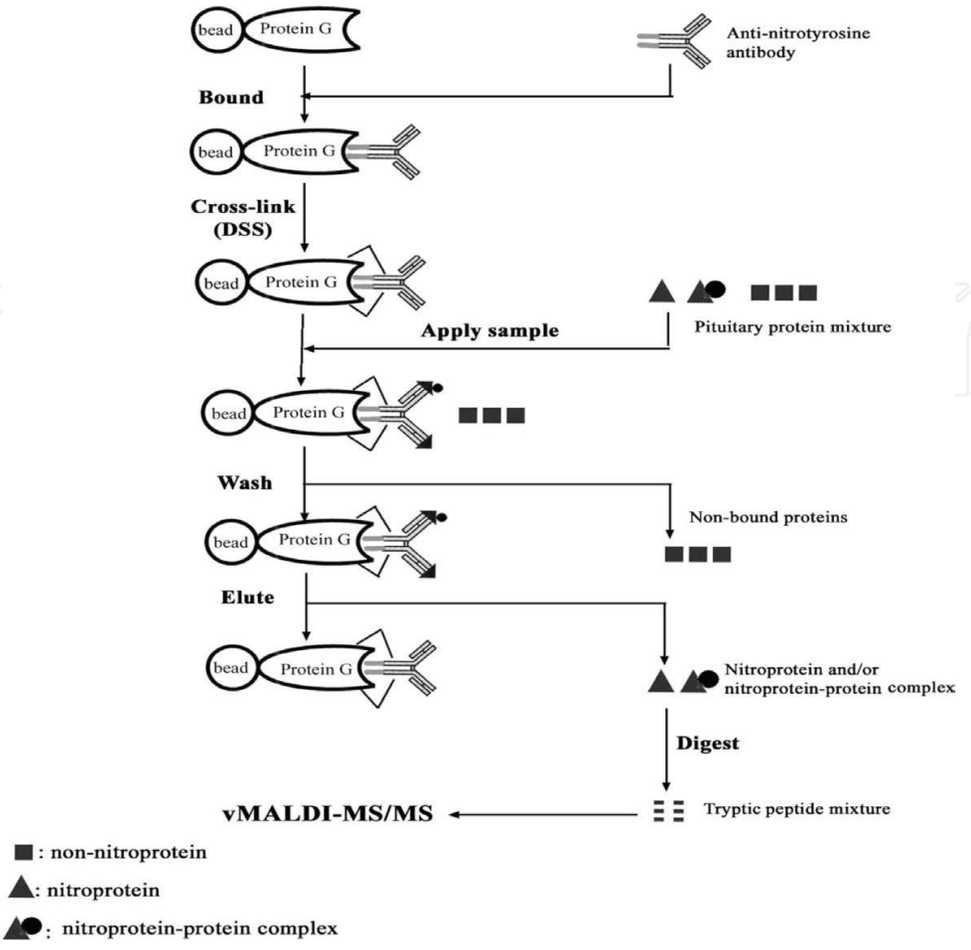

Figure 3. The use of NTAC to characterize nitroproteins and their complexes. A parallel control experiment was carried out without any anti-3-nitrotyrosine antibody. Reproduced from Zhan and Desiderio [11], with permission from Elsevier Science, copyright 2006.

tissue was diluted (1,1, v/v) with binding/washing buffer. Then, 500 μl diluted sample was incubated with the prepared NTAC to enrich and isolate nitroproteins and nitroprotein–protein complexes, followed by tripsin digestion and MS/MS identification.

3.3. MS/MS identification of nitroproteins and nitrated sites in nervous system tumors

MS/MS is the mainstream technique to identify protein species and PTMs products with verification of amino acid sequence, splicing sites, and modification site [31]. However, the very low abundance of tyrosine nitration in a proteome (one nitration in ~10^6 tyrosine residues) and the complicated mass spectrometry behaviors of a nitro group that we studied in nitroproteins complicate analyses [34, 38]. Different mass spectrometry has different advantages and complicated mass spectrometry behaviors, so choosing the proper method or combination with each other is necessary. For example, the MS behaviors of a nitropeptide differ significantly between matrix-assisted laser desorption ionization (MALDI)-and electrospray ionization (ESI)-MS on process of photochemical decompositions of the nitro group (–NO_2)

[33, 41]. Additionally, Specificity and sensitivity of the MALDI–LTQ MS/MS analytical system to characterize each nitroprotein and nitroprotein–protein complex should be considered. According to our previous experience [42], MALDI–LTQ has a number of advantages: (1) highly sensitive; (2) high accuracy measurement on amino acid sequence and nitration sites; (3) a prepared sample could be reanalyzed in several weeks.

For human astrocytoma and pituitary adenomas nitroproteomics studies, 2DGE-based nitrotyrosine Western blot analysis with MALDI-TOF were used to obtain endogenous nitroproteins from human pituitary control and adenoma tissues, and 2DGE-based nitrotyrosine Western blot analysis with liquid chromatography-electrospray ionization-quadrupole ion trap (LC-ESI-Q-IT) were used to obtain endogenous nitroproteins from human astrocytoma brain tissues, flowed by identification of nitroproteins, proteins interacted with nitroproteins and nitrotyrosine sites. A representative MS/MS spectrum was shown to identify nitropeptide (ITFDDnYIAC*C*VK) that is derived from sorcin (C9J0K6) in human astrocytoma tissue (**Figure 4**).

A total of eight nitroproteins was identified in human normal pituitary tissues with 2DGE-MS/MS [9, 36], and nine nitroproteins were identified in a human nonfunctional pituitary adenoma tissue with NTAC-MS/MS [11], and 18 nitroproteins and their 20 nitrotyrosine sites was identified in a human astrocytoma tissue with 2DGE-MS/MS [35] (**Table 1**). Three nitroprotein–protein complexes were also identified in a human nonfunctional pituitary adenoma tissue: the nitrated beta-subunit of cAMP-dependent protein kinase (PKA) complex, the nitrated proteasome–ubiquitin complex, and the nitrated interleukin 1 family member 6–interleukin 1 receptor–interleukin 1 receptor-associated kinase-like 2 (IL1-F6–IL1-R–IRAK-2) complex [37].

Furthermore, the 2DE-MS/MS-identified nitrosorcin in astrocytoma tissues was confirmed with immunoprecipitation coupled with 1D gel-Western blot experiments (**Figure 5**). The tyrosine nitration of sorcin was measured by immunoprecipitation coupled with Western blotting between IV-grade astrocytoma and normal control (N) tissues. Thus overall status of tyrosine nitration in the whole tissues could be displayed with anti-nitrotyrosine antibody. The results confirmed the tyrosine nitration of sorcin in astrocytoma, and the level of tyrosine nitration of sorcin in astrocytoma is obviously higher than the control tissues.

3.4. Functional characteristics of the nitroproteins

Comprehensive analysis of the functional characteristics of those nine nitroproteins and three nitroproten-protein complexes in a pituitary adenoma biological system revealed several important functional pathways involved in protein tyrosine nitration (**Figure 6**): Nitrated RHOGAP5 and nitrated rhophilin 2 are involved in the GTPase signal pathway. Nitrated CENT-beta 1 and nitrated PKAR1-beta are involved in the PKA signal pathway. IRAK-2 in the IL1-R complex and nitrated IL1-F6 are involved in the cytokine system. The nitrated proteasome–ubiquitin complex is an important enzymatic complex involved in the intracellular nonlysosomal proteolytic pathway. Nitrated LIRA4 might be involved in the immune system. Nitrated ZFP432 is involved in transcription regulatory systems. The nitrated S1P lyase 1 participates in sphingolipid metabolism to regulate cell proliferation, survival, and cell death as well as the immune system.

Comprehensive analysis of the functional characteristics of 18 astrocytoma nitroproteins revealed those nitroproteins participated in multiple cancer-related biological processes (**Figure 7**): (a) microtubule dynamic stabilization and cytoprotection, which mainly involvedβVIII-tubulin, and β-tubulin; (b) tumor migration and metastasis, which mainly

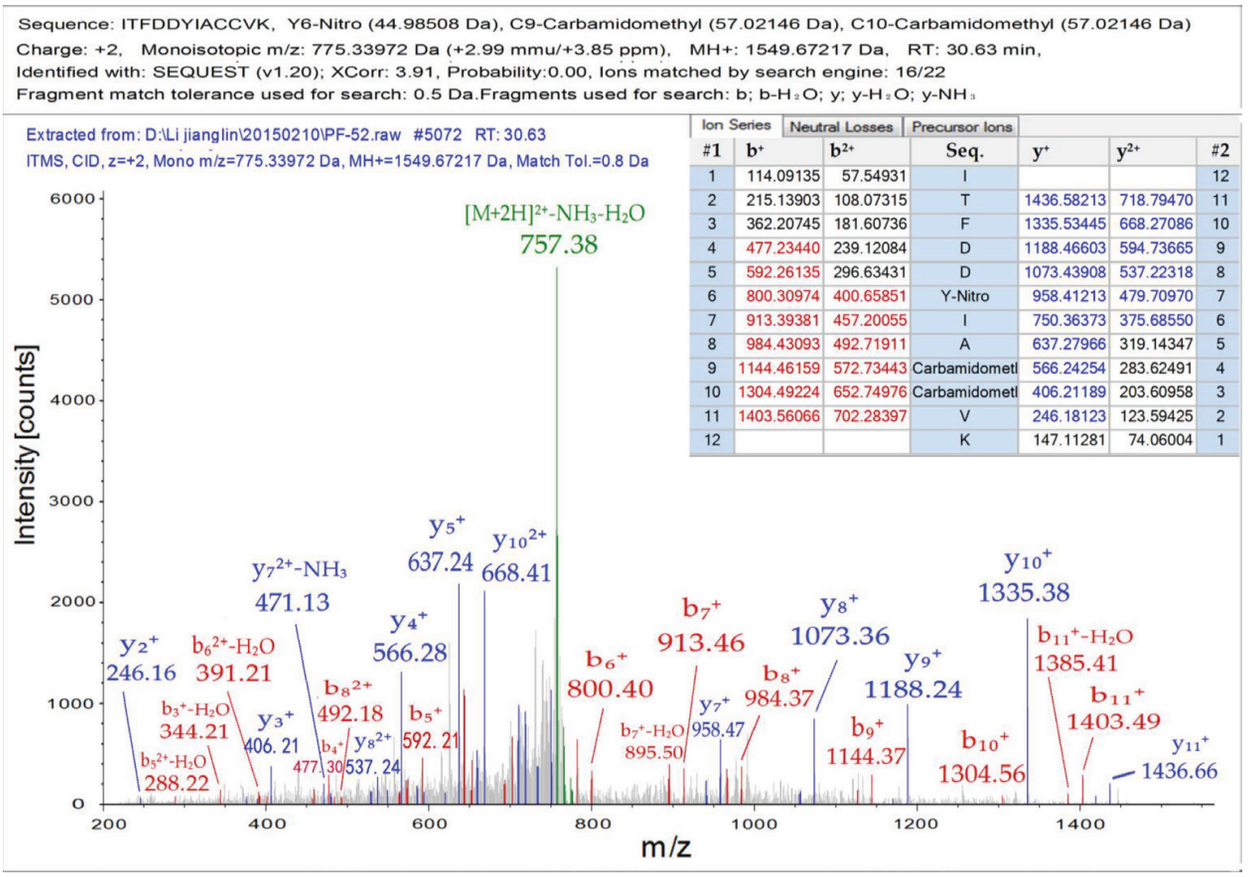

Figure 4. MS/MS spectrum of a nitropeptide (ITFDDnYIAC*C*VK) that is derived from sorcin (C9J0K6) in astrocytoma tissue. nY = nitrotyrosine residue. Reproduced from Peng and Zhan [35], with permission from Springer, copyright 2015.

Nervous system tumors/ control	Nitrated protein name	nY site
Pituitary adenoma	Rho-GTPase-activating 5 [Q13017] (ARHGAP5)	nY^{550}
	Leukocyte immunoglobulin-like receptor A4 [P59901]	nY^{404}
	Zinc finger protein 432 [O94892]	nY^{41}
	PKA beta regulatory subunit [P31321] (PRKAR1B)	nY^{20}
	Sphingosine-1-phosphate lyase 1 [O95470]	nY^{356}, Y^{366}
	Centaurin beta 1 [Q15027]	nY^{485}
	Proteasome subunit alpha type 2 [P25787] (PSMA2)	nY^{228}
	Interleukin 1 family member 6 [Q9UHA7] (IL1F6)	nY^{96}
	Rhophilin 2 [Q8IUC4] (RHPN2)	nY^{258}

Nervous system tumors/ control	Nitrated protein name	nY site
Pituitary control	Proteasome subunit alpha type 2 (PSMA2)	nY^{228}
	Mitochondrial co-chaperone protein HscB [Q8IWL3]	nY^{128}
	Actin [P03996] (ACTA2, ACTG2, ACTC1)	nY^{296}
	Synaptosomal-associated protein (SNAP91)	nY^{237}
	Ig alpha Fc receptor [P24071] (FCAR)	nY^{223}
	Progestin and adipoQ receptor family member III [Q6TCH7] (PAQR3)	nY^{33}
	PKG 2 [Q13237] (PRKG2)	nY^{354}
	Stanniocalcin 1[P52823] (STC1)	nY^{159}
Astrocytoma	Ras-related protein Rab-8B (H0YMN7)	nY^{77};nY^{78}
	Isoform 2 of Signal-induced proliferation-associated 1-like protein 2 (Q9P2F8–2)	nY^{1369}, nY^{1387}
	Regulating synaptic membrane exocytosis protein 1 (Q86UR5)	nY^{926}
	Isoform 2 of Grainyhead-like protein 1 homolog (Q9NZI5–2)	nY^{400}, nY^{402}
	Probable G-protein coupled receptor 52 (Q9Y2T5)	nY^{281}, nY^{284}
	Sorcin (C9J0K6)	nY^{116}
	Tubulin beta chain (P07437)	nY^{106}
	Tubulin beta-2A chain (Q13885)	nY^{106}, nY^{183}, nY^{200}
	Tubulin beta-2B chain (Q9BVA1)	nY^{106}
	Tubulin beta-3 chain (Q13509)	nY^{106}
	Ig kappa chain V-I region WAT (P80362)	nY^{49}
	Coiled-coil domain-containing protein 105 (Q8IYK2)	nY^{372}
	Helicase ARIP4 (E7EU19)	nY^{407}
	General transcription factor 3C polypeptide 3 (Fragment) (H7C0C0)	nY^{110}
	Isoform 2 of Arf-GAP with SH3 domain, ANK repeat and PH domain-containing protein 2 (O43150–2)	nY^{75}
	Isoform 2 of Toll-like receptor 9 (Q9NR96–2)	nY^{419}
	Transducin-like enhancer protein 2 (B4DE62)	nY^{369}

Note: nY = nitrotyrosine. Pituitary adenoma and control modified from Zhan and Desiderio [9, 11, 36], with permission from Elsevier Science, copyright 2004, 2006 and 2007. Astrocytoma modified from Peng and Zhan [35], with permission from Springer, copyright 2015.

Table 1. Nitroproteins and non-nitrated proteins identified from nervous system tumor.

involved sorcin, isoform 2 of Toll-like receptor 9, isoform 2 of Arf-GAP with SH3 domain/ ANK repeat and PH domain-containing protein 3, transducin-like enhancer protein 2, and GPR52; (c) chemotherapy resistance, which mainly involved Ras-related protein Rab 8,

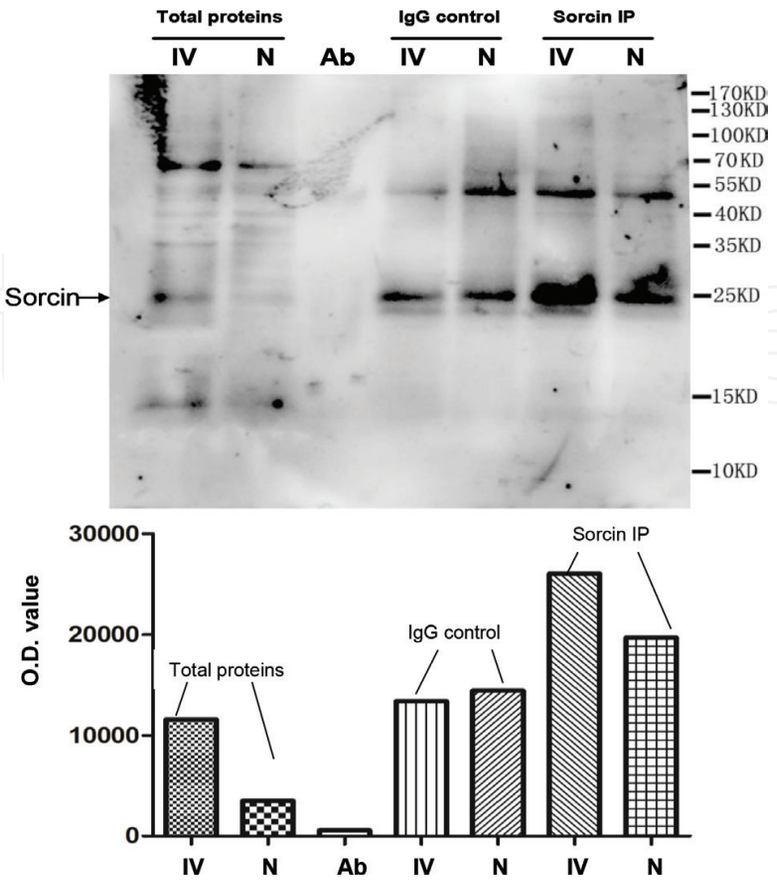

Figure 5. Nitrotyrosine immune activities in nitrosorcin-immunoprecipitated products from IV-astrocytoma and normal control (N) tissues. Reproduced from Peng and Zhan [35], with permission from springer, copyright 2015.

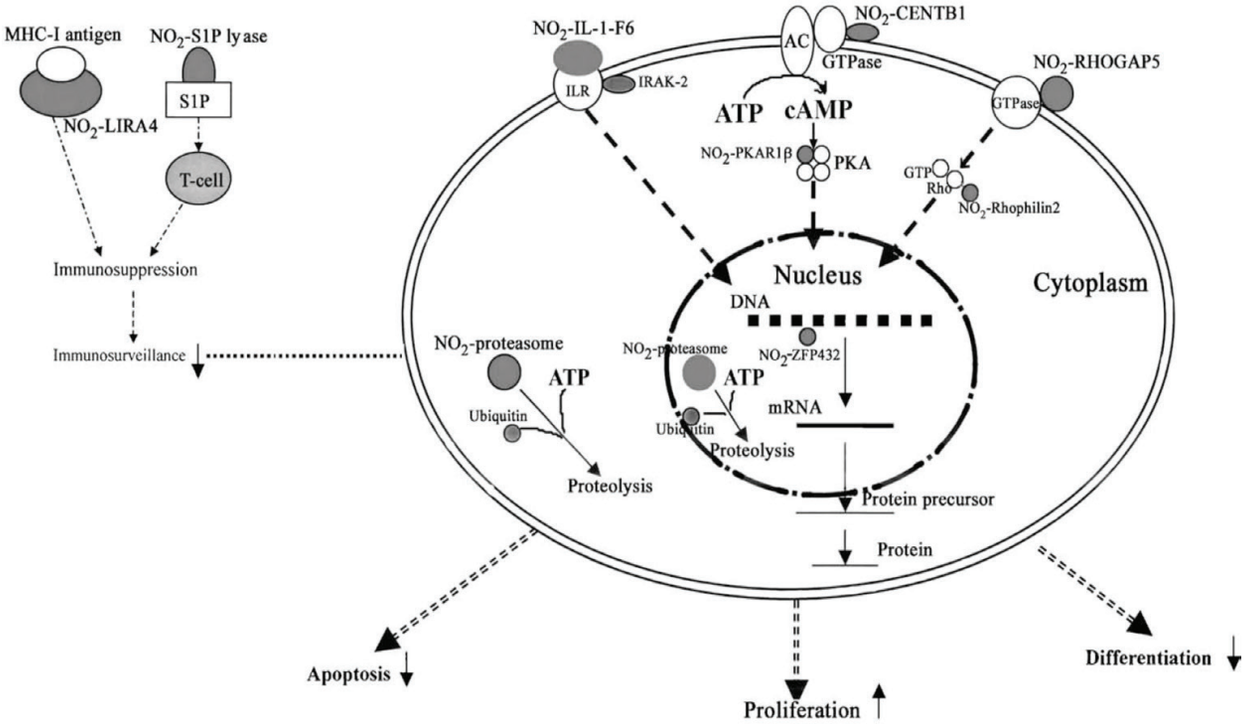

Figure 6. Experimental data-based model of nitroproteins and their functions in human nonfunctional pituitary adenomas. NO_2^-, nitroprotein. Reproduced from Zhan and Desiderio [11], with permission from Elsevier Science, copyright 2006.

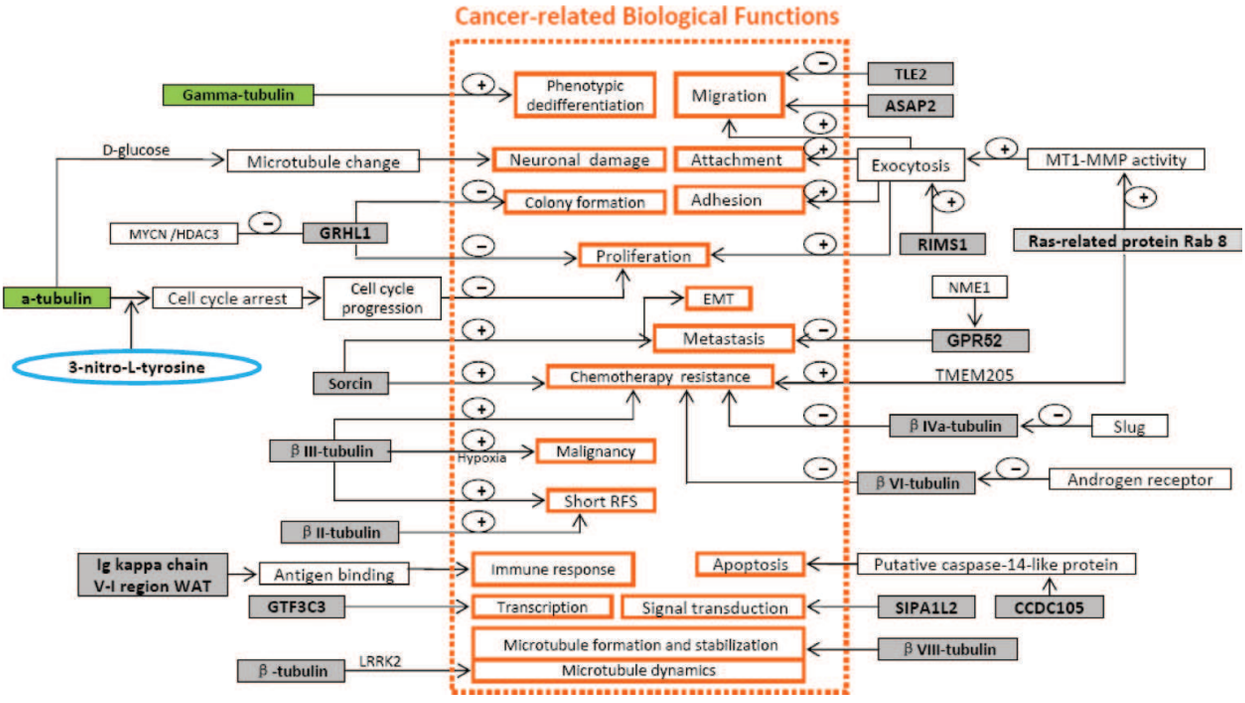

Figure 7. Experimental data-based diagram that rationalizes nitrotyrosine-containing proteins in the glioma biological system. Reproduced from Peng and Zhan [35], with permission from Springer, copyright 2015.

βIII-tubulin, βIVa-tubulin, βVI-tubulin, and sorcin; (d) cell proliferation and apoptosis, which mainly involved a-tubulin, isoform 2 of Grainyhead-like protein 1, Ras-related protein Rab 8, regulating synaptic membrane exocytosis protein 1, and coiled-coil domain-containing protein 105; (e) phenotypic dedifferentiation, which involved gamma-tubulin; (f) signal transduction, which mainly involved isoform 2 of signal-induced proliferation-associated 1-like protein 2; (g) others such as transcription, immune response, and transformation; (h) tumor malignancy and recurrence-free survival, which involvedβII-tubulin and βIII-tubulin.

4. Conclusion(s)

Protein tyrosine nitration, as one of an important PTMs generated in nervous system tumor, participates in multiple complex biological processes, including cell proliferation and apoptosis, metastasis, migration, drug-resistance, cytoskeleton, signal transduction, immune response and cellular differentiation [43]. What is the mechanism for protein tyrosine nitration in carcinogenesis and development of malignant tumors? How to identify the protein targets and exact modified sites of tyrosine nitration? One/two-dimensional gel electrophoresis-based nitrotyrosine Western blot analysis and tandem mass spectrometry have been successfully applied in the analysis of nitroproteins in nervous system tumors.

Further study is needed to solve those limits on protein nitration: (1) Protein tyrosine nitration and heterogeneity of neoplasm. (2) Three-dimensional spatial structure of a nervous system tumor-related nitroprotein. (3) Consistency issues between body fluid and tissue. With the clarification of those issues on nitroproteins, protein tyrosine nitration will have a significant impact on the field of nervous system tumors.

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Conflict of interest

We declare that we have no financial and personal relationships with other people or organizations.

Acronyms and abbreviations

CNS	central nervous system
ELISA	enzyme-linked immunosorbent assay
ESI	electrospray ionization
FDR	false discovery rates
IEF	isoelectic focusing
MALDI	matrix-assisted laser desorption ionization
MS/MS	tandem mass spectrometry
NTAC	nitrotyrosine affinity column
PAGE	polyacrylamide gel electrophoresis
PTM	posttranslational modification
Q-IT	quadrupole ion trap
RNS	reactive nitrogen species
SDS	sodium dodecyl sulfate
S/N	signal-to-noise ratio
TEMED	tetramethylethylenediamine
1DGE	one-dimensional gel electrophoresis
2DGE	two-dimensional gel electrophoresis

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