ORIGINAL ARTICLE

Functional analysis of recently identified mutations in eukaryotic translation initiation factor 2B ϵ (eIF2B ϵ) identified in Chinese patients with vanishing white matter disease

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Vanishing white matter disease (VWM) is the first human hereditary disease known to be caused by defects in initiation of protein synthesis. Gene defects in each of the five subunits of eukaryotic translation initiation factor 2B (eIF2B α – ϵ) are responsible for the disease, although the mechanism of the pathogenesis is not well understood. In our previous study, four novel eIF2B ϵ mutations were found in Chinese patients: p.Asp62Val, p.Cys335Ser, p.Asn376Asp and p.Ser610-Asp613del. Functional analysis was performed on these mutations and the recently reported p.Arg269X. Our data showed that all resulted in a decrease in the guanine nucleotide exchange (GEF) activity of the eIF2B complex. p.Arg269X and p.Ser610-Asp613del mutants displayed the lowest activity, followed by p.Cys335Ser, p.Asn376Asp and p.Asp62Val. p.Arg269X and p.Ser610-Asp613del could not produce stable eIF2B ϵ , leading to almost complete loss-of-function. No evidence was obtained for the three missense mutations in changes in eIF2B ϵ protein level or eIF2B ϵ Ser⁵⁴⁰ phosphorylation, and disruption of holocomplex assembly, or binding to eIF2. All patients in our study had the classical phenotype. p.Asp62Val and p.Asn376Asp mutations caused only mildly decreased GEF activity, were probably responsible for relatively mild phenotype in cases of classical VWM. *Journal of Human Genetics* (2011) 56, 300–305; doi:10.1038/jhg.2011.9; published online 10 February 2011

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INTRODUCTION

Vanishing white matter disease (VWM, OMIM 306896), also called childhood ataxia with central nervous system hypomyelination or eukaryotic translation initiation factor 2B (eIF2B)-related disorder, is one of the most prevalent inherited childhood leukoencephalopathies. The phenotype varies from a severe antenatal form to a mild adult-onset variant. The classical phenotype is characterized by early childhood onset of progressive neurological regression, usually with episodic deterioration provoked by febrile infection, minor head trauma or sudden fright.^{1–3} Death occurs after a period, which varies from several years to decades. The remarkable feature in brain magnetic resonance imaging is symmetric and diffuse fluid-like signal in cerebral white matter, which is consistent with the cystic degeneration found by pathological analysis.⁴

VWM is inherited in an autosomal recessive manner. Mutations in the five subunits of eIF2B (α , β , γ , δ and ϵ), encoded by *EIF2B1*–*5*,

were identified in VWM in 2001–2002 (Leegwater *et al.*,⁵ van der Knaap *et al.*⁶). The eIF2B complex has a key role in mRNA translation initiation through its guanine nucleotide exchange (GEF) activity. The eIF2B complex promotes guanosine diphosphate (GDP)/guanosine triphosphate (GTP) exchange on eIF2, and is required to regenerate active GTP-eIF2 from its inactive GDP-bound form to allow continued translation initiation. In the GTP-bound state, eIF2 recruits the initiator Met-tRNA to the 40S subunit for translation initiation. Although the pathogenic pathway from the eIF2B defects to the degeneration of cerebral white matter is far from understood, VWM is considered the first human hereditary disease caused by defects in the initiation of protein synthesis.^{7,8}

To date, >120 VWM mutations have been identified in *EIF2B1*–*5*. More than half were found in *EIF2B5* (57%), followed by *EIF2B4* (16%), *EIF2B2* (16%), *EIF2B3* (7%) and *EIF2B1* (4%).^{9–11} Most of the published studies were carried out in the Caucasian population. Since

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Table 1 *EIF2B* mutations identified in 12 Chinese VWM patients

Patient	Gene	Nucleotide change	Amino-acid change	Parental derivation
1	<i>EIF2B5</i>	c.1126A>G	p.Asn376Asp	Mother
		c.1340C>T	p.Ser447Leu	Father
2	<i>EIF2B5</i>	c.805C>T	p.Arg269X	Mother
		c.1004G>C	p.Cys335Ser	U
3	<i>EIF2B5</i>	c.185A>T	p.Asp62Val	Father
		c.1016G>C	p.Arg339Pro	Mother
4	<i>EIF2B5</i>	c.1157G>T	p.Gly386Val	Mother
		c.1827–1838del	p.Ser610–Asp613del	U
5	<i>EIF2B5</i>	c.943C>T	p.Arg315Cys	Mother
		c.943C>T	p.Arg315Cys	Father
6	<i>EIF2B5</i>	c.337C>T	p.Arg113Cys	Mother
		c.806G>A	p.Arg269Gln	Father
7	<i>EIF2B3</i>	c.140 G>A	p.Gy47Glu	Father,
		c.1037 T>C	p.Ile346Thr	Mother
8	<i>EIF2B3</i>	c.1037T>C	p.Ile346Thr	Father
		c.1037T>C	p.Ile346Thr	U
9	<i>EIF2B3</i>	c.674G>A	p.Arg225Gln	Mother
		c.674G>A	p.Arg225Gln	Father
10	<i>EIF2B3</i>	c.1037T>C	p.Ile346Thr	Mother
		ND	ND	
11	<i>EIF2B3</i>	c.1037T>C	p.Ile346Thr	Mother
		c.1037T>C	p.Ile346Thr	Father
12	<i>EIF2B2^a</i>	c.254 T>A	p.Val85Gly	Father
		c.922 G>A	p.Val308Met	Mother

Abbreviations: EIF2B, eukaryotic translation initiation factor 2B; ND, not identified; U, unavailable; VWM, vanishing white matter disease.

^aAll mutations identified in Chinese patients were described in Wu *et al.*¹², except for *EIF2B2* mutations.

2006, 12 Chinese children have been diagnosed with VWM that was confirmed by *EIF2B1–5* gene sequencing.¹² A total of 16 *EIF2B* mutations were found (Table 1) in the Chinese patients, with 11 in *EIF2B5* (68.8%), 3 in *EIF2B3* (18.8%) and 2 in *EIF2B2* (12.5%). Eight mutations were not previously reported in other populations, and of these four are in *EIF2B5*.

Of the five eIF2B subunits, eIF2Bε is the largest and the most important, as it exhibits GEF activity when expressed alone. eIF2Bε is encoded by *EIF2B5*. *EIF2B5* mutations impair the function of the eIF2B complex in diverse ways, including causing the formation of truncated proteins, disrupting holocomplex assembly and causing partial loss of GEF activity.^{13–15} The *EIF2B5* mutations that have been functionally investigated are p.Val73Gly, p.Thr91Ala, p.Leu106Phe, p.Arg113His, p.Arg195His, p.Arg299His, p.Arg315His, p.Arg339Pro, p.Gly386Val, p.Val430Ala and p.Trp628Arg.^{13,16–20} The *EIF2B5* mutations we found in Chinese patients, which were not previously reported in other population, were p.Asp62Val (c.185A>T), p.Cys335Ser (c.1004G>C), p.Asn376Asp (c.1126A>G) and p.Ser610–Asp613del (c.1827–1838del), they locate from N- through C-terminus of eIF2Bε.¹² Analysis of how these mutations give rise to the functional defects will help in define the critical sites in eIF2Bε involved in its function and regulation. Information about the functional defects that result from specific mutations will be helpful for delineating the genotype–phenotype correlations.

In this study, we analyzed how these *EIF2B5* mutations, which recently described in Chinese patients, resulted in functional defects in eIF2Bε. Mutants were compared with wild type in terms of the level of eIF2Bε protein, interaction of the mutant subunit with the others to form the eIF2B complex, interaction with the eIF2 substrate, the

phosphorylation level of eIF2Bε at the regulatory GSK3 (glycogen synthase kinase-3) site, and the GEF activity. In addition to the four recently described mutations, p.Arg269X, which was reported in 2008 (Maletkovic *et al.*¹¹), was also studied. We summarized the phenotypes of four Chinese children who harbored these *EIF2B5* mutations, and searched for genotype–phenotype correlations.

MATERIALS AND METHODS

Patients

The research was approved by Medical Ethics Committee of Peking University First Hospital. Clinical diagnosis of VWM was based on the criteria of van der Knaap *et al.*²¹ *EIF2B1–5* mutation screening was performed for each patient and the parents. Of 12 confirmed VWM patients, *EIF2B5* mutations not previously reported in other population were found in four. Data on these patients are in Table 2.

Expression vectors

Expression vectors for full-length wild-type human eIF2Bα–ε are as described previously.¹³ The eIF2Bα–δ complementary DNA in expression vectors was N-terminally tagged with Myc, and the vector with full-length eIF2Bε complementary DNA was tagged with both Myc and hexahistidine (His₆) at the N-terminus. eIF2Bε mutations (p.Asp62Val, p.Arg269X, p.Cys335Ser, p.Asn376Asp and p.Ser610–Asp613del) were generated using the Quick-change site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions, and were confirmed by sequencing.

Cell culture, transfection, lysis and pull-down purification

Human embryonic kidney 293 (HEK 293) cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Transfection with the mutants or wild type was conducted using Lipofectamine2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. Cells were lysed 48 h after transfection. To study eIF2Bα–ε complex assembly, the interaction with eIF2, phosphorylation level or the GEF activity, recombinant complex containing His-Myc-tagged eIF2Bε was purified from HEK 293 lysates containing endogenous eIF2B. Purification was by pull-down with Ni-nitrilotriacetic acid-agarose (Qiagen, Shanghai, China). Pulled-down protein was analyzed by western blot, with anti-Myc to assess the eIF2Bα–ε complex formation, anti-eIF2 for eIF2B–eIF2 interaction, and anti-eIF2BεSer⁵⁴⁰ (P) for phosphorylation level of eIF2BεSer⁵⁴⁰.

Quantitative real-time reverse transcriptase-PCR

To determine whether transcript levels of the mutants resulted in reduced protein, quantitative real-time reverse transcriptase-PCR was performed. Total RNA was isolated from transfected HEK 293 cells with TRIzol reagent (Invitrogen). We used the TaqMan probe technology for analysis. PCR products are located in 5'-upstream of both two mutations, primers are 5'-CTTCTTCCCCATCTCCAAGGA-3' (sense) and 5'-TACACCTGTGGCAGTCAGGAAT-3' (anti-sense). TaqMan probe for *EIF2B5*, with 5'-FAM and 3'-TAMRA, is 5'-CAGCCTC GGGTCCTCTTGCCC-3'. Glyceraldehyde 3-phosphate dehydrogenase is used as internal control, primers pairs are 5'-CAGTCAGCCGCATC TTCTTTT-3' (sense) and 5'-GTGACCAGGCGCCCAATAC-3' (anti-sense), TaqMan probe for glyceraldehyde 3-phosphate dehydrogenase is 5'-CGTCGCCAGCCGAGCCACA-3'.

GEF activity measurement

The activity of recombinant eIF2B complex, with His-Myc-tagged eIF2Bε, was tested by purification by pull-down as described above. GTP/GDP exchange activity was measured using the standard assay,¹³

Table 2 Clinical data and EIF2B5 mutations in four VWM patients

Patient	Gender	Developmental milestone before disease onset	Disease onset		Disease progression					Disease course ^b	Family history	EIF2B5 mutations
			Age of disease onset	Initial symptoms	Age at last follow- up	Major symptoms ^a						
1	Male	Mildly delayed	2y 11m	Motor signs	5y	M2	C1	S	D	P+E	—	p.Asn376Asp p.Ser447Leu
2	Female	Mildly delayed	2y	Motor signs	9y 4m	M3	C1	S	D	P+E	+	p.Arg269X p.Cys335Ser
3	Male	Normal	6y 8m	Motor signs	8y 3m	M1	C0	S	—	P	—	p.Asp62Val p.Arg339Pro
4	Male	Normal	4y 6m	Seizure	5y 11m	M2	C1	S	D	P	—	p.Gly386Val p.Ser610-Asp613 del

Abbreviations: EIF2B, eukaryotic translation initiation factor 2B; m, months; VWM, vanishing white matter disease; y, years; +, positive; —, negative.
^aMajor symptoms: M0, no motor handicap; M1, mild motor handicap (ataxia or gait difficulty, ambulate without assistant); M2, moderate motor handicap (ambulate with assistant); M3, severe motor handicap (wheelchair bound or confined to bed); C0, no cognitive handicap; C1, mild cognitive handicap (mild learning and language difficulty); C2, moderate cognitive handicap (obvious learning and language difficulty, still be able to communicate with others); C3, severe cognitive handicap (severe learning and language difficulty, lost of communication with others); S, seizures; D, dysarthria.
^bDisease course: P, progressive; E, episodic aggravation; P+E, progressive with episodic aggravation.

Figure 1 Effects of five EIF2B5 mutations on the eukaryotic translation initiation factor 2B (eIF2B) guanine nucleotide exchange (GEF) activity. GEF activity was measured using purified recombinant eIF2B heteropentamers containing either mutant or wild-type eIF2Bε (n=3). All five mutant eIF2Bε consistently displayed decreased GEF activity compared with the wild type.

with purified human eIF2. [³H]GDP as substrate. Amounts of purified recombinant eIF2B complex were determined by quantitative western blot using the LiCor Odyssey system, (LI-COR Biotechnology-UK Ltd, Cambridge, UK) and the amounts of protein in GEF activity assays adjusted accordingly.

RESULTS

All five eIF2Bε mutations decrease eIF2B GEF activity

To determine whether the eIF2Bε mutations affected GEF activity, we measured this activity using purified recombinant eIF2B heteropentamers containing either mutant or wild-type eIF2Bε. All five mutant eIF2Bε consistently displayed decreased GEF activity compared with the wild type. The impairment varied from mild to severe. p.Arg269X and p.Ser610-Asp613del mutants displayed the lowest GEF activity, followed by p.Cys335Ser, p.Asn376Asp and p.Asp62Val (Figure 1).

The p.Arg269X and p.Ser610-Asp613del mutations significantly reduce eIF2Bε protein level

Vectors containing the five eIF2Bε variants were transfected into HEK 293 cells for analysis by *in vitro* studies. The levels of eIF2Bε-Asp62Val,

Figure 2 Protein expression of five mutant eukaryotic translation initiation factor 2B (eIF2B)ε. The five eIF2Bε variants were transfected into human embryonic kidney 293 (HEK 293) cells for analysis by *in vitro* studies (n=5). The levels of eIF2Bε-Asp62Val, Cys335Ser and Asn376Asp were similar to the level of wild-type eIF2Bε, while eIF2Bε-Arg269X and Ser610-Asp613del were substantially reduced and were almost undetectable.

eIF2Bε-Cys335Ser and eIF2Bε-Asn376Asp were similar to the level of wild-type eIF2Bε, while eIF2Bε-Arg269X and eIF2Bε-Ser610-Asp613del were substantially reduced and were almost undetectable, which shown as very weak bands only when exposure was enhanced (Figure 2). To understand whether the reduction in the mutant protein resulted from a decrease in mRNA level, quantitative real-time reverse transcriptase-PCR was conducted. The level of the mRNA for p.Arg269X was comparable to the wild-type mRNA, while signal for p.Ser610-Asp613del mRNA was not detected suggesting that its stability may be impaired.

Interaction of eIF2Bε with other eIF2B subunits is not affected by the p.Asp62Val, p.Cys335Ser or p.Asn376Asp mutations

eIF2Bε alone can mediate GEF, but GEF activity is greatly enhanced when eIF2Bε combines with the other four subunits to form a heteropentamer.^{22,23} The binary subcomplex of eIF2Bε and eIF2Bγ is needed for holocomplex formation. Without the γ subunit, eIF2Bε was unable to bind a complex of eIF2Bαβδ. To determine whether the mutations affected the assembly of eIF2B complex, we compared eIF2Bεγ subcomplex formation between the mutants and the wild type. HEK 293 cells were co-transfected with His-Myc-tagged eIF2Bε and Myc-tagged eIF2Bγ. Protein pulled down with Ni-nitrilotriacetic acid-agarose, containing recombinant eIF2Bεγ, was analyzed by western blot, using anti-Myc. The amount of the pulled-down Myc-tagged eIF2Bγ was comparable between the three missense mutations and the wild type, while no Myc-tagged eIF2Bγ was pulled down from p.Arg269X or p.Ser610-Asp613del transfected cells (Figure 3). Therefore, p.Asp62Val, p.Cys335Ser and p.Asn376Asp did not affect the assembly of the eIF2Bεγ subcomplex. The failure to detect

Figure 3 Effects of three *EIF2B5* mutations on the interaction of eukaryotic translation initiation factor 2B (eIF2B)ε with other eIF2B subunits. Human embryonic kidney 293 (HEK 293) cells were co-transfected with His-Myc-tagged eIF2Bε and Myc-tagged eIF2Bγ. Protein pulled down with Ni-nitrilotriacetic acid (NTA)-agarose, containing recombinant eIF2Bεγ, was analyzed by western blot, using anti-Myc. p.Asp62Val, p.Cys335Ser and p.Asn376Asp did not affect the assembly of the eIF2Bεγ subcomplex (Figure 3a). Then we expressed the His-Myc-tagged eIF2Bε subunit together with all four other Myc-tagged subunits. Similar amounts of Myc-tagged α, β, γ, δ or ε subunits were pulled down from cells with the three missense mutations and the wild type (Figures 3b and c). The failure to detect pulled-down other eIF2B subunits with p.Arg269X and p.Ser610-Asp613del is probably because of the significantly reduced amount of protein in the lysate ($n=4$).

pulled-down eIF2Bγ with p.Arg269X and p.Ser610-Asp613del probably reflects the greatly reduced amount of protein in the lysate coupled, perhaps, with inability of the truncated polypeptide to bind stably to eIF2Bγ. We further studied whether the mutations affected formation of the eIF2B heteropentamer by expressing the His-Myc-tagged eIF2Bε subunit together with all four other Myc-tagged subunits. Similar amounts of Myc-tagged α, β, γ, δ or ε subunits were pulled down from cells with the three missense mutations, and the wild-type eIF2Bε (Figure 3).

The p.Asp62Val, p.Cys335Ser and p.Asn376Asp mutations do not affect the binding of eIF2B complex to eIF2

Binding of eIF2B complex to eIF2 (its substrate) is critical for its GEF activity. The eIF2 trimeric complex consists of α, β and γ subunits. Phosphorylation of eIF2α can inhibit the GEF activity of eIF2B, and is an important regulation mechanism for eIF2B.^{23,24} We studied whether the binding of eIF2B complex to eIF2 or eIF2α (P) was affected by the three missense mutations. HEK 293 cells were co-transfected with His-Myc-tagged eIF2Bε and the other four Myc-tagged eIF2B subunits. Protein pulled down with Ni-nitrilotriacetic acid-agarose, containing recombinant eIF2B, was analyzed by western blot with anti-eIF2 or anti-eIF2α (P). The amount of eIF2 and eIF2α (P), was similar for the three missense mutations and the wild type (Figure 4). Thus, the reduced activity of the missense mutants is not due to impaired binding to substrate or enhanced binding to eIF2α(P).

The p.Asp62Val, p.Cys335Ser and p.Asn376Asp mutations do not affect the phosphorylation level of eIF2BεSer⁵⁴⁰

Phosphorylation of eIF2Bε itself is one of the mechanisms that regulates its function, and eIF2BεSer⁵⁴⁰, which is the target of

Figure 4 Effects of three *EIF2B5* mutations on the binding of eukaryotic translation initiation factor 2B (eIF2B) complex to eIF2. Human embryonic kidney 293 (HEK 293) cells were co-transfected with His-Myc-tagged eIF2Bε and the other four Myc-tagged eIF2B subunits. Protein pulled down with Ni-nitrilotriacetic acid (NTA)-agarose, containing recombinant eIF2B, was analyzed by western blot with anti-eIF2 or anti-eIF2α (P). The amount of eIF2 and eIF2α (P), was similar for the three missense mutations and the wild type ($n=3$).

Figure 5 Phosphorylation level of eukaryotic translation initiation factor 2B (eIF2B)εSer⁵⁴⁰ with three missense mutations. We analyzed purified His-Myc-tagged eIF2Bε by western blot, using anti-eIF2BεSer⁵⁴⁰ (P) and anti-eIF2Bε, and calculated a semi-quantitative eIF2BεSer⁵⁴⁰ (P)/eIF2Bε ratio for the mutants and the wild type. None of three missense mutations, p.Asp62Val, p.Cys335Ser and p.Asn376Asp, affected the phosphorylation level of eIF2Bε Ser⁵⁴⁰ ($n=3$).

GSK3, is a key regulatory phosphorylation site in eIF2Bε.^{23–25} To determine whether the mutations changed the phosphorylation level of the GSK-3 site, we analyzed purified His-Myc-tagged eIF2Bε by western blot, using anti-eIF2BεSer⁵⁴⁰ (P) and anti-eIF2Bε, and calculated a semi-quantitative eIF2BεSer⁵⁴⁰ (P)/eIF2Bε ratio for the mutants and the wild type. None of three missense mutations, p.Asp62Val, p.Cys335Ser and p.Asn376Asp, affected the phosphorylation level of eIF2Bε Ser⁵⁴⁰ (Figure 5).

DISCUSSION

eIF2Bε is a multidomain protein (721 amino acids) that is widely expressed in eukaryotic cells. It is the largest subunit in the eIF2B heteropentamer complex, and contains the catalytic domain.²⁶ The C-terminal residues (518–712) of eIF2Bε contain a minimal functional unit that interacts with eIF2, and exhibits GEF activity. Within the C-terminal region, residues 581–712 can interact with eIF2, and residues 518–580 are required for GEF activity, and are thus considered the ‘catalytic center’. The role of the N-terminal 500 residues of eIF2Bε is unclear, but is probably involved in interaction with the other eIF2B subunits.^{23,24}

Measurement of eIF2B GEF activity in patients’ transformed-lymphocytes was reported to be an important tool for the diagnosis of eIF2B-related disorders.²⁷ We found that all these recently described eIF2Bε mutations in Chinese VWM children resulted in a decrease in eIF2B complex GEF activity. The p.Arg269X and p.Ser610-Asp613del

mutants displayed the lowest GEF activity, followed by p.Cys335Ser, p.Asn376Asp and p.Asp62Val. Therefore, all the eIF2Bε mutations impaired the eIF2Bε catalytic function, and consequently affected normal translation initiation.

Our data showed that both p.Arg269X and p.Ser610-Asp613del resulted in significant produce of stable eIF2Bε, either because of a decrease in mRNA level or posttranslational degradation. Therefore, these two mutations lead to an almost loss-of-function and should be considered null mutations. The other three mutations did not change the protein level. As all of them are in the N-terminus, it is unlikely that they impair the catalytic function of eIF2Bε by interfering directly with GEF activity or substrate binding. On the basis of proposed pathways for regulating eIF2B, we next attempt to define the reason for the decreased GEF activity in the p.Asp62Val, p.Cys335Ser and p.Asn376Asp mutants.

Although eIF2Bε itself exhibits GEF activity, the formation of a five-subunit eIF2B complex stimulates GEF activity 10- to 40-fold in yeast or mammalian cells.²² The N-terminus of eIF2Bε is considered to be critical for complex formation. The subcomplex eIF2Bεγ is reported to be required for holocomplex formation. We examined whether the N-terminal mutations interfered with assembly of eIF2Bεγ or the heteropentamer. All the mutants examined here efficiently formed both types of complex indicating that p.Asp62, p.Cys335 and p.Asn376 in eIF2Bε are probably not critical residues for subunit interaction.

Direct phosphorylation of eIF2Bε has been described for mammalian eIF2B, which is another way of functional regulation.^{23,24,28} At least four protein kinases have been found to phosphorylate eIF2Bε, including CK1 (casein kinase1), CK2 (casein kinase2), DYRK (dual-specificity tyrosine phosphorylated and regulated kinase) and GSK3. Of these, GSK3 is thought to have greatest effect on regulation of eIF2B activity. Phosphorylation of human eIF2Bε by GSK3 at Ser⁵⁴⁰ inhibits eIF2B activity. In the presence of insulin, GSK3 activity is inhibited, thus eIF2Bε is dephosphorylated and therefore becomes more active. In our study, the p.Asp62Val, p.Cys335Ser and p.Asn376Asp mutations showed no effects on the phosphorylation of the GSK3 site. Their N-terminal location makes it unlikely that these mutations affect the other known phosphorylation sites directly, as most of them are in the C terminus. Indirect effects or changes in other potential phosphorylation sites are possible.

Another mechanism of regulation is phosphorylation of the eIF2α subunit. Phosphorylated eIF2α-Ser⁵¹ has a high affinity for eIF2Bε, but instead of promoting GEF function, the increased affinity reduces the GTP/GDP exchange function, and thus inhibits eIF2B activity.²³ We previously showed that certain mutants in eIF2Bε lead to stronger association with eIF2α, perhaps explaining the lower GEF activity of complexes containing these mutants.¹³ We examined whether these missense mutations affect eIF2Bε binding to either total eIF2 or phosphorylated eIF2. Our data showed that the heteropentamer containing mutant eIF2Bε had binding similar to the wild type, so these mutations are unlikely abolish regulation of eIF2B by eIF2α.

The phenotype of VWM varies from a severe antenatal form to a mildest adult-onset variant. More than 120 eIF2Bα-ε mutations have been found in VWM since 2001 (Maletkovic *et al.*¹¹). Nonsense mutations are rare and observed only in heterozygotes, associated with a missense mutation allele. The correlation between eIF2B genotype and phenotype is not clear. It is reported that the disease severity is not correlated with either the type of the subunit mutated, or the position of the mutation within the protein.²⁹ Recently p.Arg113His in the ε and p.Glu213Gly in the β subunits have been associated with milder phenotypes, and a relationship between severe phenotype with early infantile onset and p.Arg195His in the ε has

been found.^{29,30} One hypothesis is that severe phenotypes occur in the presence of homozygotic mutations in highly conserved regions, with mutations in non-conserved amino acids leading to mild phenotype. However, the genotype-phenotype correlation is not consistent.

All patients in our study had the classical phenotype, with disease onset between 2 and 6 years of age. However, even within this phenotype, patients differed from each other in age of onset, disease progression and clinical manifestations. Horzinski *et al.*²⁷ found a weak correlation between GEF activity measured in the eIF2B-mutated lymphoblastoid cell line and age at disease onset. Patient 2, with compound heterozygous mutations p.Arg269X/p.Cys335Ser, showed the most severe phenotype. Disease symptoms began as early as 2 years of age, with mild developmental delay even before disease onset. As p.Arg269X is almost functional null, the allele with p.Cys335Ser was the only copy available to produce eIF2Bε in this patient. Although as a non-conserved amino acid, eIF2Bε-Cys335Ser allele resulted in a significant decrease in eIF2B GEF activity, at only half the activity of wild type, and may not provide enough protection from the severe phenotype. GEF activity was also reported to be significant low ($39 \pm 7\%$ of control) in a patient's lymphoblast with p.Arg269X heterozygous mutation.²⁷ Patient 1, with an onset at around 3 years of age, harbored the heterozygous genotype p.Asn376Asp/p.Ser447Leu. p.Ser447Leu was reported in a patient with the most severe antenatal form of VWM, who had a compound heterozygous genotype with another missense mutation, thus may have contributed to the severe phenotype.³¹ eIF2Bε-Asp376Asp, which had GEF activity that was approximately 80% of wild type, is probably not related to a severe classical phenotype in the classical form. Patient 3 showed the mildest phenotype, with disease symptoms beginning after 6 years of age, and progressing relatively slowly. This patient harbored the mutations p.Asp62Val/p.Arg339Pro. p.Arg339Pro is reported to lead to a significant reduction in GEF activity to approximately 30% of wild type,¹³ while we found only slightly reduced activity for p.Asp62Val. Therefore, p.Asp62Val is probably related to the milder phenotype in this patient. Like p.Arg269X, p.Ser610-Asp613del is nearly a functional null, so it is not surprising that this mutation occur in a compound heterozygous manner in patient 4.

In summary, our functional study on eIF2Bε mutations recently identified in Chinese VWM patients provides further evidence toward an understanding of the functional defects that result from different mutations. Although we were unable to determine the mechanisms by which the three missense mutations impaired eIF2Bε function, we found that they caused at least partial loss of the GEF activity, when part of the eIF2B complex. They seem to impair the intrinsic activity of the eIF2B holocomplex, pointing to interactions between the C-terminal catalytic domain and the more N-terminal regions of eIF2Bε in which these mutations occur. Complicated mechanisms, like extensive cross-talk between different domains of eIF2Bε and other subunits, and phosphorylation and dephosphorylation of eIF2B and eIF2, appear to be required to regulate eIF2B function. GEF activity of eIF2B may be a preliminary indicator for assessing the severity of functional impairment, and may correlate with phenotype severity in some cases.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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