ORIGINAL RESEARCH ARTICLE | Genetics inMedicine

Homozygosity for aquaporin 7 G264V in three unrelated children with hyperglyceroluria and a mild platelet secretion defect

Christophe Goubau, MD[1,2], Jaak Jaeken, MD, PhD[2], Elena N Levtchenko, MD, PhD[2], Chantal Thys, MSc[1], Michela Di Michele, PhD[1], Geert A Martens, MD, PhD[3], Erik Gerlo, MD, PhD[3], Rita De Vos, PhD[4], Gunnar M Buyse, MD, PhD[2], Nathalie Goemans, MD[2], Chris Van Geet, MD, PhD[1,2] and Kathleen Freson, PhD[1]

Purpose: Aquaporin 7 (AQP7) belongs to the aquaglyceroporin family, which transports glycerol and water. AQP7-deficient mice develop obesity, insulin resistance, and hyperglyceroluria. However, AQP7's pathophysiologic role in humans is not yet known.

Methods: Three children with psychomotor retardation and hyperglyceroluria were screened for *AQP7* mutations. The children were from unrelated families. Urine and plasma glycerol levels were measured using a three-step enzymatic approach. Platelet morphology and function were studied using electron microscopy, aggregations, and adenosine triphosphate (ATP) secretion tests.

Results: The index patients were homozygous for AQP7 G264V, which has previously been shown to inhibit transport of glycerol in *Xenopus* oocytes. We also detected a subclinical platelet secretion defect with reduced ATP secretion, and the absence of a secondary aggregation wave after epinephrine stimulation. Electron microscopy revealed round platelets with centrally located granules. Immunostaining showed AQP7 colocalization, with dense granules that seemed to be released after strong platelet activation. Healthy relatives of these patients, who were homozygous (not heterozygous) for G264V, also had hyperglyceroluria and platelet granule abnormalities.

Conclusion: The discovery of an association between urine glycerol loss and a platelet secretion defect is a novel one, and our findings imply the involvement of AQPs in platelet secretion. Additional studies are needed to define whether AQP7 G264V is also a risk factor for mental disability.

Genet Med 2013:15(1):55–63

Key Words: AQP7; G264V; hyperglyceroluria; platelet; secretion

INTRODUCTION

Aquaporins (AQPs) are a family of evolutionarily conserved membrane proteins that facilitate the transport mainly of water but also of some other small molecules across the cell membrane[1,2] and between organelles.[3,4] Multiple (patho)physiologic functions have been attributed to AQPs. These include the urine-concentrating mechanism, epithelial fluid secretion, cell migration, mediation of cerebral edema, and neural signal transduction (reviewed in ref. 5). AQPs have six transmembrane domains, the NH2- and COOH-terminal domains being in the cytoplasm. The channel pore is made of two highly conserved short hydrophobic regions with a typical asparagine–proline–alanine (NPA) motif.[6,7] To date, 13 AQP family members (AQP0–12) have been identified in various mammalian tissues.[2] AQPs are classified, according to their sequence homology and permeability, into three subfamilies: (i) the water-specific "classical" AQPs, (ii) the aquaglyceroporins, and (iii) the unorthodox AQPs.[4,8–11] The aquaglyceroporin subfamily transports glycerol in addition to water because of the presence of an aspartic residue near the second NPA box, resulting in expansion of the pore to accept a larger molecule such as glycerol. The four subtypes of the aquaglyceroporin subfamily are: AQP3, AQP7, AQP9, and AQP10 (reviewed in refs. 9,10). Unorthodox AQPs are highly similar to the other AQP subclasses, but additional three-dimensional structure analyses are required for a better understanding of the pore structure of this subclass.[7]

Aquaporin 7 (AQP7; MIM602974) was previously referred to as "aquaporin adipose" because it was originally identified in human adipose tissue.[12] Subsequently, other researchers found AQP7 expression in testis and renal proximal tubule cells.[13,14] The various AQP7 mouse models that were developed (for a phenotype overview, see **Supplementary Table S1** online) showed a role for AQP7 in controlling fat and in glucose metabolism.[15–17] AQP7-knockout mice were shown to develop obesity and insulin resistance, with excess glycerol concentrations in the adipocytes. In addition, it was shown that these mice have reduced water permeability in the renal proximal straight tubule brush border membrane and a marked elevation of glycerol in the urine, possibly indicating the presence of a novel glycerol reabsorption pathway in the proximal straight tubules.[18,19]

[1]Department of Cardiovascular Sciences, Center for Molecular and Vascular Biology, University of Leuven, Leuven, Belgium; [2]Department of Pediatrics, University Hospital Leuven, Leuven, Belgium; [3]Department of Clinical Chemistry and Radioimmunology, University Hospital Brussels, Brussels, Belgium; [4]Department of Pathology, University of Leuven, Leuven, Belgium. Correspondence: Kathleen Freson (kathleen.freson@med.kuleuven.be)

Submitted 12 March 2012; accepted 14 June 2012; advance online publication 16 August 2012. doi:10.1038/gim.2012.90

In humans, however, the pathophysiologic role of AQP7 has been less obvious. In a Japanese cohort, three *AQP7* missense mutations (R12C, V59L, and G264V) were detected that did not show any association with obesity or with type 2 diabetes.[20] Of note, the G264V (rs62542743) *AQP7* mutation was shown (in *Xenopus* oocytes) to inhibit transport of water and glycerol. The only subject who was homozygous for G264V in the Japanese study had no obesity, no diabetes, and no loss of fertility. Another study found the G264V *AQP7* heterozygous variant in 8% of a Caucasian population (in contrast to 3.75% in the Japanese population), but again no association was found with obesity or diabetes.[21] This later study showed that AQP7 was downregulated in subcutaneous adipose tissue of women with severe obesity but without type 2 diabetes. It was recently suggested that AQP3 and AQP9 represent additional pathways for transport of glycerol in human adipocytes and that these AQPs are therefore potentially responsible for AQP7 redundancy.[22]

Here we present the cases of three children from unrelated families. All three children had psychomotor retardation of unknown etiology, pronounced renal glycerol loss (hyperglyceroluria), and a subclinical, platelet-dense granule secretion defect. The homozygous *AQP7* G264V mutation was detected in these children. The relevance of the mutation in relation to their clinical phenotype, and the role and subcellular localization of AQP7 in platelets were studied.

MATERIALS AND METHODS

Glycerol measurements in blood plasma and urine samples

Blood samples were collected from the three patients and from 21 age-matched healthy controls, EDTA-anticoagulated, processed, and stored, as described earlier.[23] Urine samples were taken at the same time and immediately stored at −80 °C. Glycerol concentrations were measured using a three-step enzymatic reaction with glycerol kinase, as previously described.[23]

Genetic screening for *AQP3*, *AQP7*, and *AQP9*

Genomic DNA was isolated from leukocytes by a salting-out method. Platelet RNA was extracted with Trizol (Life Technologies, Merelbeke, Belgium). Approximately 1 µg of total RNA was used in the presence of RNAseI inhibitor (Promega, Leiden, The Netherlands) for oligo(dT)-primed first-strand complementary DNA synthesis with Moloney murine leukemia virus reverse transcriptase (Life Technologies). *AQP3* (NG_007476) and *AQP9* (NG_011975) were PCR-amplified from the patients' complementary DNA, using primers AQP3-1 (forward) and AQP3-2 (reverse), and AQP9-1 (forward) and AQP9-2 (reverse), respectively (see **Supplementary Table S2** online for primer sequences) and sequenced on an ABI310 automatic sequencer (Applied Biosystems, London, UK).

The *AQP7* gene (NG_027764) was screened using genomic DNA from the index cases. The entire exonic sequence with exon/intron boundaries was amplified as five fragments, using specific primer sets (see **Supplementary Table S2** online for primer sequences) to avoid coamplification of the *AQP7* pseudogene (NR_002817).[20]

Hematologic counts and functional platelet studies

EDTA-anticoagulated blood was analyzed on an automated cell counter (Cell-Dyn 1300; Abbott Laboratories, Abott Park, IL) to determine blood cell counts and mean platelet volume. Platelet-rich plasma was prepared by centrifugation (15 min at 150*g*) of whole blood anticoagulated with 3.8% trisodium citrate (9:1). The platelet-rich plasma was used for functional platelet studies and electron microscopy studies, as described previously.[24] In short, aggregation studies were carried out by adding Horm collagen (0.5, 1, and 2 µg/ml), epinephrine (1.25 and 2.5 µmol/l), ristocetin (0.5 and 1.2 mg/ml), TRAP6 (thrombin receptor activating peptide) (18 µmol/l), arachidonic acid (1 mmol/l), or adenosine diphosphate (ADP) (5 and 10 µmol/l). ATP secretion tests were performed after stimulation of platelets with Horm collagen (2 µg/ml) and ADP (10 µmol/l).

The effects of the polyclonal rabbit anti-AQP7 antibody (sc-28625; Santa Cruz Biotechnology, Santa Cruz, CA) and a control polyclonal rabbit anti-FOG1 antibody[25] on platelet ATP secretion were evaluated using a modified method of the one described by Cho *et al.* for AQP1.[26] First, the monoclonal Fc receptor–blocking antibody IV.3 (purified from hybridoma ATCC-HB217; American Type Culture Collection, Molsheim, France; 10 µg/ml) was added to platelet-rich plasma for 5 min at 37 °C, after which the platelets were permeabilized with 50 µg/ml saponin (Merck, Darmstadt, Germany) for 10 min at 37 °C together with the anti-AQP7 or control anti-FOG1 antibody (at a final concentration of 5 µg/ml). Platelet ATP secretion was measured after stimulation with 20 µmol/l ADP.

Immunoblot analysis

The platelet releasate, microvesicle fraction, and remaining insoluble pellet after stimulation of washed platelets with strong agonists (TRAP6 with A23187 or thrombin) were obtained as described previously.[24,27] Equal amounts of protein extracts (10 µg) were resolved by SDS–polyacrylamide gel electrophoresis on 10% gels and transferred onto a Hybond ECL-nitrocellulose membrane (GE Healthcare, Diegem, Belgium). After blocking in 5% milk powder in Tris-buffered saline–Tween 20 (10 mmol/l Tris-HCl pH 8.0, 150 mmol/l sodium chloride, 0.1% Tween 20) for 1 h at room temperature, the membranes were incubated overnight at 4 °C with the anti-AQP7, anti-integrin β3 antibody (no. 4702, Cell Signaling Technology, Leiden, The Netherlands), or anti-β–actin (no. 4970, Cell Signaling Technology) antibody. The membranes were then incubated for 3 h at room temperature with an horseradish peroxidase-conjugated secondary antibody (at 1:1,000). The subsequent staining was performed with western blotting-enhanced chemiluminescence detection reagent (GE Healthcare).

Immunostaining of platelets and CHRF cells

Human megakaryocytic CHRF-288-11 cells (ATCC-CRL10107) were grown as previously described.[28] Platelets from the platelet-rich plasma were washed in the presence of Apyrase 1 U/ml and 10 mol/l prostaglandin E1 (PGE1), and resuspended in modified Tyrode-HEPES buffer so as to obtain a concentration

of 2×10^{10} platelets/l. CHRF cells were spread for 1 h and platelets for 30 or 45 min at 37 °C on glass cover slips coated with human fibrinogen (100 µg/ml). Thirty minutes after being spread, the platelets were further stimulated with 20 mol/l TRAP6 for 15 minutes at 37 °C. Adherent cells were fixed with 4% paraformaldehyde in cytoskeleton buffer (0.1 mol/l PIPES, 2 mol/l glycerol, 1 mmol/l EDTA, 1 mmol/l $MgCl_2$, pH 6.9), and permeabilized for 15 min with 0.2% triton X-100 (Roche, Mannheim, Germany) at room temperature. After blocking with 1% bovine serum albumin (Albumax; Life Technologies) for 30 min at room temperature, the cells were incubated with a 1:50 anti-AQP7, anti-von Willebrand factor, or anti-CD63 (BD biosciences PharMingen, San Diego, CA) antibody overnight at 4 °C. After five washing steps with phosphate-buffered saline, the cells were incubated with a specific secondary antibody diluted 1:200 (Alexa Fluor 488, or 568, or 647 conjugated; Life Technologies) together with phalloidin rhodamine (Sigma-Aldrich, Poole, UK) for F-actin staining, for 45 min at 37 °C. Analysis was done using a Zeiss Axiovert 100M confocal microscope (Carl Zeiss, Gottingen, Germany). Fluorescence intensities were quantified using the Java image processing program ImageJ 1.34 g (National Institutes of Health image software).

Statistical analysis

Statistical differences between the two groups were evaluated using the Student's *t*-test (two-tailed). For evaluating the effect of the anti-AQP7 antibody on platelet secretion, the nonparametric Mann–Whitney U-test was used because of the low number of comparisons. Differences were considered significant for values of $P < 0.05$.

RESULTS

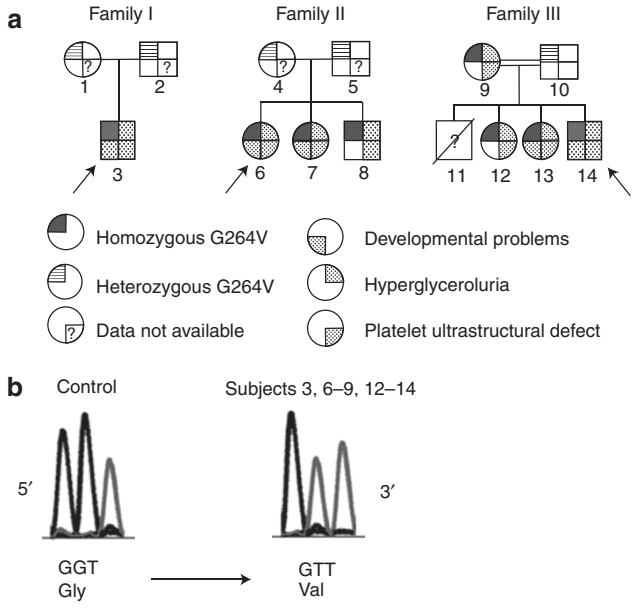
Clinical description

We describe three children (from unrelated families) with psychomotor retardation of unknown etiology. The pedigrees of the patients are shown in **Figure 1a** and the detailed clinical descriptions of the index patients and relatives are listed in **Table 1**.

The first child (index subject 3) was the only child of healthy, unrelated parents. He presented to the outpatient clinic at the age of 14 months because of recurrent vomiting, and subsequently developed gross motor delay (walking commenced only at the age of 17 months) and autistic characteristics, and had an IQ of 108. At the age of 2 years, an episode of seizures was reported, with normal electroencephalogram. The child's facial features were not dysmorphic and he was not overweight (body mass index 14.8 kg/m^2 at the age of 5 years, −0.6 SD score, ref. 29). He was taking no medications.

The second patient (index subject 6) was a girl with obvious psychomotor retardation (skill level of a 2-year-old child at a calendar age of 4 years), hypotonia, and discrete dysmorphic facial features (large mouth with small widely spaced teeth and dysplastic left ear). The brain magnetic resonance imaging result was normal. Her weight and length were at the third percentile (body mass index 15.5 kg/m^2 at the age of 5 years, +0.0 SD score). One older sister (subject 7) had learning difficulties at school, and a younger brother (subject 8) had a normal phenotype at 18 months of age. The parents are healthy and unrelated.

The third child in our study (index subject 14) was a boy, the fourth child of consanguineous Turkish parents. He presented with psychomotor retardation and profound hypotonia. From the age of 6 months he had developed generalized seizures, and had been treated with valproic acid and levetiracetam. The brain magnetic resonance imaging was normal. His



c

AQP7 orthologs	
Homo sapiens	VAPLL GAYLGG
Mus musculus	VAPLL GAYLGG
Danio rerio	VAPFI GGVLGA
Aquaglyceroporins (AQP7 paralogs)	
AQP3	VSPLL GSIAGV
AQP9	VGPLV GAVIGG
AQP10	VAPLV GATVGT
Classical AQPs	
AQP0	VGPII GGGLGS
AQP1	VGPFI GGALAV
AQP2	IGPLV GAILGS
AQP4	VGPII GAVLAG
AQP5	VGPIV GAVLAA
AQP6	VGPLM GALLAS
Unorthodox AQPs	
AQP11	LAPSL GILLMI

Figure 1 Clinical and genetic characterization of patients. (a) Pedigrees of the three unrelated families, indicating the *AQP7* G264V genotype and phenotype characterized by the absence or presence of hyperglyceroluria, platelet ultrastructural abnormalities as detected by electron microscopy, and neurodevelopmental problems. The index patients are indicated by arrows. Lack of data is indicated by a question mark in the box. (**b**) Genetic analysis of *AQP7*. At nucleotide position 963, a homozygous G-to-T substitution was found in the index subjects (right panel), causing a glycine-to-valine substitution at codon 264 (G264V). (**c**) Evolutionary conservation of the affected AQP7 codon 264 in various species and AQP subclasses. The highly conserved residue is underlined. AQP7, aquaporin 7.

Table 1 Genotype, clinical characteristics, and metabolic screen in index patients and their relatives

Subject (male/female)	Age (years)	AQP7 G264V	Clinical details	U glycerol (mmol/mol creat) (normal <1)	U protein (g/g creat) (normal <0.50 if <2 years[a]; <0.17 if >2 years)[b]	U glucose (g/l) (normal <0.2)	TSH (mIU/l) (normal 0.27–4.20)	BMI (SDS)	TG (mg/dl) (normal <180)	P glucose (mg/dl) (normal 55–100)
Family I										
1 (F)	46	+/–	Asymptomatic	<1	(Dipstick–)[a]	<0.1				78
2 (M)	50	+/–	Asymptomatic	<1	(Dipstick–)[a]	<0.1				
Index 3 (M)	5	+/+	Delayed rough motoric development, autistic features, epileptic insult at age 2 years	948, 916, 495	0.21; <0.1[b]	0.0	4.7; 3.1; 6.1	–0.6	53	102
Family II										
4 (F)	28	+/–	Asymptomatic	<1	0.09; 0.09[a]					
5 (M)	29	+/–	Asymptomatic	<1	0.04; 0.03[b]	<0.1	1.6			
Index 6 (F)	5	+/+	Hypotonia, PMR, discrete facial dysmorphy	2,806, 1,293, 1,000	2.17; 4.58; 1.11[a,¶]	<0.1	6.5; 18.8; 10.4[¶¶]	0.0	45; 65; 45	71; 80; 85
7 (F)	5	+/+	Learning problems at school	1,385, 1,175, 505	0.14; 0.19; <0.1[b]	<0.1	4.0; 3.8; 2.3	–0.7	34; 84	163
8 (M)	1.5	+/+	Asymptomatic	1,432	0.05[a]	<0.1		0.2		
Family III										
9 (F)	34	+/+	Asymptomatic	681				+0.5		
10 (M)	34	+/–	Asymptomatic	<1				+1.4		
11 (M)		NA	Hypotonia, epilepsy, died at age 8 months							
12 (F)	14	+/+	Asymptomatic	411				+1.1		
13 (F)	8	+/+	Learning problems at school	1,238				–1.8		
Index 14 (M)	5	+/+	Hypotonia, PMR, epilepsy from age 6 months, internal strabismus	1,814, 1,009, 877	0.15[b]	0.1	4.41; 8.75	+0.3	120; 134	112; 87; 83

Age at most recent clinical examination is given in years. The *AQP7* genotype is presented in this table as +/– for heterozygous carriers, and +/+ for homozygosity. Up to three values are given when available: either most recent, or values before the intake of medication (¶before ACE inhibitor, ¶¶before L-thyroxine). Urine protein levels are given in grams per gram creatinine when available, or as below the threshold when a dipstick analysis was used (<50 mg/l). The most recent body mass index (BMI, kg/m^2) is given as SD score (SDS).[29] Plasma glucose levels are random nonfasting glucose values.
AQP7, aquaporin 7; creat, creatinine; NA, not available; P, plasma; PMR, psychomotor retardation; TG, triglyceride; TSH, thyroid-stimulating hormone; U, urine.
[a]U protein normal values <2 years. [b]U protein normal values >2 years.

weight and length were at percentiles 25 and 50, respectively (body mass index 15.9 kg/m^2 at the age of 5 years, +0.3 SD score). Clinical examination showed internal strabismus in addition to dolichocephaly and small testes. An older brother (subject 11) had shown a similar constellation of symptoms (psychomotor retardation, hypotonia, and epilepsy) but had died at the age of 8 months. No further data or DNA were available relating to this subject. One sister (subject 13) had learning difficulties at school, attributed to inadequate mastering of language skills. Another older sister (subject 12) and both parents were asymptomatic.

Extensive diagnostic testing was performed in these index patients but failed to pinpoint a particular neurodevelopmental diagnosis (**Supplementary Table S3** online for overview of all tests).

Glycerol measurements and other metabolic parameters

Metabolic screening revealed normoglycerolemic hyperglyceroluria in the three index patients (**Table 1**). Plasma glycerol levels were similar in the patients (mean 91 µmol/l, range 46–162 µmol/l) and the controls (mean 79 µmol/l, range 34–229 µmol/l), ruling out prerenal causes of severe glyceroluria (495–2,806 mmol/mol creatinine in the patients as compared with <1 mmol/mol creatinine levels in the controls). Urine glycerol excretion was within normal ranges in the parents of the index cases, except for the mother (subject 9) of index patient 14, who had a urine creatinine level of 681 mmol/mol. Of note, urine glycerol levels were elevated in the clinically normal siblings of the index patients (mean 1,024 mmol/mol creatinine, range 4111,432). The patients' levels of blood glucose, serum cholesterol, and triglycerides were within normal ranges. Elevated levels of thyroid-stimulating hormone but normal thyroid hormone levels were also noted in the patients, except for index patient 6, who also had a low free thyroxine level (0.82 ng/dl, normal 0.93–1.70 ng/dl). She was started on L-thyroxine therapy.

Genetic screening for *AQP3*, *AQP7*, and *AQP9*

Based on the finding of pronounced urine glycerol loss, the *AQP3, AQP7*, and *AQP9* genes were screened for mutations.[30,31] No mutations were detected in *AQP3* and *AQP9*. In the *AQP7* gene, a homozygous nucleotide substitution (G to T in exon 8) was detected in all three index cases, converting glycine into valine at codon 264 (G264V) in the sixth transmembrane domain of the protein (**Figure 1b**). The parents were heterozygous carriers of the mutation, except for the mother of the third patient (subject 9) who was homozygous (**Figure 1a** and **Table 1**). All siblings of the index patients were also homozygous for this mutation. The mutated residue is well conserved in AQP7 of different species but also among the other AQP subfamily members (classical AQPs, aquaglyceroporins and unorthodox AQPs; **Figure 1c**), suggesting that it is important for AQP functioning. It is obvious that the homozygous G264V variant cosegregates with the hyperglyceroluria in the three families. The G264V *AQP7* variant had previously been screened in Japanese and Caucasian subjects with obesity and/or type 2 diabetes, but no association between the genotype and these phenotypes could be demonstrated.[20,21] These studies did not include measurement of the urine glycerol levels, but normal plasma glycerol levels were reported for the homozygous carrier in the Japanese study,[20] in line with the findings in our study.

Morphologic and functional platelet studies

Hematologic analysis showed a normal platelet count with a mildly increased mean platelet volume (9.7 ± 1.0 fl vs. 7.8 ± 1.0 fl, $P = 0.001$) in all homozygous carriers of G264V *AQP7* carriers relative to heterozygous carriers (**Table 2**). Electron microscopy revealed enlarged and especially more rounded platelets with centrally localized granules in some of the platelets and a pronounced open canalicular system (**Figures 1a** and **2a**). Subject 10, a heterozygous carrier who was the father of index patient 14, had structurally normal platelets. We

Table 2 Platelet studies in homozygous G264V subjects

Subject	Platelet count (× 10^9/l)	MPV (fl)	Aggregation (%amplitude)		ATP secretion	
			Col 2 g/ml	Epi 1.25 mol/l	Col 2 g/ml	ADP 10 mol/l
Normal	150–450	6.8–8.8**	69–88	48–100**	1.2–2.8	0.5–2.1*
Index-3	282	9.0	84	14	2.0	0.3
Index-6	280	9.3	71	62 (Delayed second wave)	1.7	0.6
7	360	9.4	77	33	1.7	0.0
8	307	7.8	67	NA	0.3	0.0
9	291	10.5	70	19	1.2	0.8
12	206	9.7	83	17	1.0	1.0
13	208	11.6	80	65	0.9	1.2
Index-14	217	11.0	80	8	2.4	0.5

Normal values for the different platelet characteristics are given in the table as tested in 40 healthy subjects for mean platelet volume (MPV), aggregations, and ATP secretions. Statistics were performed using the average values of all homozygous individuals and healthy controls.
ADP, adenosine diphosphate; ATP, adenosine triphosphate; Col, collagen; NA, not available.
*$P < 0.05$, **$P < 0.01$.

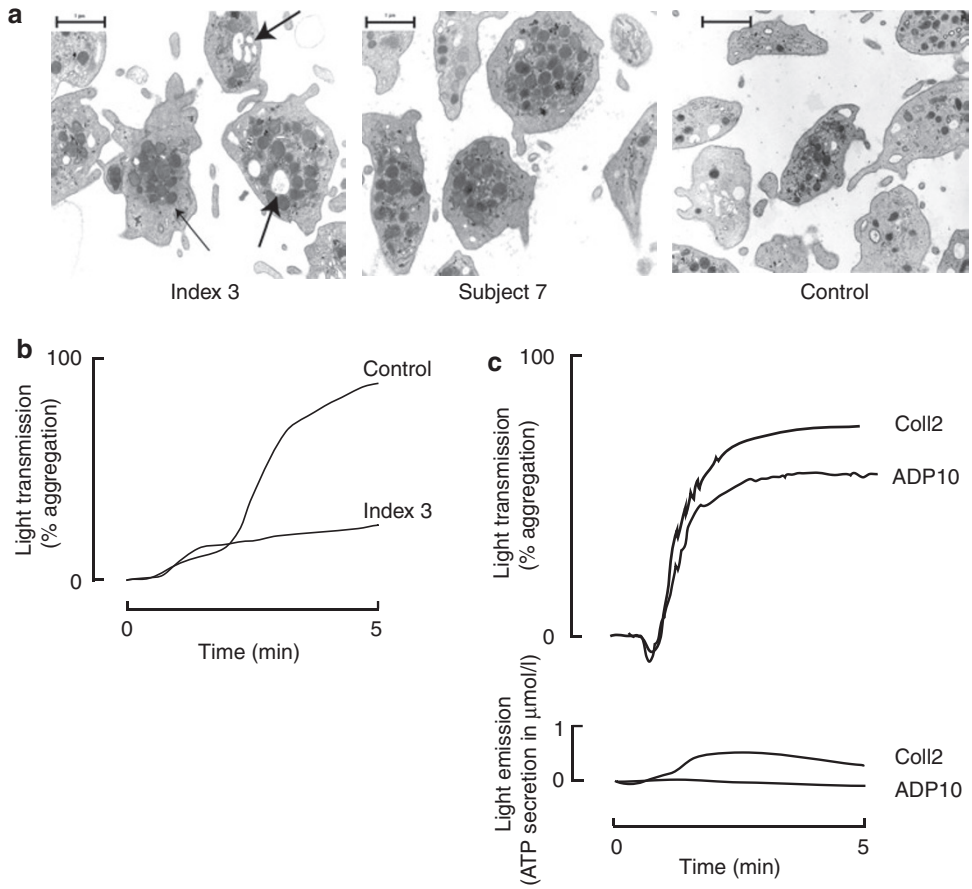


Figure 2 Platelet morphology and functioning. (**a**) Electron microscopy studies of platelets from individuals who were homozygous for *AQP7* G264V showed enlarged, more rounded platelets as compared with the typical discoid control platelets. Some platelets also presented with centralized granules and prominent open canalicular systems, as indicated by arrows. Bars represent 1 µm. (**b**) Absence of the secondary aggregation wave after platelet stimulation with epinephrine (1.25 µmol/l) in index patient 3 as compared with a control individual. This curve is representative of those obtained for the other homozygous G264V carriers. (**c**) ADP (10 µmol/l) and collagen (2 µg/ml) induced aggregation (upper curves) and ATP secretion (lower curves) in platelets of index patient 6. These curves are representative of those obtained for the other homozygous G264V carriers. ADP, adenosine diphosphate; AQP7, aquaporin 7; ATP, adenosine triphosphate.

also detected a mild subclinical platelet dense granule secretion defect in the homozygous carriers, as highlighted by the absence of a secondary aggregation response to epinephrine stimulation (Figure 2b and Table 2). This type of aggregation defect is typically present in patients with reduced platelet dense granule release, as we have earlier described with respect to patients with storage pool disease.[24] The use of a stronger agonist such as Horm collagen showed no defects in aggregation response in the patients because this type of platelet stimulation is less dependent on secretion.[24] In line with this finding, the standard platelet aggregation tests with different agonists (ristocetin, arachidonic acid, U46619, ADP, and Horm collagen) that are generally used to detect a platelet-related clinical bleeding problem were all normal for subject 6. In addition, a reduction in ATP secretion was found in response to stimulation with ADP, the response being less pronounced with Horm collagen in the patients (Figure 2c and Table 2). These findings further supported the likelihood that the mild dense granule secretion defect was present in these subjects. Although these homozygous G264V carriers presented with a platelet secretion defect, none of them had any obvious clinical bleeding problems.

Subcellular localization of AQP7 in human platelets

Given that the *AQP7* G264V variant affects platelet morphology and functioning, we further studied the subcellular localization of the AQP7 protein in normal human platelets. After full stimulation of platelets with strong agonists (A23187/TRAP6 or thrombin), AQP7 was found mainly in the platelet releasate, using immunoblot analysis (Figure 3a). Integrin β3 is a typical platelet cell membrane protein that was, in line with expectations, detected mainly in the pellet and microvesicle membrane fractions. Actin is bound to the membranes but, after stimulation, can also be detected in the platelet releasate, as described by other proteomic studies.[27] This is probably because the actin that is bound to the platelet granule-membrane is partially released during stimulation; however, the underlying mechanism for this is not known. We hypothesize that AQP7 as a membrane protein is released through a similar mechanism. Indeed, evidence exists of the unexpected presence of cytoskeletal and

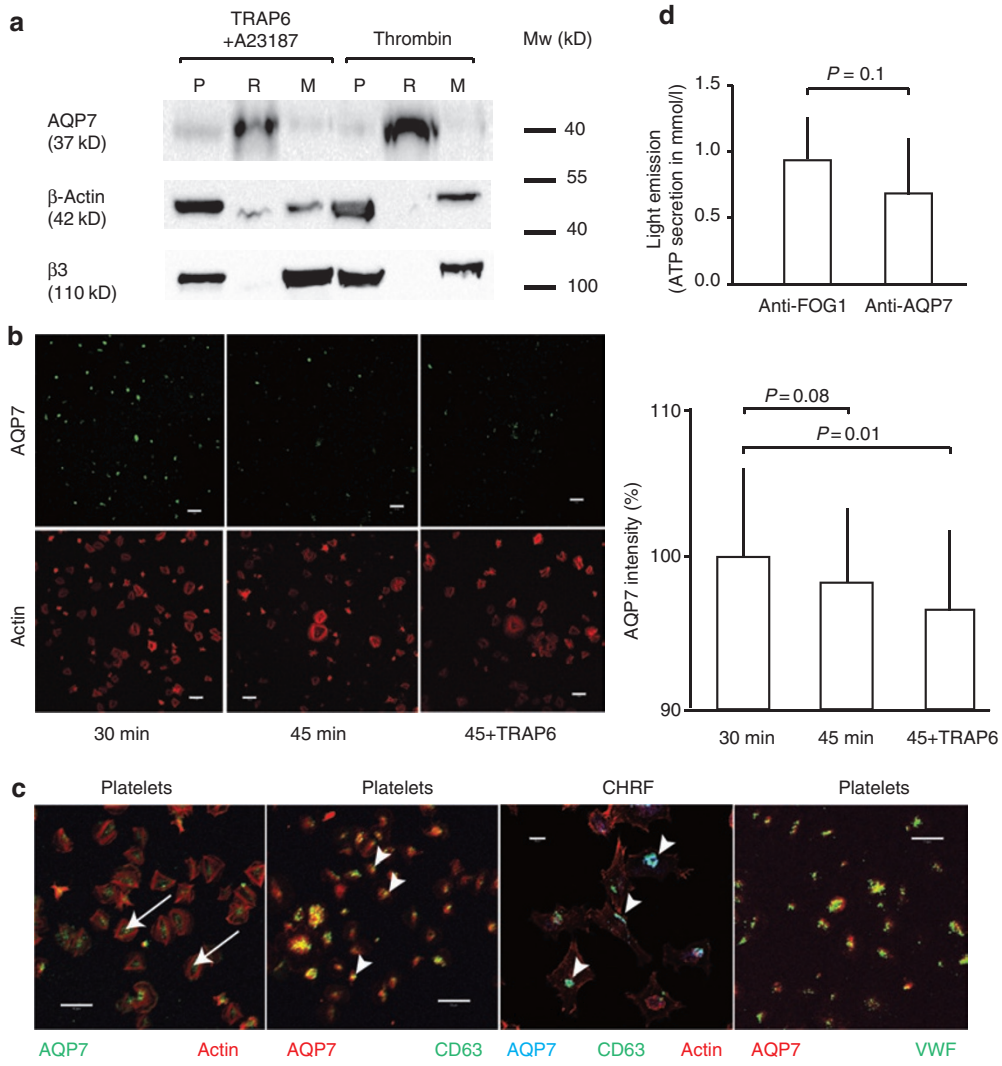


Figure 3 AQP7 localization and functioning in normal human platelets. (**a**) AQP7 is present mainly in the platelet releasate (R) after full stimulation of platelets from a healthy control subject. Integrin β3, used as a control membrane protein, was present in the pellet (P) and microvesicle (M) membrane fractions. (**b**) Left: AQP7 immunostaining in platelets spread for 30 and 45 min without adding an agonist, and at 45 min after costimulation with TRAP6. Bars represent 10 µm. Right: Quantification of AQP7 staining during platelet spreading. Mean intensities are given as percentages of the starting fluorescence intensity. Error bars represent SDs. The data are from two independent experiments. (**c**) AQP7 immunostaining in spread platelets shows a granular pattern (arrows in left panel). AQP7 presents a strong colocalization with the dense granule marker CD63 in platelets (arrowheads in middle left panel), which was also seen in megakaryocytic CHRF cells (arrowheads in middle right panel). However, only weak colocalization could be seen with the α-granule marker von Willebrand factor (VWF) (right panel). A negative control with the secondary antibody excluded aspecific binding (data not shown). Bars represent 10 µm. (**d**) ADP-induced ATP secretion after incubation with the polyclonal rabbit anti-AQP7 or anti-FOG1 antibody in permeabilized platelets. Mean values along with SD are depicted. The data are from three independent experiments. ADP, adenosine diphosphate; AQP7, aquaporin 7; ATP, adenosine triphosphate; TRAP6, thrombin receptor activating peptide.

actin-binding proteins in the platelet releasate.[24,27] In addition, immunostaining of platelets spread on fibrinogen showed a reduced expression of AQP7 for the duration of platelet spreading, and an even more pronounced reduction after TRAP6 stimulation (**Figure 3b**). These experiments again indicate that AQP7 is partially released during spreading, initiating mild platelet activation and secretion, and that it is further released when stimulated by TRAP6, which induces full activation and granule release. To further specify the exact subcellular location of AQP7, immunostaining was performed. It showed colocalization of AQP7 with CD63 (a marker for platelet dense granules) but not with von Willebrand factor (a marker for platelet alpha granules) (**Figure 3c**). The colocalization of AQP7 with CD63 could also be demonstrated in the human megakaryocytic cell line CHRF.

To evaluate the role of AQP7 in platelet-dense granule release, permeabilized platelets were incubated with anti-AQP7 or a control antibody before ATP secretion was measured. This anti-AQP7 antibody was raised against the amino acid sequence where the G264V mutation is located (the epitope being amino acids 169–269 of the C-terminus of AQP7). Although not significant ($P = 0.1$), a trend toward a decreased ATP secretion was

found after stimulation with 20 µmol/l ADP for the anti-AQP7 antibody as compared with the aspecific control anti-FOG1 antibody (**Figure 3d**).

DISCUSSION

We describe, for the first time, the cosegregation of the homozygous G264V *AQP7* variant with a marked loss in urine glycerol. A pronounced loss of glycerol in the urine coincident with normal plasma concentration values has never been described in humans, although AQP7-deficient mice have been shown to have hyperglyceroluria.[19] Renal AQP7 is responsible for reabsorption of glycerol at the renal proximal tubules in coordination with AQP3.[30,31] All the homozygous carriers of G264V *AQP7* in our study had hyperglyceroluria, whereas no glycerol loss was present in the heterozygous parents. The hypothesis that this mutation has functional relevance was earlier supported by the findings of Kondo *et al.*,[20] who could not show any association between the presence of the mutation and the occurrence of obesity or type 2 diabetes; however, they did find the first homozygous G264V subject with impaired plasma glycerol release from fat tissue after exercise tests, although urine glycerol levels were not determined. AQPs as a family have a conserved structure,[8,32] existing in membranes as homotetramers. Each subunit monomer forms a separate permeable pore composed of six α-helix transmembrane domains with inverted symmetry between the first and last three domains, and a pore-forming loop with the signature NPA motif.[33] The G264V mutation is located in the highly conserved GxxxG motif of the sixth transmembrane helix in nearly all AQPs (**Figure 1c**).[33–35] Glycine in the motif can sometimes be replaced by alanine.[34] It should be noted that the presence of a mutation in another genetic locus, cosegregating with the aforementioned mutation, still remains a possibility.

Four independent AQP7-knockout mouse models that are viable and healthy have been described (**Supplementary Table S1** online). Based on these models, AQP7 was found to be crucial in fat metabolism as well as in renal glycerol reabsorption. Except for the fact that AQP7-knockout mice as well as the human patients in our study had profound urine glycerol loss, there is nothing in common between the two phenotypes. This can be explained by the difference between having a complete loss of *AQP7* and having only an *AQP7* missense mutation with probable partial loss of activity. In addition, significant differences have been noted between the different AQP7-knockout models, probably related to their genetic backgrounds and possibly indicating redundancy by other AQPs. The latter factor could also play a role in humans. To our knowledge, no study exists that describes a role for AQP7 in platelets or links AQP7 deficiency to behavior changes using AQP7-knockout mice.

Platelets are easy to isolate in a nonactive state and are ideal cells in which to evaluate G-protein signal transduction, secretion, and adhesion in hemostatic disorders. In addition, we have shown that these types of functional platelet studies are also very useful in characterizing an unknown disorder that includes only a subclinical platelet defect.[36] We therefore performed various platelet tests in these patients after all other diagnostic tests (see **Supplementary Table S3** online) were negative. Our study indicates that AQP7 is also important for platelet morphology and functioning. Homozygous carriers have enlarged and more rounded platelets with centrally localized granules. This results in a reduced release of granule content when platelets are stimulated with a weak agonist. Even though AQP7 was detected in the releasate after platelet stimulation, it is not clear whether it is actually secreted, given that the releasate also captures other membrane-bound proteins such as actin.[37] The published literature contains extremely limited support for the hypothesis that AQPs have a role in the morphology and functioning of platelets. It was only very recently that AQP6 was shown to be involved in the regulation of platelet volume through a G protein–mediated pathway.[38] Although most AQPs reside at the plasma membrane, their presence in intracellular organelles such as secretory granules has been demonstrated in other types of cells, wherein they also play a role in secretion through regulation of the vesicle volume.[39,40] Regulated secretion is a feature of specialized cells such as neurons and neuroendocrine cells, but platelets also secrete their granules at sites of vascular damage in response to stimulation. A role for AQPs in platelet granule secretory function has never been reported. AQP7 could interfere with platelet-dense granule formation and functioning, as previously described with respect to AQP1, which was found to be associated with exosomes during reticulocyte maturation.[41]

A largely variable neurologic phenotype was observed among the homozygous G264V carriers, ranging from severe hypotonia, psychomotor retardation, and/or epilepsy needing medication in the index cases to normal functioning in their healthy siblings. More studies are needed to define whether mental disability is also related to the AQP7 G264V variant, or whether a separate genetic factor is responsible for the neurological phenotype in the affected homozygous carriers. AQP7 was found to be expressed during perinatal development of the mouse brain.[42] AQP1 and AQP6 are associated with synaptic vesicles and participate in their swelling,[43] implying a possible role for AQPs in neuronal granule secretion. The functions of AQPs in the central nervous system seem to be diverse; studies have shown a role for AQPs in bidirectional transport of water between brain and blood vessels, in cerebrospinal fluid formation, in neural signal transduction, in osmoreception, and in some brain pathologies such as brain edema, brain tumors, and autism (reviewed in refs. 8,44). Given that AQP7 also facilitates glycerol transport, it might have a role in the energy metabolism of neurons, as was previously found with respect to AQP9. The latter showed increased expression in human glioblastoma, and this increased expression was associated with increased energy metabolism of glioma cells.[45]

In conclusion, this study is the first to show a cosegregation of the G264V defect in AQP7 with hyperglyceroluria, and to imply the involvement of a new category of proteins, the AQPs, in platelet morphology and granule secretion.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/gim

ACKNOWLEDGMENTS

This work was supported by the "Excellentie financiering KULeuven" (EF/05/013), by research grants G.0490.10N and G.0743.09 from the Fund for Scientific Research–Flanders (FWO-Vlaanderen, Belgium), grant GOA/2009/13 from the Research Council of the University of Leuven (Onderzoeksraad KULeuven, Belgium), Heimburger Award 2010 (CSL Behring). C.G. has a research grant of the "Stichting Marguerite-Marie Delacroix", Belgium. G.M.B. and C.V.G. are senior clinical investigators of the FWO—Vlaanderen. We thank Pieter Vermeersch for his contribution in measuring urine glycerol.

DISCLOSURE

The authors declare no conflict of interest.

REFERENCES

1. Denker BM, Smith BL, Kuhajda FP, Agre P. Identification, purification, and partial characterization of a novel Mr 28,000 integral membrane protein from erythrocytes and renal tubules. *J Biol Chem* 1988;263:15634–15642.
2. King LS, Kozono D, Agre P. From structure to disease: the evolving tale of aquaporin biology. *Nat Rev Mol Cell Biol* 2004;5:687–698.
3. Gena P, Fanelli E, Brenner C, Svelto M, Calamita G. News and views on mitochondrial water transport. *Front Biosci* 2009;14:4189–4198.
4. Nozaki K, Ishii D, Ishibashi K. Intracellular aquaporins: clues for intracellular water transport? *Pflugers Arch* 2008;456:701–707.
5. Verkman AS. More than just water channels: unexpected cellular roles of aquaporins. *J Cell Sci* 2005;118(Pt 15):3225–3232.
6. Agre P, King LS, Yasui M,et al. Aquaporin water channels–from atomic structure to clinical medicine. *J Physiol (Lond)* 2002;542(Pt 1):3–16.
7. Ishibashi K, Kondo S, Hara S, Morishita Y. The evolutionary aspects of aquaporin family. *Am J Physiol Regul Integr Comp Physiol* 2011;300:R566–R576.
8. Barbara B. Aquaporin biology and nervous system. *Curr Neuropharmacol* 2010;8:97–104.
9. Fujiyoshi Y, Mitsuoka K, de Groot BL,et al. Structure and function of water channels. *Curr Opin Struct Biol* 2002;12:509–515.
10. Rojek A, Praetorius J, Frøkiaer J, Nielsen S, Fenton RA. A current view of the mammalian aquaglyceroporins. *Annu Rev Physiol* 2008;70:301–327.
11. Verkman AS. Aquaporins: translating bench research to human disease. *J Exp Biol* 2009;212(Pt 11):1707–1715.
12. Kuriyama H, Kawamoto S, Ishida N,et al. Molecular cloning and expression of a novel human aquaporin from adipose tissue with glycerol permeability. *Biochem Biophys Res Commun* 1997;241:53–58.
13. Ishibashi K, Kuwahara M, Gu Y,et al. Cloning and functional expression of a new water channel abundantly expressed in the testis permeable to water, glycerol, and urea. *J Biol Chem* 1997;272:20782–20786.
14. Ishibashi K, Yamauchi K, Kageyama Y,et al. Molecular characterization of human Aquaporin-7 gene and its chromosomal mapping. *Biochim Biophys Acta* 1998;1399:62–66.
15. Hara-Chikuma M, Sohara E, Rai T,et al. Progressive adipocyte hypertrophy in aquaporin-7-deficient mice: adipocyte glycerol permeability as a novel regulator of fat accumulation. *J Biol Chem* 2005;280:15493–15496.
16. Hibuse T, Maeda N, Funahashi T,et al. Aquaporin 7 deficiency is associated with development of obesity through activation of adipose glycerol kinase. *Proc Natl Acad Sci USA* 2005;102:10993–10998.
17. Maeda N, Funahashi T, Hibuse T,et al. Adaptation to fasting by glycerol transport through aquaporin 7 in adipose tissue. *Proc Natl Acad Sci USA* 2004;101:17801–17806.
18. Skowronski MT, Lebeck J, Rojek A,et al. AQP7 is localized in capillaries of adipose tissue, cardiac and striated muscle: implications in glycerol metabolism. *Am J Physiol Renal Physiol* 2007;292:F956–F965.
19. Sohara E, Rai T, Miyazaki J, Verkman AS, Sasaki S, Uchida S. Defective water and glycerol transport in the proximal tubules of AQP7 knockout mice. *Am J Physiol Renal Physiol* 2005;289:F1195–F1200.
20. Kondo H, Shimomura I, Kishida K,et al. Human aquaporin adipose (AQPap) gene. Genomic structure, promoter analysis and functional mutation. *Eur J Biochem* 2002;269:1814–1826.
21. Ceperuelo-Mallafré V, Miranda M, Chacón MR,et al. Adipose tissue expression of the glycerol channel aquaporin-7 gene is altered in severe obesity but not in type 2 diabetes. *J Clin Endocrinol Metab* 2007;92:3640–3645.
22. Rodríguez A, Catalán V, Gómez-Ambrosi J, Frühbeck G. Aquaglyceroporins serve as metabolic gateways in adiposity and insulin resistance control. *Cell Cycle* 2011;10:1548–1556.
23. Li R, Keymeulen B, Gerlo E. Determination of glycerol in plasma by an automated enzymatic spectrophotometric procedure. *Clin Chem Lab Med* 2001;39:20–24.
24. Di Michele M, Thys C, Waelkens E,et al. An integrated proteomics and genomics analysis to unravel a heterogeneous platelet secretion defect. *J Proteomics* 2011;74:902–913.
25. Freson K, Thys C, Wittewrongel C, Vermylen J, Hoylaerts MF, Van Geet C. Molecular cloning and characterization of the GATA1 cofactor human FOG1 and assessment of its binding to GATA1 proteins carrying D218 substitutions. *Hum Genet* 2003;112:42–49.
26. Cho SJ, Sattar AK, Jeong EH,et al. Aquaporin 1 regulates GTP-induced rapid gating of water in secretory vesicles. *Proc Natl Acad Sci USA* 2002;99:4720–4724.
27. Coppinger JA, Cagney G, Toomey S,et al. Characterization of the proteins released from activated platelets leads to localization of novel platelet proteins in human atherosclerotic lesions. *Blood* 2004;103:2096–2104.
28. Di Michele M, Peeters K, Loyen S,et al. Pituitary adenylate cyclase-activating polypeptide (PACAP) impairs the regulation of apoptosis in Megakaryocytes by activating NF-κB: a proteomic study. *Mol Cell Proteomics* 2012;11:M111.007625.
29. Cole TJ, Freeman JV, Preece MA. Body mass index reference curves for the UK, 1990. *Arch Dis Child* 1995;73:25–29.
30. Verkman AS. Roles of aquaporins in kidney revealed by transgenic mice. *Semin Nephrol* 2006;26:200–208.
31. Verkman AS. Dissecting the roles of aquaporins in renal pathophysiology using transgenic mice. *Semin Nephrol* 2008;28:217–226.
32. Walz T, Fujiyoshi Y, Engel A. The AQP structure and functional implications. *Handb Exp Pharmacol* 2009:31–56.
33. Heymann JB, Engel A. Structural clues in the sequences of the aquaporins. *J Mol Biol* 2000;295:1039–1053.
34. Murata K, Mitsuoka K, Hirai T,et al. Structural determinants of water permeation through aquaporin-1. *Nature* 2000;407:599–605.
35. Russ WP, Engelman DM. The GxxxG motif: a framework for transmembrane helix-helix association. *J Mol Biol* 2000;296:911–919.
36. Freson K, Labarque V, Thys C, Wittevrongel C, Geet CV. What's new in using platelet research? To unravel thrombopathies and other human disorders. *Eur J Pediatr* 2007;166:1203–1210.
37. Piersma SR, Broxterman HJ, Kapci M,et al. Proteomics of the TRAP-induced platelet releasate. *J Proteomics* 2009;72:91–109.
38. Lee JS, Agrawal S, von Turkovich M, Taatjes DJ, Walz DA, Jena BP. Water channels in platelet volume regulation. *J Cell Mol Med* 2012;16:945–949.
39. Sugiya H, Matsuki M. AQPs and control of vesicle volume in secretory cells. *J Membr Biol* 2006;210:155–159.
40. Kelly ML, Cho WJ, Jeremic A, Abu-Hamdah R, Jena BP. Vesicle swelling regulates content expulsion during secretion. *Cell Biol Int* 2004;28:709–716.
41. Blanc L, Liu J, Vidal M, Chasis JA, An X, Mohandas N. The water channel aquaporin-1 partitions into exosomes during reticulocyte maturation: implication for the regulation of cell volume. *Blood* 2009;114:3928–3934.
42. Shin I, Kim HJ, Lee JE, Gye MC. Aquaporin7 expression during perinatal development of mouse brain. *Neurosci Lett* 2006;409:106–111.
43. Jeremic A, Cho WJ, Jena BP. Involvement of water channels in synaptic vesicle swelling. *Exp Biol Med (Maywood)* 2005;230:674–680.
44. Benga O, Huber VJ. Brain water channel proteins in health and disease. *Mol Aspects Med* 2012; e-pub ahead of print 7 April 2012.
45. Warth A, Mittelbronn M, Hülper P, Erdlenbruch B, Wolburg H. Expression of the water channel protein aquaporin-9 in malignant brain tumors. *Appl Immunohistochem Mol Morphol* 2007;15:193–198.