

Identification and Characterization of Membrane Proteins from Mouse Brain Tissue

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Abstract

Membrane proteins are attractive targets for proteomics research because of their vital roles in numerous processes of the cell including: cell adhesion, immune response, metabolism and signal transduction... At the start of proteomics research, two-dimensional gel electrophoresis (2D-PAGE) was routinely used to separate complex proteomic samples. However, this method faces difficulties in separating membrane proteins due to their hydrophobicity. In this study, mouse brain membrane fractions were prepared using carbonate extraction and ultracentrifugation. The separation and identification of the membrane proteins by using a gel-based approach in combination with two-dimensional nano liquid chromatography coupled online with tandem mass spectrometry (2DNanoLC-Q-TOF-MS/MS) were presented. In total, 298 identified membrane proteins from mouse brain tissues were verified and predicted by UniProt database, SOSUI and TMHMM algorithms. Among them, 129 (43.3%) proteins that have at least one transmembrane domain were predicted by SOSUI and TMHMM. Furthermore, the function, subcellular location and hydrophobicity value of the identified membrane proteins were also categorized.

Keywords: Membrane proteins; Proteomics; Mouse brain; NanoLC-ESI-MS/MS

Introduction

According to recent published studies, membrane proteins are the most attractive targets in proteomics research. It is estimated that, approximately 30% of proteins encoded by the mammalian genome are transmembrane proteins [1,2]. These proteins participate in a variety of cellular processes such as cell adhesion, immuno-protection, metabolism and signal transduction [3]. Differential expression of membrane proteins can be the main cause of pathological states such as: Alzheimer's disease, diabetes, Hodgkin's disease and liver cirrhosis [4]. Membrane proteins are the usual candidates for drug development (about 60% of approved drugs target membrane proteins) due to their roles as transporters, receptors and structural proteins as well as their impacts on intracellular processes [5]. Therefore, the study of membrane proteins holds promises not only for the understanding of disease mechanism but also for the search of new biomarkers that can be the targets for the drug development.

Up to this day, a numerous of methods have been used to separate and identify membrane proteins from different samples. Two-dimensional electrophoresis (2D-PAGE) has been emerged as a primary method to separate these proteins [6,7]. However, this method has shown several drawbacks, preparation for the first dimension is time-consuming, and the whole process is hard to automate. Furthermore, 2D-PAGE faces many difficulties in analyzing several types of proteins, such as low-abundance proteins, hydrophobic proteins, very large as well as very small proteins, and proteins with extreme pI values. Since the analysis of membrane proteins remains a significant challenge in proteomics, other techniques need to be established to address these problems. In recent years, two-dimensional nano liquid chromatography (2DNanoLC) has been employed as an alternative separation technique for 2D-PAGE. Using in-solution digestion and nano liquid chromatography coupled online with tandem mass spectrometry (NanoLC-MS/MS), many proteomic studies have been reported [8-11]. Mammalian brain is one of the most significant challenges not only in proteomics research but also in current biomedical science due to its immense complexity. Mice have been widely used as brain research models due to their

genetic accessibility and suitability as models for many aspects of human biology [12]. In this study, the fractionation, purification and identification of membrane proteins from mouse brain tissue using a gel-based approach in combination with two-dimensional nano liquid chromatography coupled online with tandem mass spectrometry (2DNanoLC-Q-TOF-MS/MS) are presented. Other characteristics of identified membrane proteins were also categorized by using SOSUI, TMHMM algorithms and UniProt database.

Materials and Methods

Materials

Formic Acid (FA), Dithiothreitol (DTT), Iodoacetamide (IAA), ammonium bicarbonate, ammonium acetate, trypsin (proteomics sequencing grade), sodium bicarbonate, protease inhibitor and triton X-100 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Formic acid (FA) and Trifluoroacetic acid (TFA) were obtained from Fluka (Fluka Chemie GmbH, Buchs, Switzerland). Acetonitrile (ACN, chromatogram grade) and other chemicals (analytical grade) were obtained from Barker (Pittsburgh, USA). The Bradford assay kit, acrylamide, bis-acrylamide, urea, glycine, Tris, CHAPS, and SDS were purchased from Bio-Rad (Hercules, CA, USA). The other basic chemicals, equipments and apparatus were supplied by The National Key Laboratory of Gene Technology, Institute of Biotechnology (IBT), Vietnam Academy of Science and Technology (VAST). Mice were provided by the National Institute of Hygiene and Epidemiology (Hanoi, Vietnam).

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Preparation of membrane fractions

The preparation of membrane fractions was carried out according to our previous studies [13]. Briefly, mouse brains were taken from ten-week-old mice and stored in liquid nitrogen until analysis. Mouse brains were minced into small pieces using a clean scalpel and then washed three times with the ice-cold PBS buffer (0.2 g KCl, 8 g NaCl, 1.44 g Na₂HPO₄, 0.24 g KH₂PO₄) before being suspended in 3 volumes of the homogenization buffer (0.25 M sucrose in 5 mM Tris-HCl, pH 7.4 with 1mM tetrasodium EGTA, 1mM sodium orthovanadate (Na₃VO₄) and 2 mM sodium fluoride in deionized filter-sterilized Milli-Q water) containing protease inhibitors (Calbiochem Protease Inhibitor Cocktail Set 111, catalog number 39134, contains AEBSE, aprotinin, bestatin, E-64, leupeptin, pepstatin A). Subsequently, the sample was homogenized in a Potter-Elvehjem homogenizer on ice with a motor driven teflon pestle at approximately 1,000 x g for about 5 min. Completely homogenized samples were centrifuged at 10,000xg for 15 min at 4°C. The supernatant was collected and centrifuged at 100,000 x g at 4°C for 1 hr using Sorvall Ultra Pro 80 refrigerated ultra centrifuge (DuPont, USA). After discarding the clear supernatant, the crude membrane pellets from this harvest were washed, suspended in ice-cold sterile 0.1 M Na₂CO₃, pH 11.4, containing protease inhibitors, and incubated with gentle stirring at room temperature for 1 hr. The mouse brain membrane protein pellets were obtained by centrifugation at 100,000xg for 1 hr at 4°C and again suspended in ice-cold 0.1 M Na₂CO₃, pH 11.4, containing protease inhibitors. The membrane sample was then divided and stored at -80°C until analysis. The protein concentration of the extracted membrane fraction was measured using the Bio-Rad Bradford assay kit (Bio-Rad, Hercules, CA, USA).

SDS-PAGE and in-gel digestion

Membrane proteins (about 25 µg protein/lane) were loaded into 10% SDS-PAGE gels for sample separation. The gel was stained with Coomassie Brilliant Blue G-250. The stained protein bands were excised from gels and placed into 2-ml Eppendorf tubes for trypsin digestion. The in-gel digestion was carried out based on our previous publication [13]. Briefly, gel were destained with 50% ACN in 25 mM NH₄HCO₃, pH 8.0. The gel pieces were then reduced by incubating with 5 mM DTT solution at 56°C for 45 min and alkylated for 1 hr with 20 mM IAA solution in darkness at room temperature. The membrane proteins were digested by adding trypsin with weight ratio 1:50 and incubating overnight at 37°C. The tryptic peptides were extracted with 60% ACN in 1% TFA (v/v). All extracts were saved and dried, and re-dissolved in 0.1% FA for 2D-NanoLC-MS/MS analysis.

Multidimensional chromatography and mass spectrometry

Tryptic peptides were re-dissolved in 30 µl of 0.1% formic acid and loaded onto Strong Cation Exchange (SCX) Chromatography Column (LC Packing, Dionex, Netherland) at a flow rate of 30 µl/min for separation in the first dimension. The bound peptides were then eluted by the ammonium acetate gradients from 10 mM to 2M: 10 mM, 20 mM, 40 mM, 60 mM, 80 mM, 100 mM, 200 mM, 500 mM, 1M and 2M. After desalting and concentrating on a C18 trap column (LC Packing, Dionex, Netherland), the peptides were loaded onto a C18 Reversed Phase (RP) Column (GraceVydac, Hesperia, CA, USA) with mobile phases consisting of 0.1% formic acid in water (buffer A) and 0.1% formic acid in 85% acetonitrile (buffer B). Peptides were then eluted using a linear gradient of buffer B from 0% to 100% at a flow rate of 0.2 µl/min for 90 min.

Tandem mass spectrometry analysis was performed using an ABI

QSTARXL hybrid quadrupole/TOF MS/MS instrument (Applied Biosystems/MDS SCIEX, Ontario, Canada) equipped with a nano-ESI ion source. The MS instrument was operated in the positive ion mode with a spray voltage of 2.5 kV. MS and MS/MS spectra were recorded and processed in the IDA (Information Dependent Acquisition) mode controlled by Analyst QS software (Matrix Science Ltd., London, UK). The scan range of each full MS was from 400 to 1200 amu followed by MS/MS fragmentation of the three most intense precursor peptide ions for 1 s each.

Identification and characterization of membrane proteins

The tandem MS spectra were searched using MASCOT software (Matrix Science Ltd., London, UK) against the SWISS-PROT protein sequence database. For Mascot searching, the following parameters were used: trypsin was chosen for protein digestion; carbamidomethylation (C) was set as fixed modification and oxidation (M) was set as variable modification. The peptide mass tolerance was 100 ppm and the MS/MS fragment tolerance was limited to 0.5 Da. For further verification, proteins were validated by MSQuant software, available at <http://msquant.alwaysdata.net/>. The MSQuant software is used as a validation and quantitation tool that produces the Mascot peptide identifications (HTLM files) and allows manual verification against the raw MS data (QSTARXL raw files) [14]. Membrane proteins were sorted from total identified proteins based on UniProt protein database [15] and TMHMM [16] algorithms. The average hydrophobicity values and Transmembrane Domains (TMDs) of the identified proteins were calculated using the SOSUI algorithm [17]. In addition, the mapping of TMDs in the identified proteins could be predicted by TMHMM algorithm. The subcellular location, function and post-translational modification of these membrane proteins were obtained by searching in the UniProt database that is available at <http://www.uniprot.org/>.

Results

Enrichment and identification of mouse brain membrane proteins

The main difficult tasks of membrane proteomics come from membrane protein enrichment and separation. As mentioned previously, three-dimensional separation method combined SDS-PAGE prefractionation with trypsin digestion of gel slices to generate peptides and continuous two-dimensional LC/MS/MS analysis, was proved to be the best for significantly enhancing protein identifications including single and multiple pass transmembrane proteins [18-20]. In our study, carbonate extraction and ultracentrifugation were also applied before SDS-PAGE prefractionation to enrich mouse brain membrane proteins. As it was evaluated by SDS-PAGE, a large number of membrane proteins were enriched and extracted (data not shown). The SDS-PAGE gel was then cut into approximately 10 bands for in-gel digestion. After desalting and cleaning up with Zip tips, the peptides were applied to NanoLC-MS/MS analysis as it was shown in the previous publication [13]. Subsequently, the bioinformatics tools and softwares were used for membrane protein identification and characterization. The obtained mass spectra were searched using the Mascot software against the Swiss-Prot protein sequence database. In addition, MSQuant software was employed for protein validation [14]. Finally, membrane proteins were sorted from the total identified proteins based on UniProt database, SOSUI and TMHMM prediction algorithms. In total, 298 membrane proteins from mouse brain were detected. Of which, 129 proteins were predicted to have at least one TMD according to SOSUI and TMHMM.

Transmembrane domains (TMDs) and hydrophobicity value

TMDs are one of the most distinctive features of membrane proteins. In our study, two algorithms including SOSUI and TMHMM were used to predict TMDs from the identified proteins. It has to be noted that several differences between the SOSUI and TMHMM results were observed. Going into details, 114 and 101 proteins were predicted to have at least one TMD according to SOSUI and TMHMM algorithms, correspondingly. In summary, 129 integral membrane proteins were detected by combining the results from two algorithms, of which 86 proteins having at least one TMD were predicted by both SOSUI and TMHMM (see the diagram in Figure 1). The detail results of TMDs prediction according to SOSUI and TMHMM are shown in the bar chart (Figure 1). Proteins with one TMD formed the largest proportion (40 proteins), followed by the proteins with two TMDs (28 proteins by SOSUI and 19 proteins by TMHMM). It is notable that, a significant number of proteins were predicted to have more than 10 TMDs (9 proteins by SOSUI and 8 proteins by TMHMM).

As for the prediction results of each protein, It is interesting that 43 integral membrane proteins were predicted by either SOSUI or TMHMM (Table 1). Of these, 28 proteins were predicted as transmembrane or integral membrane proteins based only on SOSUI while the rest were predicted to have at least one TMD according to just TMHMM (15 proteins). For instance, ADP/ATP translocase 1 has 3 TMDs based on TMHMM while it is a peripheral membrane protein according to SOSUI. In contrast, Protein FAM40B is a transmembrane protein with 4 TMDs following SOSUI while it is a peripheral membrane protein based on TMHMM.

In this study, SOSUI algorithm was also used to evaluate the average hydrophobicity of identified membrane proteins. The average hydrophobicity values of all membrane proteins were calculated based on their amino acid sequences. It is shown that the majority of identified membrane proteins (248 proteins, 83.2%) have hydrophobicity values below zero. Only 49 proteins have positive hydrophobicity values, of which 38 proteins are transmembrane proteins. Meanwhile, some UniProt annotated membrane proteins could not be analyzed by

SOSUI prediction algorithm because their size consists of more than 5000 amino acids.

Functional and subcellular classifications of membrane proteins

The function and subcellular location of identified membrane proteins were determined based on UniProt database according their accession numbers (Figure 2). As illustrated in Figure 2a, proteins have a certain distribution or are shuttled between organelles: 114 proteins (38.3%) are from plasma membrane and a range of proteins are from other cell components, such as mitochondrion (91 proteins, 30.5%), vesicle (21 proteins, 5%), endoplasmic reticulum (13 proteins, 4%), extracellular space (10 proteins, 3.3%), Golgi apparatus (6 proteins, 2%). Based on UniProt database, evidently, a significant number of membrane proteins have multiple locations (28 proteins, 9.4%), while the subcellular location could not be identified for a number of membrane proteins (15 proteins, 5%).

Regarding to the functions of membrane proteins, as shown in Figure 2b, enzymes make up the largest group (107 proteins, 35.9%), follow up are regulators, such as inhibitors, activators, transcription factors, etc (70 proteins, 23.5%). In contrast, proteins that participate in transport processes have much smaller proportions: channel (9 proteins, 3%) and transporter (16 proteins, 5.5%). It should be noted a significant number of proteins with underfined function according to UniProt database (37 proteins, 12.4%).

Discussion

The genome-wide mapping of gene expression obtaining by in situ hybridization, voxelation technology combined with cDNA microarray approach is one of the most comprehensive data from mouse brain [21-23]. However, genome annotation using currently available tools is likely to underpredict representation of membrane-associated gene products/proteins that are critical for intra- and intercellular communication, especially in the nervous system. Recently, the development of proteomic techniques has been established new strategies to profile complicated proteome. Membrane proteins are difficult to analyze due to their complex properties including the hydrophobicity and high molecular weight. At the beginning of proteomic research, 2D-PAGE was used routinely as the basic method for membrane protein analysis [24-26]. However, the limitation of this method [9], especially in the number of identified proteins, have impuled the development of new approach for the effective analysis of membrane proteins. Up to this time, the combination of nano liquid chromatography (NanoLC) and mass spectrometry (MS) has been emerged as the alternative method for proteome research, especially for the analysis of membrane proteins. For the profiling of brain membrane proteins, a number of studies using NanoLC-MS have been carried out. Using Global Proteomic Analysis Complemented with CysteinyI-Peptide Enrichment, Wang et al identified 7792 non-redundant proteins (34% of the predicted mouse proteome). A among them, approximately 26% of the identified proteins were annotated as membrane proteins by gene ontology (GO) annotations; 1447 proteins were predicted to have transmembrane domains, and many of the membrane proteins were found to be involved in transport and cell signaling [12]. In the other study, Nielsen et al identified 862 proteins from the mouse brain cortex and 1685 proteins from the mouse hippocampus [27]. Actually, the completed proteome of complex brain tissue has not been extensively characterized using LC-MS/MS approaches and the results from independent surveys are also

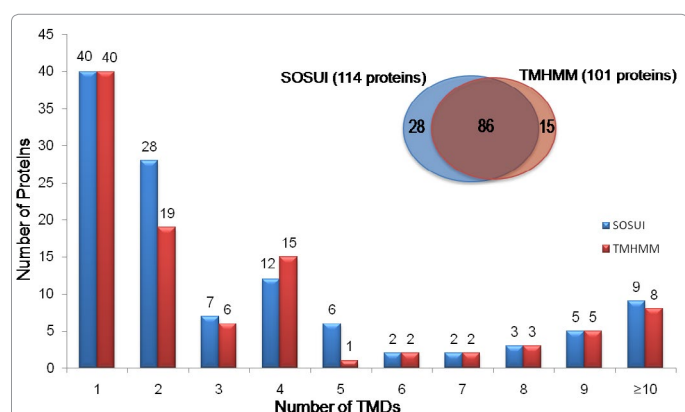
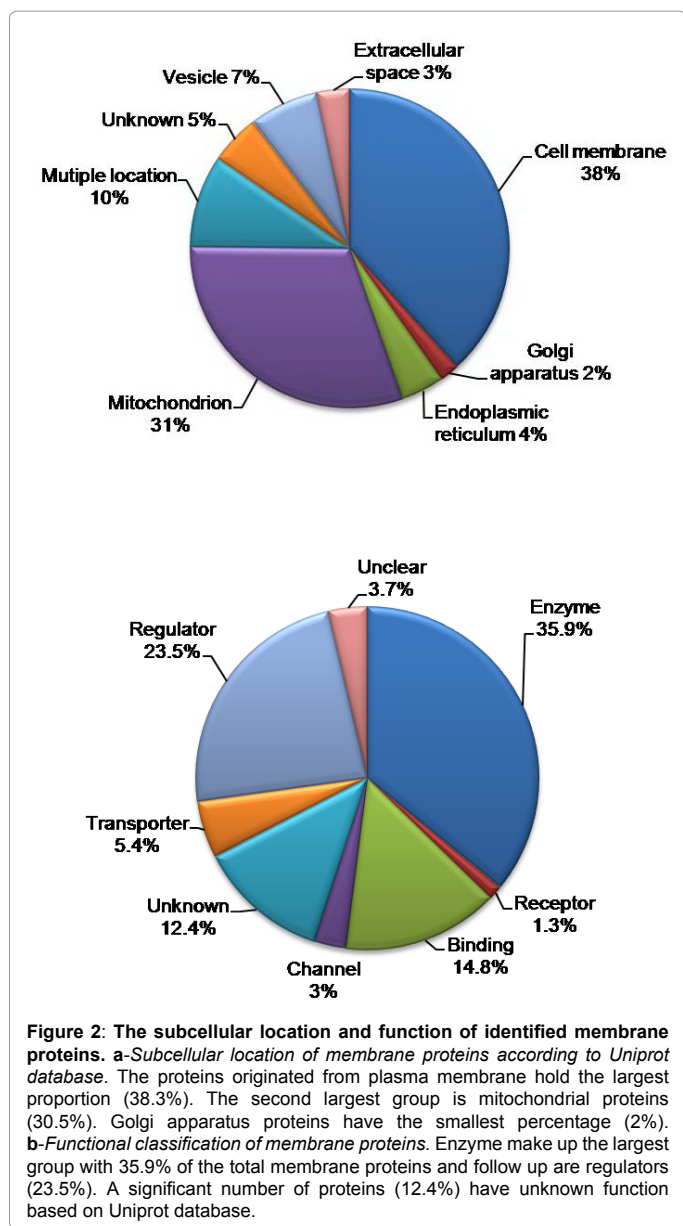


Figure 1: Transmembrane domains prediction of the identified membrane proteins by SOSUI and TMHMM algorithms. In total, 129 membrane proteins were predicted to have at least one TMD according to SOSUI and TMHMM algorithms. Of these, 86 transmembrane proteins were predicted by both SOSUI and TMHMM while the rest were proteins predicted by either SOSUI or TMHMM. In the set of proteins having TMDs, proteins with one TMD occupy the largest proportion (40 proteins based on SOSUI and TMHMM). The second largest group are proteins with two TMDs (28 proteins by SOSUI and 19 proteins by TMHMM). Notably, a significant number of proteins have more than 10 TMDs (9 proteins by SOSUI and 8 proteins by TMHMM).

Nº.	Accession number	Protein name	Matched peptides	Score	TMDs (SOSUI)	TMDs (TMHMM)
1	ERP29_MOUSE	Endoplasmic reticulum resident protein 29	1	52	0	1
2	ADT1_MOUSE	ADP/ATP translocase 1	1	35	0	3
3	ADT2_MOUSE	ADP/ATP translocase 2	3	104	0	2
4	ADT4_MOUSE	ADP/ATP translocase 4	2	58	0	2
5	APOO_MOUSE	Apolipoprotein O	1	65	0	1
6	CY1_MOUSE	Cytochrome c1, heme protein, mitochondrial	7	263	0	1
7	GDIA_MOUSE	Rab GDP dissociation inhibitor alpha	2	67	0	2
8	GPDM_MOUSE	Glycerol-3-phosphate dehydrogenase, mitochondrial	2	58	0	1
9	MCU_MOUSE	Calcium uniporter protein, mitochondrial	1	62	0	2
10	MFF_MOUSE	Mitochondrial fission factor	1	45	0	1
11	NDUA4_MOUSE	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 4	3	139	0	1
12	PPIB_MOUSE	Peptidyl-prolylcis-transisomerase B	4	191	0	1
13	PTH2_MOUSE	Peptidyl-tRNA hydrolase 2, mitochondrial	1	63	0	1
14	QCR9_MOUSE	Cytochrome b-c1 complex subunit 9	2	37	0	1
15	SFXN3_MOUSE	Sideroflexin-3	5	166	0	2
16	DLDH_MOUSE	Dihydrolipoyl dehydrogenase, mitochondrial	8	206	1	0
17	GDAP1_MOUSE	Ganglioside-induced differentiation-associated protein 1	2	100	1	0
18	TINAL_MOUSE	Tubulointerstitial nephritis antigen-like	2	101	1	0
19	UBA1Y_MOUSE	Ubiquitin-like modifier-activating enzyme 1 Y	1	28	1	0
20	RPTOR_MOUSE	Regulatory-associated protein of mTOR	4	29	2	0
21	TANC2_MOUSE	Protein TANC2	4	24	2	0
22	UBA1_MOUSE	Ubiquitin-like modifier-activating enzyme 1	2	53	2	0
23	FA40A_MOUSE	Protein FAM40A	2	38	3	0
24	FA40B_MOUSE	Protein FAM40B	1	33	4	0
25	SERPH_MOUSE	Serpin H1	5	183	2	0
26	LAMA4_MOUSE	Laminin subunit alpha-4	1	51	1	0
27	LAMB2_MOUSE	Laminin subunit beta-2	4	198	1	0
28	LGI1_MOUSE	Leucine-rich glioma-inactivated protein 1	1	32	1	0
29	THY1_MOUSE	Thy-1 membrane glycoprotein	1	64	2	0
30	GRP78_MOUSE	78 kDa glucose-regulated protein	1	69	1	0
31	NID1_MOUSE	Nidogen-1	2	40	1	0
32	NID2_MOUSE	Nidogen-2	2	64	1	0
33	AP3D1_MOUSE	AP-3 complex subunit delta-1	1	26	1	0
34	ATP5L_MOUSE	ATP synthase subunit g, mitochondrial	1	42	1	0
35	NDUB6_MOUSE	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 6	1	27	1	0
36	MTCH2_MOUSE	Mitochondrial carrier homolog 2	1	98	3	0
37	AOFA_MOUSE	Amine oxidase [flavin-containing] A	3	57	2	0
38	KCC2A_MOUSE	Calcium/calmodulin-dependent protein kinase type II subunit alpha	17	614	1	0
39	MELK_MOUSE	Maternal embryonic leucine zipper kinase	2	15	1	0
40	CBPM_MOUSE	Carboxypeptidase M	1	53	2	0
41	KCC2G_MOUSE	Calcium/calmodulin-dependent protein kinase type II subunit gamma	4	181	1	0
42	KCC2B_MOUSE	Calcium/calmodulin-dependent protein kinase type II subunit beta	7	248	1	0
43	KCC2D_MOUSE	Calcium/calmodulin-dependent protein kinase type II subunit delta	7	240	1	0

Table 1: List of integral membrane proteins predicted by either SOSUI or TMHMM.



different due to the membrane enrichment methods, MS systems and used softwares. In order to improve the protein identification efficiency, several studies have proposed a gel-based approach using SDS-PAGE before applying the tryptic peptides to NanoLC-MS analysis for membrane protein identification [7,16,18]. The important purpose of proteomics research is to profile the proteomes/subproteomes from specific samples including both biological fluids and cells/tissues. So, the results of identification and characterization of membrane proteins shown in present study may contribute as an important and special reference data for the database of mouse brain membrane proteome that were initially established by the previous projects [12,27-29].

Prediction of TMDs of membrane proteins is of interest in current proteomics and bioinformatics research. So far a number of methods have been applied for this mission, using a variety of specific algorithmic techniques [30,31]. In our study, 86 transmembrane proteins (66.7%) were confirmed by both TMHMM and SOSUI while

the rest (43 proteins) were predicted by either TMHMM or SOSUI prediction software. Different predicted softwares have their own error rate due to particular algorithms. Eukaryotic sequences have much lower prediction accuracies than their prokaryotic counterparts. One plausible explanation to this observation is that prokaryotic TM sequences have more conserved features than eukaryotic TM sequences due to simpler cellular structure, therefore are less likely to be wrongly predicted [31].

UniProtKB/Swiss-Prot is a high-quality, manually annotated, non-redundant protein sequence database because it combines information extracted from scientific literature and biocurator-evaluated computational analysis [15,32]. In this work, several characters of the identified membrane proteins including subcellular location, function were categorized based on UniProt database. Analyzing the subcellular location of these proteins, we found that the highest percentage (38.3%) of membrane proteins comes from plasma membrane and this outcome corresponds with the previous survey from Wang's group [12]. The analysis of functional and subcellular classification of the identified protein in this study showed that only few participated in transport process (channel 3%, transporter 5.5%). This may indicate two things: (i) they are not present in the membrane sample because of the poor extraction; (ii) these proteins are really difficult to detect. Probably, proteins located in different cellular compartments could be equally represented among the identified proteins if they were extracted, separated and identified according to the mentioned methods. In our study, for obtaining more information about the mouse brain membrane proteins, their functions were evaluated based on UniProt database. The highest percentage of the identified membrane proteins (35.9%) have catalytic activity and these answer reflects the vital role of membrane proteins in the signal transduction pathway of the cell [31,33,34].

Conclusion

By using a gel-based approach combined with NanoLC-MS and Bioinformatics tools (UniProt database, SOSUI and TMHMM prediction algorithms), 298 membrane proteins from mouse brain tissues were identified and characterized. Of these, 129 (43.3%) proteins have at least one transmembrane domain according to SOSUI and TMHMM prediction algorithms. Furthermore, the function, subcellular location, transmembrane domains (TMD) and average of hydrophobicity of the identified membrane proteins were categorized and analyzed.

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