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ORIGINAL ARTICLE

Screening of Substrates of Protein Arginine Methyltransferase 1 in Glioma[△]

Shan Wang, Xiao-chao Tan, Bin Yang, Bin Yin, and Xiao-zhong Peng*

National Laboratory of Medical Molecular Biology, Institute of Basic Medical Sciences,
Chinese Academy of Medical Sciences & Peking Union Medical College,
Beijing 100005, China

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Objective To screen the asymmetric dimethyl arginines (ADMA)-containing proteins which could combine with protein arginine methyltransferase 1 (PRMT1).

Methods Western blot was adopted to identify the expression of PRMT1 and the proteins with ADMA in glioma cell lines and normal brain tissues, and then to detect the changes of ADMA level after knock-down of PRMT1 with RNAi transfection in U87MG cells. Co-Immunoprecipitation (Co-IP), western blot, and sliver staining were employed to screen the candidate binding proteins of PRMT1. Then liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used to identify the binding proteins of PRMT1.

Results The expression of PRMT1 and some levels of ADMA were higher in glioma cell lines than in normal brain tissues. After knocking down PRMT1, some ADMA levels were found declined. After screening the binding proteins of PRMT1 with Co-IP and LC-MS/MS, 26 candidate binding proteins were identified. Among them, 6 candidate proteins had higher ions scores (>38) and bioinformation analysis predicted that SEC23-IP, ANKHD1-EIF4EBP3 protein, and 1-phosphatidylinositol-3-phosphate 5-kinase isoform 2 had possible methylated arginine sites.

Conclusions The high expression of PRMT1 in glioma may induce the change of ADMA levels. Altogether 26 candidate proteins were identified, which contain ADMA and specifically bind with PRMT1.

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SINCE the research on post-translational modification has become more and more crucial, the importance of arginine methylation of proteins has been recognized. Arginine methylation is

involved in many biochemical and biological processes, such as signal transduction, cell proliferation, transcriptional regulation, and RNA splicing.^{1,2} The protein arginine methyltransferase (PRMT) family is the main group of enzymes to methylate arginine residues. PRMTs catalyze the post-translational transfer of a methyl group from the donor S-adenosyl-L-methionine to arginine residues.¹ In the mammalian cells, 8 PRMT members have been identified so far to have enzymatic activity among 11 members

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*Corresponding author Tel: 86-10-65296411, E-mail: pengxiaozhong@pumc.edu.cn

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of PRMT family. PRMT1 is the most important member and could contribute to as much as 85% of all the cellular PRMT activities.³ It can catalyze the arginine residues to asymmetric dimethyl arginine (ADMA), which might play a role in many diseases, especially tumorigenesis.⁴ For example, apoptosis signal-regulating kinase 1, one of the important regulators in apoptosis signaling pathway, can be methylated by PRMT1 and implicated in the apoptosis of breast tumor.⁵ Estrogen receptor α (ER α) is also one of the substrates of PRMT1. Methylated ER α can interact with Src/FAK and p85, and then activate Akt pathway to promote cell proliferation and inhibit apoptosis.⁶ In all the brain tumors, glioma is the most common and malignant one.⁷ The present study investigated the relationship between PRMT1 and proteins with ADMA in glioma, and screened the proteins binding with PRMT1, so as to lay the foundation for exploring the molecular mechanism of PRMT1 in glioma.

MATERIALS AND METHODS

Tissue samples

Two human normal brain tissue samples were provided by Department of Anatomy, Peking Union Medical College. The study protocol was approved by Institutional Ethics Committee.

Glioma cell culture and RNAi transfection

The human glioma cell lines (T98G, U87MG, and A172) were obtained from American Type Culture Collection (ATCC) and all cultured according to the guidelines recommended by ATCC. The Stealth™ RNAi Duplexes of *PRMT1* were purchased from Invitrogen Life Technologies Inc. (Grand Island, NY, USA). The sequence of PRMT1-RNAi was 5'-GCCUGCAAGUGAAGCGGAAUGACUA-3'. The Stealth™ RNAi Negative Control Duplexes (RNAi-NC, Cat. NO. 12935-300) were also synthesized by Invitrogen. Before transfection, glioma cells were seeded in DMEM containing 10% fetal bovine serum without antibiotics overnight. In the next day, according to the manufacturer's protocol for Lipofectamin™ 2000-based transfections (Invitrogen), the cells were transfected with RNAi-PRMT1 or RNAi-NC, respectively. The final concentration was 100 nmol/L.

Western blot

Total protein of glioma cells or normal brain tissue samples were extracted with lysis buffer [50 mmol/L Tris-HCl (pH 8.0), 1% NP-40, 150 mmol/L NaCl, 2 μ g/mL leupeptin, 2 μ g/mL pepstatin, 2 μ g/mL aprotinin, and 2 μ g/mL PMSF]. The protein lysate was separated by 12% or 8% sodium

dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred electrophoretically onto nitrocellulose membrane for 1 or 2 hours. After blocking with 5% skimmed milk for at least 1 hour, the membranes were incubated with specific primary antibodies for PRMT1 (Millipore, Billerica, MA, USA), ASYM24 (specifically to proteins containing ADMA, Millipore), or GAPDH (Abmart, Shanghai, China). Then the membranes were washed with Tris-buffered saline, further incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG antibody or goat anti-mouse IgG antibody (Zhongshan Goldenbridge Biotechnology Co., Beijing, China) for 1 hour, and visualized by enhanced chemiluminescence.

Co-immunoprecipitation (Co-IP)

U87MG, a representative glioma cell line, was harvested in ice-cold phosphate-buffered saline and lysed with lysis buffer, the same buffer as used in western blot. After centrifuging at 13 800 $\times$ g for 30 minutes, the cleared supernatants were pre-cleaned with protein G agarose (Roche Co., Mannheim, Germany) for 1 hour at 4°C. The cleared lysates were incubated for 16 hours at 4°C with the antibodies for PRMT1 (Millipore, experimental group) and normal rabbit IgG (Zhongshan Goldenbridge Biotechnology Co., negative control group). Protein G agarose was then added and incubated for 3 hours at 4°C. After washing with washing buffer [50 mmol/L Tris-HCl (pH 7.5), 500 mmol/L NaCl, 0.1% Nonidet 40, 0.05% SDS] for four times, the Co-IP proteins were denatured and analyzed with SDS-PAGE.

Silver-staining

The protein samples from Co-IP were separated using 10% SDS-PAGE, and the gel was then incubated with the stationary liquid (30% alcohol, 10% acetic acid) for 20 minutes. After washing with washing buffer for 10 minutes, the gel was further incubated with sensitization buffer (0.05 g Na₂S₂O₃, 250 mL H₂O) for 30 minutes. It was later incubated with silver staining buffer for 30 minutes and then color reagent (4 g Na₂CO₃, 80 μ L formaldehyde, 200 mL H₂O) for 6 minutes.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

The stained protein bands after silver staining on the gel were carved, digested, and analyzed by mass spectrometry in the Center of Equipment Testing, Academy of Military Medical Sciences. Sequence analysis was performed with MASCOT (version 2.2, Matrix Sciences, Boston, MA, USA) using the non-redundant protein database from Mascot's

web site (<http://www.matrixscience.com/cgi/search-from.pl?FORMVER=2&SEARCH=MIS>). Then we utilized 2 methods to screen PRMT1's binding proteins from the result of MASCOT. One was ions scores, in which a number greater than 38 indicates identity or extensive homology ($P < 0.05$). The other was the same proteins, which existed in IP-PRMT1 group (Co-IP with PRMT1 antibody) and also in IP-IgG control group (Co-IP with rabbit IgG). Judging from the ions scores and eliminating the same proteins, we selected the final candidate proteins.

Bioinformation analysis

We put the sequences of final candidate proteins into MeMo web site, which focuses on predicting the protein methylation including arginine methylation or lysine methylation with an accuracy for prediction of arginine methylation reaching 86.7%.⁸ MeMo's web site is <http://www.bioinfo.tsinghua.edu.cn/~tigerchen/memo.html>.

RESULTS

PRMT1 expression and ADMA levels in glioma cells

After performing western blot in two normal brain tissues and three glioma cell lines, PRMT1 was found substantially up-regulated in glioma cells compared with normal brain tissues. Some ADMA levels were also higher in glioma cells, but a few ADMA levels were reduced (Fig.1)

Relationship between PRMT1 and proteins with ADMA

Western blot results showed that after suppression of PRMT1 expression with RNAi-PRMT1 transfection, a lot of ADMA levels were up-regulated to different degrees (Fig. 2A), but others were not changed. Furthermore, Co-IP results revealed that there were some proteins binding to PRMT1 and being dimethylated at arginine site (Fig. 2B).

Binding proteins of PRMT1 screened by LC-MS/MS

Sliver staining visualized target proteins of PRMT1 (Fig. 3A). After analysis of the carved bands by LC-MS/MS and MASCOT database, 26 proteins were identified in the experimental group (Table 1). Based on the ions scores and after eliminating the same proteins, 6 candidate proteins were selected from the 26 proteins. According to the analysis result of MeMo web site, SEC23-interacting protein (SEC23-IP) might have 1 arginine methylated site, ANKHD1-EIF4EBP3 protein and 1-phosphatidylinositol-3-phosphate 5-kinase isoform 2 might have 11 sites each (Table 2). Because the bands of high molecular weight proteins were not clear in Figure 2B, we repeated western

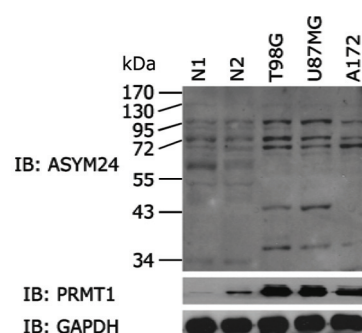


Figure 1. Western blot of PRMT1 expression and asymmetric dimethyl arginine (ADMA) levels (by ASYM24 antibody) in normal brain tissues (N1 and N2) and human glioma cell lines (T98G, U87MG, and A172).

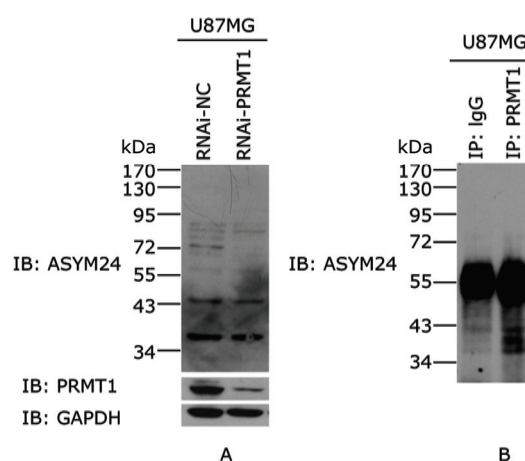


Figure 2. Detection of the relationship between PRMT1 and proteins containing ADMA.

A. Western blot analysis of ASYM24 antibody in glioma cell line U87MG after knock-down of PRMT1. Cells transfected with RNAi Negative Control Duplexes (RNAi-NC) served as control.

B. ASYM24 antibody was used for western blot in the protein samples of co-immunoprecipitation (Co-IP). IP:IgG lane is the control and IP:PRMT1 lane is the experimental one.

blot, changing the concentration of SDS-PAGE to 8% and prolonging the time of transferring to 2 hours. The results demonstrated that there were some high molecular weight proteins (95-170 kD), which could bind to PRMT1 and contained ADMA (Fig. 3B). There was indeed a band around 130 kD, at a position similar to the one of carved band 1 (Fig. 3A). Judging from the results of LC-MS/MS and western blot, SEC23-IP was the most possible candidate protein of PRMT1, because the ions score of SEC23-IP is the highest (about 123) in the database, and the molecular weight of SEC23-IP is 111 kD, which is the closest to the carved band 1 in Figure 3A and the colored band around 130 kD in Figure 3B.

Table 1. The result of LC-MS/MS analysis for candidate proteins

No.	Protein name	GI	Mass	Ions score	emPAI
1'	Q1Z 7F5 [Homo sapiens]	gi 184407	24561	38	0.29
	F-box only protein 21 isoform 2 [Homo sapiens]	gi 16306500	71433	38	0.05
	synaptonemal complex central element protein 1 isoform 1 [Homo sapiens]	gi 18640754	32581	35	0.10
	ATP synthase subunit alpha, mitochondrial precursor	gi 4757810	59714	31	0.06
	tetratricopeptide repeat protein 25	gi 13899233	76608	25	0.04
1	SEC23-interacting protein [Homo sapiens]	gi 6005824	111007	123	0.30
	ANKHD1-EIF4EBP3 protein [Homo sapiens]	gi 37620163	277004	43	0.01
	cytoplasmic dynein 1 heavy chain 1 [Homo sapiens]	gi 33350932	532072	43	0.01
	tetratricopeptide repeat protein 25 [Homo sapiens]	gi 13899233	76608	29	0.04
	immunoglobulin gamma 3 heavy chain variable region [Homo sapiens]	gi 304561932	13459	29	0.25
	GTP-binding protein Di-Ras2 [Homo sapiens]	gi 21703367	22471	20	0.15
2'	ATP synthase subunit alpha, mitochondrial precursor [Homo sapiens]	gi 4757810	59714	51	0.06
	SWI/SNF complex 155 KDa subunit [Homo sapiens]	gi 1549239	122379	42	0.03
	brefeldin A-inhibited guanine nucleotide-exchange protein 2 [Homo sapiens]	gi 5052123	201964	42	0.02
	Ig gamma-1 chain C region	gi 121039	36083	23	0.09
2	Ig gamma-1 chain C region	gi 121039	36083	30	0.09
	KIAA0368 [Homo sapiens]	gi 2224677	159376	24	0.02
	tetratricopeptide repeat protein 25 [Homo sapiens]	gi 13899233	76608	18	0.04
3'	SWI/SNF complex 155 KDa subunit [Homo sapiens]	gi 1549239	122379	46	0.03
	F-box only protein 21 isoform 2 [Homo sapiens]	gi 16306500	71433	39	0.05
	L21 ribosomal protein [Homo sapiens]	gi 619788	17646	28	0.19
	a disintegrin-like and metalloprotease with thrombospondin type 1 motifs 20 [Homo sapiens]	gi 27463367	214515	18	0.02
3	enolase [Homo sapiens]	gi 31179	49446	53	0.07
	SWI/SNF complex 155 KDa subunit [Homo sapiens]	gi 1549239	122379	42	0.03
	immunoglobulin kappa heavy chain [Homo sapiens]	gi 2765421	51971	34	0.06
4'	F-box only protein 21 isoform 2 [Homo sapiens]	gi 16306500	71433	39	0.05
	zinc finger protein ZNF134 [Homo sapiens]	gi 488553	40271	24	0.09
	KIAA0264 [Homo sapiens]	gi 1665795	47767	24	0.07
	tetratricopeptide repeat protein 25 [Homo sapiens]	gi 13899233	76608	22	0.04
4	1-phosphatidylinositol-3-phosphate 5-kinase isoform 2 [Homo sapiens]	gi 121583483	236986	42	0.01
5'	F-box only protein 21 isoform 2 [Homo sapiens]	gi 16306500	71433	47	0.05
	dermcidin preproprotein [Homo sapiens]	gi 16751921	11277	32	0.30
	RecName: Full=Ig gamma-1 chain C region	gi 121039	36083	26	0.09
5	kallikrein-11 isoform 2 precursor [Homo sapiens]	gi 21618357	31039	30	0.11
	28S ribosomal protein S26, mitochondrial precursor [Homo sapiens]	gi 14269586	24197	22	0.14
6'	RecName: Full=Ig gamma-1 chain C region	gi 121039	36083	29	0.09
6	hCG2040015, isoform CRA_a [Homo sapiens]	gi 119631099	47124	39	0.07
	obscurin [Homo sapiens]	gi 15026974	721240	14	0
	integrin alpha-V precursor variant [Homo sapiens]	gi 62089374	121600	14	0.03
	phosphoprotein 75 - human	gi 1362890	42188	14	0.08
	syndecan 3 [Homo sapiens]	gi 13810649	45468	14	0.07
	FHL2 isoform 5 [Homo sapiens]	gi 82408395	44148	14	0.07
	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor-like 2, isoform CRA_b [Homo sapiens]	gi 119602499	80989	14	0.04
	hCG1984864 [Homo sapiens]	gi 119610361	11025	14	0.31
	arylsulfatase E (chondrodysplasia punctata 1), isoform CRA_c [Homo sapiens]	gi 119619125	12254	14	0.28
7'	glyceraldehyde-3-phosphate dehydrogenase [Homo sapiens]	gi 31645	36031	111	0.42
	F-box only protein 21 isoform 2 [Homo sapiens]	gi 16306500	71433	44	0.05
	Chain R, Twinning In Crystals Of Human Skeletal Muscle D- Glyceraldehyde-3-Phosphate Dehydrogenase	gi 230867	35853	36	0.19
	hCG17042, isoform CRA_b [Homo sapiens]	gi 119631704	19540	32	0.17
	tropomyosin alpha-4 chain isoform 2 [Homo sapiens]	gi 4507651	28504	18	0.12
7	dermcidin preproprotein [Homo sapiens]	gi 16751921	11277	51	0.30
	carboxypeptidase Z precursor [Homo sapiens]	gi 2160714	72458	15	0.05

LC-MS/MS: liquid chromatography-tandem mass spectrometry; emPAI: exponentially modified protein abundance index; GI: MASCOT serial number, which could link to the mascot search result and NCBI BLAST search.

Ions score is $-10 \times \log(P)$, where P is the probability that the observed match is a random event. An individual ions score > 38 indicates identity or extensive homology ($P < 0.05$). The No. refers to the band number showed by the arrows in Figure 3.

Table 2. The result of MeMo web site analysis for candidate proteins (individual ions score > 38, *P*<0.05)

No.	Protein name	Methylated position	Flanking residues
1	SEC23-interacting protein [Homo sapiens]	426	TRPRVVK R GIDDNLD
2	ANKHD1-EIF4EBP3 protein [Homo sapiens]	267	MHANVED R GNKGDIT
		1144	VVDLL L ARGANKEHR
		1314	FCELLIH R GAHIDVR
		1583	NKINGEP R GGGAGGN
		1682	LNLTSPK R GQKREEG
		1713	VVSRI M RGGCNITA
		1746	GERMIT I RGGTESTR
		1894	TWGPFV P VPVNPNT
		2140	VSHRMQ P RGSFYMSV
		2377	TERLAR I RQGGVAQ
		2612	GAEGAGD R GRDTR
3	DYNC1H1 (cytoplasmic dynein 1 heavy chain 1) [Homo sapiens]	0	0
4	enolase [Homo sapiens]	0	0
5	1-phosphatidylinositol-3-phosphate 5-kinase isoform 2 [Homo sapiens]	181	FRRRHHC R LCGQIFC
		258	PRTVPGS R KASRNIF
		842	LGCTIK L RGGSDYEL
		896	FHSLIE G RHEGAVQ
		1158	MLADYR A RGGRIQPK
		1161	DYRARG G RIQPKNSD
		1194	KNEGDEE R GLILSDA
		1531	PPSPGRL R QGEESKI
		1748	SGMLSFF R GTAGKSP
		1766	SQKRETL R GADSAYY
		1858	HSSPWQ A RGGKSGAA
6	hCG2040015, isoform CRA_a [Homo sapiens]	0	0

The bold "R"s in flanking residues indicate the arginine residues which might be methylated.

DISCUSSION

PRMTs play a role in many cellular processes, such as DNA damage, signaling, and epigenetic gene expression, by methylating arginine residues of their substrates.^{1,2} Proteomics analysis revealed more than 200 proteins that underwent arginine methylation.^{9,10} Among the family members of PRMTs, PRMT1 is the most predominant methyltransferase in mammalian cells, and the role of PRMT1 in tumor genesis is gradually attracting attention.¹¹ It was reported that PRMT1 might influence the apoptosis of breast cancer cell and the proliferation of osteosarcoma cell, and might be a potential therapeutic target for many kinds of cancers.^{5,12}

In the present study, the expression of PRMT1 and the levels of ADMA were found considerably changed in glioma cells, and the tendency of some ADMA levels was in accord with the expression of PRMT1. Additionally, ADMA levels declined after knock-down of PRMT1. The findings indicated that PRMT1 could regulate the levels of some ADMA in glioma. With Co-IP and LC-MS/MS, we screened 26 proteins that could combine with PRMT1, among which 6 had higher ions scores (>38) and only existed in the PRMT1

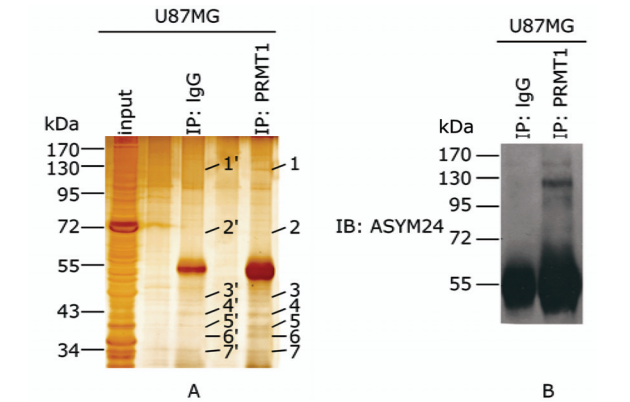


Figure 3. Screening for the putative target proteins of PRMT1. A. Sliver staining of proteins, which combined with normal rabbit IgG (IP:IgG) or PRMT1 (IP:PRMT1). The arrows show the carved bands to be identified by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The numbers of carved bands (1-7 and 1'-7') correspond with the results of LC/MS-MS in Table 1. B. Western blot results of ADMA levels of PRMT1's target proteins with higher molecular weight (95-170 kD).

experimental group. Bioinformation analysis predicted that SEC23-IP, ANKHD1-EIF4EBP3 protein, and 1-phosphatidylinositol-3-phosphate 5-kinase isoform 2 might have methylated aginines. Based on the ions score and molecular weight of candidate proteins, we supposed SEC23-IP was one of the most possible proteins, which could bind to PRMT1 and contain ADMA, in our database. As a member of the phosphatidic acid preferring-phospholipase A1 family, SEC23-IP is located in endoplasmic reticulum (ER) to play a crucial role in ER-Golgi transport. It takes part in the construction of the multimeric coat protein II complex and recognizes ER export signals on cargo proteins for inclusion in vesicles.¹³ The result of the present study indicates that PRMT1 might bind to SEC23-IP and dimethylate its arginine, suggesting that PRMT1 might play roles in ER-Golgi transport *via* arginine dimethylation of SEC23-IP. Previous studies reported significantly changed expression of SEC23-IP in some tumors, including gastric cancer and melanoma.^{14, 15} Yet few researches have been conducted on SEC23-IP in glioma so far. According to the findings of this research group, the relationship between SEC23-IP and PRMT1 might have an impact on the genesis of glioma.

In conclusion, the tendency of ADMA levels was found in accordance with PRMT1 expression in glioma. Twenty-six candidate proteins binding with PRMT1 were identified by Co-IP and LC-MS/MS, among which 3 (i.e. SEC23-IP, ANKHD1-EIF4EBP3 protein, and 1-phosphatidylinositol-3-phosphate 5-kinase isoform 2) had possible methylated aginine sites, as predicted by bioinformation analysis. Further studies on the relationship between PRMT1 and these proteins are needed to contribute to the exploration of the molecular mechanism of post-translational modification in glioma.

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