Journal of Animal and Veterinary Advances 7 (6): 736-747, 2008
ISSN: 1680-5593

Clinicopathological Studies on Aquaporin 9 (AQP 9) in Wild and AQP9 Knockout Mice

Mohamed Abd El-Azim Hashem
Department of Clinical Pathology, Faculty of Veterinary Medicine,
Zagazig University, Sharkia Province, Egypt

Abstract: Aquaporin 9 (AQP9) is a water channel membrane protein also permeable to small solutes such as urea, glycerol and 5-fluorouracil, a chemotherapeutic agent. The objective of this study were to determine the cellular and subcellular localization of AQP9 in different organs of wild and AQP9 knockout mice (males and females) by immunohistochemistry (IHC), immunofluorescence (IF) and RT-PCR. Twenty wild (normal control) and homozygous AQP9 KO-mice (C57 BL /6J) of both sexes were used in this study. Immunocytochemical analyses using rabbit- affinity-purified anti-rat AQP9 and Rabbit EnVision as primar and secondary antibodies respectively, revealed AQP9 specific labeling in the hepatocytes, kidney, spleen and epididymis of wild type mice ($AQP9^{+/+}$), but a complete absence of labeling in $AQP9^{-/-}$ mice. In liver, the labeling was strongest at the sinusoidal surface and there was little intracellular labeling. AQP9 expression was found to be sex-linked. In female's liver the expression of AQP9 was mostly confined to perivascular hepatocytes, whereas males showed a more homogeneous hepatocyte staining. Confocal immunofluorescence confirmed the localization of AQP9 immunostaining on the sinusoidal surface or basolateral plasma membrane of hepatocytes. In the absence of physiological stress, knockout mice did not display any visible behavioral or severe physical abnormalities. Compared with control mice, serum levels of glycerol and triglycerides were significantly increased, in association with hypoglycemia in the $AQP9^{-/-}$ mice, whereas total cholesterol, urea, alanine aminotransferase and alkaline phosphatase were not statistically different. These findings showed that AQP9 is a major water channel protein that is expressed throughout different organs, with high expression in the liver of male mice. Moreover, AQP9 is important for hepatic glycerol metabolism.

Key words: Aquaglyceroporins, aquaporin9, immunohistochemistry, PCR, liver

INTRODUCTION

Aquaporins (AQPs) are members of a large family of integral membrane proteins involved in the rapid movement of water and neutral solutes across cell membranes (Caperna *et al*., 2007; Krane and Goldstein, 2007) and are fundamentally important to the fluid transport in the bile ducts and ductules of the liver (Talbot *et al*., 2003). In mammalian cells, water passes across cell membranes by simple diffusion across the lipid bilayer and by bulk flow driven by an osmotic gradient through hydrophilic pores or channels (Badran and Hermo, 2002). The discovery of water channel proteins (aquaporins) has provided a molecular explanation for the way by which water can cross the cell plasma membrane of highly water permeable epithelia. Verkman and Mitra (Verkman and Mitra, 2000) stated that AQPs may be involved in membrane fluidity and structural integrity, as well as tumor growth and angiogenesis. Some AQPs are constitutively expressed, while others are regulated by hormones, pH variations, phosphorylation and binding of auxiliary proteins (Engel *et al*., 2000). Various disease states have been associated with alterations in AQP expression and targeting in cells (Van *et al*., 2000).

To date, 13 isoforms of AQPs (AQP0–AQP12) have been identified in a wide variety of mammal cells, such as liver, kidney, lung, pancreas, brain, gastrointestinal tract, eye, ear, immune system, skin, adipose, muscles, uterus and testis (Ishibashi *et al*., 2002). Based on sequence homology data, phylogenetic comparisons and permeability properties, AQPs of 0-10 are now subdivided into two major groups: orthodox AQPs and aquaglyceroporins (Agre and Kozono, 2003; Zardoya, 2005). The group of orthodox AQPs is composed of 6 members: AQPs 0, 1, 2, 4, 5 and 6, as well as AQP8 (Liu *et al*., 2007). They are water-selective channels and permeable to water alone. The group of aquaglyceroporins includes four members: AQPs 3, 7, 9 and 10. They are non-selective water channels which are permeable to glycerol, urea and other small non-electrolytes, as well as to water (Marinelli *et al*., 2004; Liu and Wintour, 2005). The original studies of Xenopus laevis oocytes expressing rat AQP9 reported permeability to a wide range of 14C-or 3H-labeled solutes including

polyols, carbamides, purines, pyrimidines, nucleosides and monocarboxylates (Carbrey *et al*., 2003). In the liver, these AQPs act in concert with ion transporters and facilitate metabolic activity associated with cell volume perturbations, osmotic balance, concentration and movement of biliary solutes and may be involved in glycogen turnover (Marinelli *et al*., 2004).

Rojek *et al*. (2007) reported AQP9 expression in many tissues, using different immunohistochemical and molecular approaches. In the liver, AQP9 is expressed in hepatocytes within the sinusoidal surfaces of hepatocyte plates, where, during starvation, it is speculated to function in glycerol uptake from the bloodstream for gluconeogenesis (Rojek *et al*., 2007; Kuriyama *et al*., 2002; Sergio *et al*., 2005). In addition, AQP9 has been proposed to be involved in urea elimination from hepatocytes (Ishibashi *et al*., 1998). AQP9 expression has been reported in other tissues including the male reproductive tract, where it localizes to the efferent ductule epithelium, epididymis and vas deferens and may be involved in sperm maturation, concentration and storage, respectively (Pastor-Soler *et al*., 2002). In addition, it has been reported in the plasma membranes of Leydig cells in rat testis, white pulp of spleen, brain (Elkjaer *et al*., 2000; Warth *et al*., 2007) and in the peripheral leukocytes (Ishibashi *et al*., 2002). It is important to emphasize that, despite an abundance of data from different studies; there are still major discrepancies between the reported expression sites. In speculation, these differences may be due to either heterogeneity of expression in different species, existence of different splice variants, or may represent artifacts related to specificity of the anti-AQP9 antibodies used in IHC, IF and immunoblotting methods.

The purposes of the present studies were: To generate AQP9 gene knockout mice with genotyping; to identify the exact tissue localization of AQP9 in mice, males or females, wild-type and/or knockout- type, to study some biochemical analyses in wild and knockout mice and to study the liver structure of wild and knockout mice by electron microscopy.

MATERIALS AND METHODS

Generation of AQP9 knockout mice: The generation was accomplished by completely removal of whole exon5 from AQP9 genomic DNA and substituted for nucleotides sequence encoding a neomycin phosphotransferase expression cassette (Fig. 1). This was done in the Structural Pathology Department, Institute of Nephrology, Niigata University, Japan. For AQP9 genotyping, the mice were tested by PCR using genomic tail DNA and 2primers.

Experimental animals: A total of 10 wild type and 10 homozygous AQP9 KO-mice (C57 BL /6J) at 2-3 months of age were utilized. Both wild-type ($AQP9^{+/+}$) and AQP9 KO-mice ($AQP9^{-/-}$) were randomly assigned to 4 subgroups based on sex type. All mice were housed at controlled temperature, had free access to food and water and were maintained on 12 h light-dark cycles. Experiments involving these mice were approved by the Animal Care Committees of Niigata School of Medical and Dental Sciences.

Antibodies: An affinity-purified rabbit polyclonal antibody against an AQP9 C-terminal peptide from rat AQP9 (DAKO, Carpentaria, CA, USA) was used as a primary antibody. The antibody was diluted 1:100 with sterile Phosphate Buffer Saline (PBS) containing 0.05% sodium azide (NaN_3) before use. A Horseradish-Peroxidase (HRP) conjugated to goat anti-rabbit immunoglobulins (EnVision, DAKO, Kyoto, Japan) was used as a secondary antibody in dilution 1:5 with sterile PBS containing 0.02% NaN_3 for Immunohistochemistry (IHC). For Immunofluorescence (IF), Fluorescein Isothiocyanate (FITC)-labeled goat anti-rabbit polyclonal IgG from IBL (Immuno-Biological Laboratories, Takasaki, Gunma, Japan) in dilution 1:100 with sterile PBS containing 0.05% NaN_3 was used as a secondary antibody.

Primers: The primer sets were obtained from Nihon Gene Res. Lab., Miyagi, Sendai city, Japan.

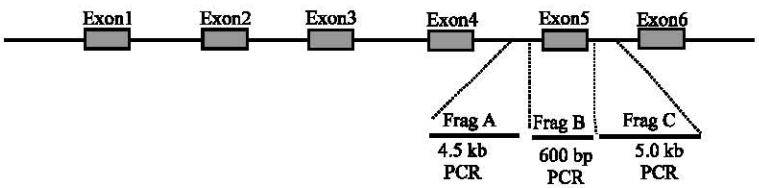

Fig. 1: Genomic structure of mouse AQP9 DNA and knickout construct. The exon5 was completely removed and subsituated by mcleotides sequence encoding a neomycin phosphotransferase expression cassette

- For AQP9 genotyping, 2primers were used: 5'-CAAATCCCAGCAACCATCTG -3' and 5'-GTAGCACATGCTTGCAATGC -3. The expected PCR product for homozygous AQP9 KO- mice is 350 bp, for wild-type mice is 605 bp and both for heterozygous mice (AQP9 $^{+/-}$).
- To amplify specifically AQP9 transcript, 2primers were used: A sense primer: 5'-CTCATCACGGGA GAAAATGG-3' and an antisense primer: 5'-GCTGGTTCTGCCTTCACTTC-3' (the expectedPCR product is 437bp).
- For a positive control, glyceraldehydes 3-phosphate dehydrogenase (GAPDH) was used as a house-keeping gene. The sense primer: 5'-CAAGATGGTGAAGGTCGGTG-3' and an antisense primer: 5'-GAAGATGGTGATGGGTTTCC-3'(PCR product is 239 bp).

Serum analysis: Blood was drawn from the supra-orbital venous plexus of AQP9$^{+/+}$and AQP9$^{-/-}$ mice under light ether anesthesia; left for clotting and clear sera were separated after centrifugation at 2000×g for 20 min at 4°C. Blood chemistry was determined by a clinical chemistry laboratory (Egypt).

Immunohistochemistry (IHC): Immunoperoxidase staining was carried out according to the procedure of Koyama *et al*. (1999). The tissues (liver, kidney, spleen, pancreas, stomach, intestine, heart, lung, skin, brain, testis, epididymis, uterus and ovary) from wild type and AQP9$^{-/-}$ mice were fixed with methyl-Carnoy's fixative (60% methanol, 30% chloroform, 10% acetic acid), embedded in paraffin and sectioned at 4 μm thickness. Sections were incubated overnight at 4°C with primary rabbit anti-rat AQP9 antibody (2 μg mL^{-1}). After being washed (PBS for 3×5 min), slides were incubated for 1 h at room temperature with secondary antibody, HRP-coupled goat anti-rabbit immunoglobulins (EnVision) and washed again as described above. The peroxidase reaction was developed with 0.05% 3, 3'-diaminobenzidine tetrahydrochloride (DAB) and counterstained with Mayer's hematoxylin for 3 min. The immunostained sections were dehydrated in ethanol series and mounted with mounting medium. Then the immunocytochemical signal was examined with an Eclipse E800 light microscope (Nikon, Niigata, Japan). For the control, sections were incubated without primary antibody and showed no staining.

Immunofluorescence (IF): Liver tissues were removed from mice and frozen at -80°C in *n*-hexane for 30 min and kept at -80°C until sectioning. They were embedded in Tissue-Tek Optimal Cutting Temperature (OCT) compound (Tissue-Tek, Miles Inc., Torrance, CA) and mounted on a cutting block. After freezing in a Reichert Frigocut microtome, the tissue was cut at 3-4 μm thickness and sections were picked up on Superfrost Plus microscope slides (Matsunami Glass Ind., LTD., Japan)). For immunostaining, sections were incubated overnight at 4°C in rabbit anti- rat AQP9 primary antibody. Slides were washed twice for 5 min in high-salt PBS (2.7% [w v^{-1}] NaCl) to reduce nonspecific staining (Elkjaer *et al*., 2000; Maria *et al*., 2005) and once in normal PBS. They were then incubated for 1 h at room temperature with secondary antibody, a goat anti-rabbit IgG coupled to Fluorescein Isothiocyanate (FITC). After being washed (PBS for 3×5 min), sections were coverslipped, mounted in Vectashield (Vector Labs, Burlingame, CA) and examined under a Zeiss LSM 510 confocal fluorescence microscope.

RNA isolation: Total cellular RNA was also isolated from the same previous tissues used for IHC by a modified acid guanidinium thiocyanate phenol-chloroform extraction method (Trizol, GIBCO BRL, Life Technologies, Rockville, MD). Total RNA concentration was quantified by spectrophotometry and its quality (purity) was checked by electrophoresis in agarose gels (Chomezynski and Sacchi, 1987).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR): For detection of AQP9 mRNA, Reverse transcription was performed using total RNA (5 μg); previously treated with DNase I buffer and DNase I amplification grade for 15 min at 2524°C; and SuperScript II reverse transcriptase enzymes. Reverse transcription was achieved by heating the reaction mixture for 1h at 42°C and then for 5 min at 95°C and chill on ice for 5 min to stop the reaction. PCR experiment was performed using 1 μL of the cDNA product (template) plus 4 μL of PCR mix containing 10 Õ PCR buffer (0.5 μL), 2.5 mM dNTPs (0.4 μL), Milli Q. (1 μL), Taq polymerase (0.1 μL) and specific primers (1pmol μL^{-1}), forward (1 μL) and reverse (1 μL). The PCR amplification cycle using PCR thermal cycler machine (TakaRa Co., Otsu, Shiga, Japan) was 35 PCR cycles, one PCR cycle consisted of initial denaturing at 95°C 5 min, denaturing at 95°C 1 min, annealing at 62°C 1 min and extension reaction at 72°C 1 min. After 35 cycles, final products were extended for 5 min at 72°C. AQP9 amplified cDNA fragments were resolved in 1.5% agarose gel containing ethidium bromide and their electrophoretic migration was compared against a 1 kp plus DNA ladder (Marker) and the products were photographed under ultraviolet illumination.

Electron microscopy (EM): For transmission EM, the liver of wild type and knockout mice were removed and fixed in 2.5% glutaraldehyde at 4°C overnight. They were then osmicated and embedded in Epon 812. Seventy nm thick sections were cut on a Reichert-E ultramicrotome and collected on formvar-coated EM grids. The ultrathin sections were stained in 5% uranyl acetate for 3 min, then in lead citrate for 1min, dried and finally examined with the electron microscope (Philips CM 400).

Statistical analysis: Data are given as mean values ±Standard Errors (SE). Statistical comparisons were accomplished by unpaired *t* test (equal variances). $p<0.05$ were considered statistically significant.

RESULTS

AQP9 knockout mice were generated as shown in Fig. 1. Under physiological conditions, the knockout mice have normal embryonic survival, fertility, appearance, behavior and plasma parameters, except for a significant change in the level of some biochemical analyses.

Expression of AQP9 protein in mouse organs: Immunohistochemistry staining was used to localize AQP9 in different tissues of mice, males, females, wild and/or KO-type. In wild mice, using rabbit anti-rat AQP9 antibodies, only liver, epididymis and kidney (not previously identified expression site) showed strong immunostaining, whereas there was a complete absence of staining in AQP9$^{-/-}$ mice. At high concentration, the AQP9 antibody also stained different structures in spleen and pancreas (not shown).

Immunocytochemical localization of AQP9 in mice liver: Liver AQP9 expression was analyzed to determine cell distribution and membrane localization and whether AQP9 expression/distribution was dependent on the sex and the

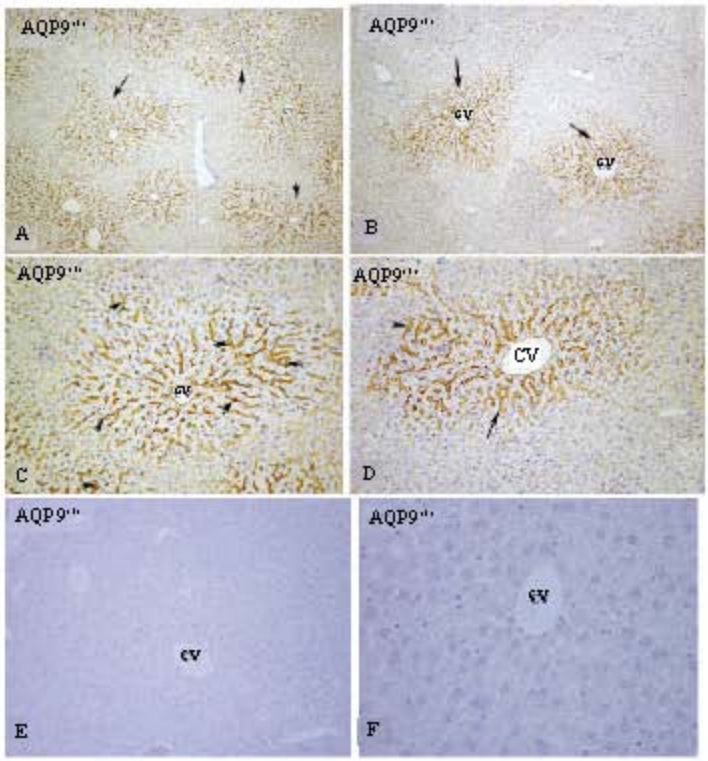

Fig. 2: Immunohistochemistry for AQP9 in liver of AQP9$^{+/+}$ and AQP9$^{-/-}$ mice stained with rabbit polyclonal antibody at dilution 1:100. In male's liver, immunoreactivity of AQP9 (brown staining) was more homogenous and widely distributed in hepatocytes (A) than female's liver in which staining was mostly confined to perivascular hepatocytes (B). Higher magnification showed staining of the sinusoidal plates (arrow heads) surrounding the central vein (CV) in the AQP9$^{+/+}$ male (C) and female mice (D). The label appears to be more uniformly distributed in all the hepatocytes of male mice. However, AQP9$^{-/-}$ mice showed completely absent of AQP9 labeling in the liver (E and F). Original magnifications: A, B, E ×100; C, D×200; F×400

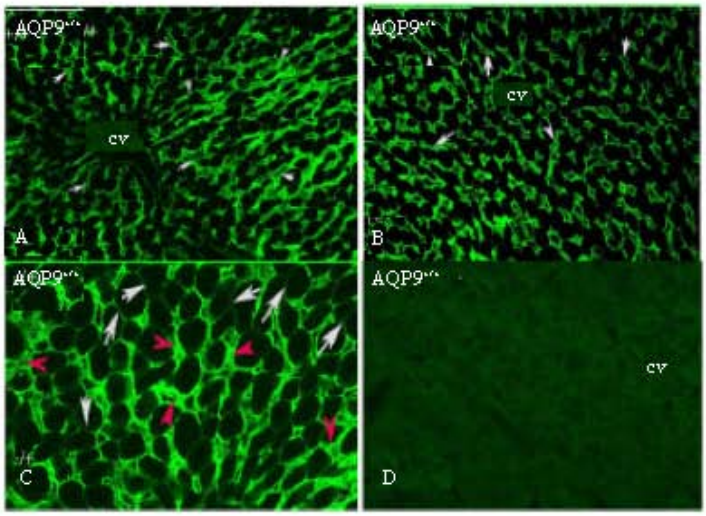

Fig. 3: Immunofluorescence localization of AQP9 in mice liver using anti-AQP9 antibodies. Frozen sections from liver of male (A) and female (B) wild- type mice ($AQP9^{+/+}$) showing hepatocytes close to the central vein (CV) intensely labeled. The label appears to be more uniformly distributed in all the hepatocytes in male mice. (C) High magnification showing the basolateral membrane expression of AQP9 (red arrows). A white arrow indicates the apical membrane of two hepatocytes. (D) $AQP9^{-/-}$ mice showed completely absence of labeling. Original magnifications: A, B, D ×200; C×400

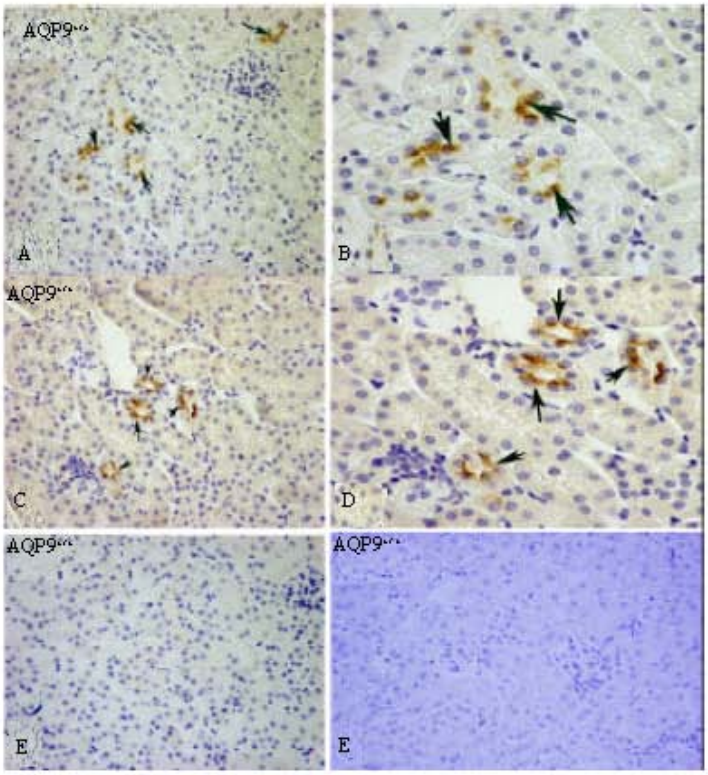

Fig. 4: Immunocytochemistry localization of AQP9 in kidney using anti-AQP9 antibodies. $AQP9^{+/+}$ mice showing anti-AQP9 labeling in the distal (A, B) and collecting (C, D) tubules. The labeling is mainly intracellular (arrows). E and F: immunolabeling $AQP9^{-/-}$ mice showing absence of AQP9 staining in all kidney tubules. Original magnifications: A-D ×200

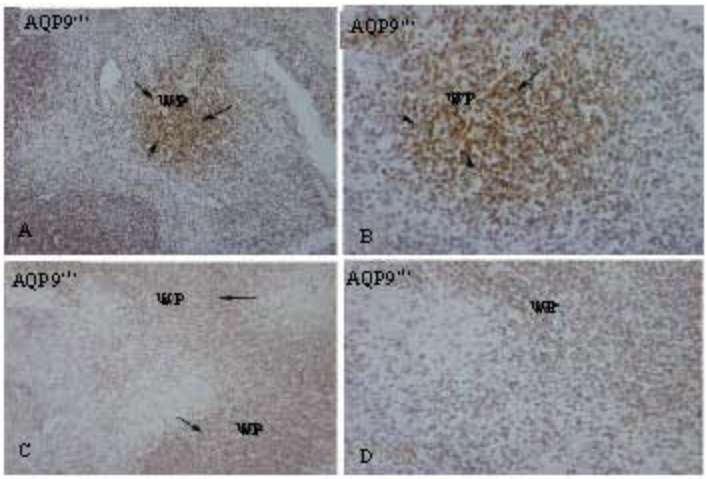

Fig. 5: Immunocytochemistry localization of AQP9 in spleen using anti-AQP9 antibodies. A, B: AQP9$^{+/+}$ mice showing anti-AQP9 labeling in the white pulp (WP). The labeling is mainly in the leukocytes (arrows). C, D: AQP9$^{-/-}$ mice showing complete absence of AQP9 immunoreactivity. Original magnifications: A-D ×200

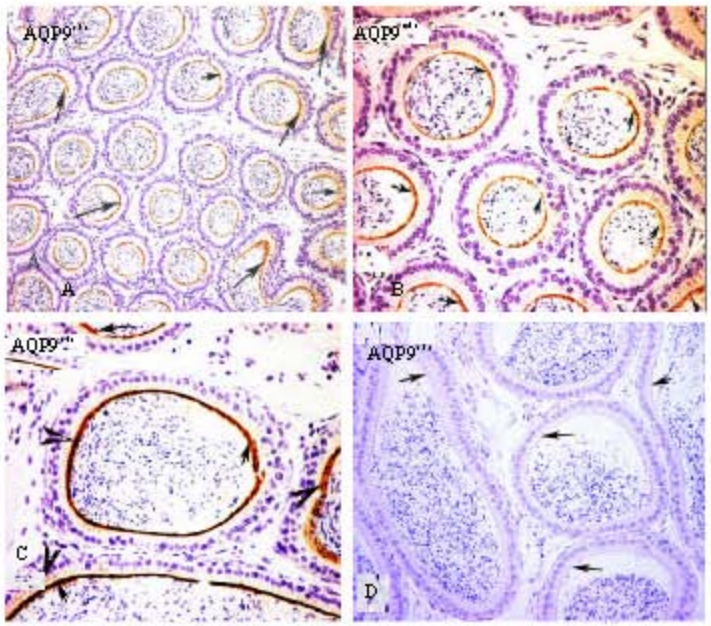

Fig. 6: Immunohistochemistry localization of AQP9 staining in the epididymis. In AQP9$^{+/+}$ mice (A- C), AQP9 staining is concentrated at the apical pole (microvilli) of principal cells of initial segment (A, B). In cauda epididymidis (C), intense reaction is seen over the microvilli (thin arrows) of principal cells and cytoplasm of clear cells (thick arrows). Sperms in the lumen are unreactive. The staining is completely absent in the AQP9$^{-/-}$ mouse (D). Original magnifications: A, ×200; B, C, D ×400

knockout effect. AQP9$^{+/+}$ male mice showed a more homogeneous hepatocyte staining by AQP9 (Fig. 2 a and c), whereas in female's liver, the expression of AQP9 was mostly confined to perivascular hepatocytes (Fig. 2 b and d). Staining was restricted to the sinusoidal surfaces of hepatocyte plates of the liver. In some cases, there was little intracellular labeling. In AQP9$^{-/-}$ mice, there was no staining of liver (Fig. 2e and f).

Immunofluorescence: Immunofluorescence staining of liver was used to confirm the subcellular localization and distribution of AQP9 in hepatocytes. Affinity-purified antibodies were used to stain cryostat liver sections from mice (Fig. 3). AQP9 was found only expressed in the hepatocytes of control mice (AQP9$^{+/+}$). In male mice the expression of AQP9 was found to be more homogeneous and involved all the hepatocytes (Fig. 3a). However, in

female mice, the expression was strongest in those cells closest to the central vein (Fig. 3b). At higher magnification, the staining was localized in the basolateral membrane of the cells in both male and female mice, whereas the apical bile canalicular membrane was not labeled (Fig. 3c). The staining is completely absent in the AQP9$^{-/-}$ mouse (Fig. 3d).

Immunocytochemical localization of AQP9 in mice kidney: Immunoperoxidase labeling of kidney sections showing anti-AQP9 labeling in the distal and collecting tubules of AQP9 $^{+/+}$ mice of both sexes. In addition, very weak labeling was observed in the proximal tubules, whereas other nephron segments and glomeruli did not exhibit labeling in excess of background. In the distal tubule, the labeling was confined to cytoplasmic domains (in apical, central and basal parts of the cells) (Fig. 4a and b) and absence of, or very weak, labeling of basolateral plasma membrane domains. Similarly, in collecting duct cells the strong labeling was associated with intracellular structures (arrow in Fig. 4b, c). The AQP9 $^{-/-}$ mice of both sexes showed completely absence of AQP9 staining in the kidney tissues (Fig. 4e and f).

Immunohistochemical localization of AQP9 in spleen: The AQP9 $^{+/+}$ mice, males and females showed AQP9 immunolabeling in the cells of white pulp (Fig. 5a and b), whereas there was no labeling of cells in the red pulp.

Immunohistochemical localization of AQP9 in reproductive organs: The testis, seminiferous epithelium, uterus and ovary of AQP$^{+/+}$ mice showed no AQP9 expression, whereas there was only expression of anti-AQP9 labeling in the epididymis of male mice. In epididymis, AQP9 was uniformly distributed over the microvilli of the principal cells of all regions, with the most intense reaction being noted in the initial segment and cauda regions. Intense labeling of the apical stereocilia was observed in principal cells of the initial (Fig. 6a and b) and distal (Fig. 6c) segments of the epididymis. This staining was completely absent in AQP9$^{-/-}$ mice (Fig. 6d).

Electron microscopy: Transmission electron microscopy showed no apparent differences in the structures of liver in wild-type (Fig. 7a) and AQP9 null (Fig. 7b) mice. However, the bile canaliculi in KO-mice were shorter than the wild-type.

RT-PCR analysis of the expression of AQP9 in mouse organs: For AQP9 genotyping, the mice were tested for the presence of the disrupted AQP9 allele by PCR using genomic tail DNA and 2 primers. The 2 Primers set produced the correct wild-type 605 bp PCR product in the

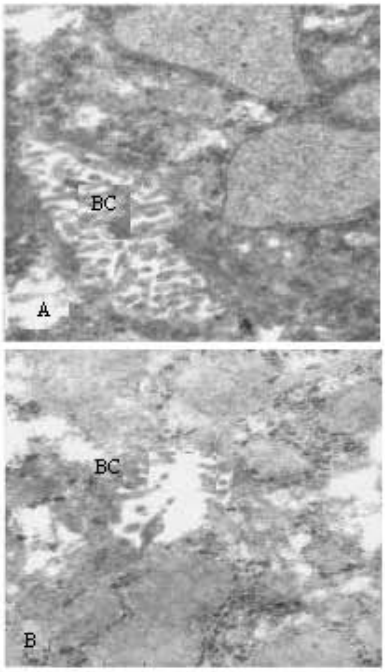

Fig. 7: Electron microscopy examination of liver in AQP$^{+/+}$ and AQP$^{-/-}$ mice. AQP$^{-/-}$ mice (B) showed short and small bile canaliculi (B C) comparatively with the AQP$^{+/+}$ control mice (A)

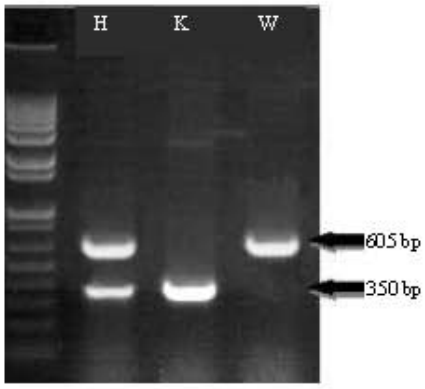

Fig. 8: Showed AQP9 genotyping. A 2Primers set were produced the correct PCR product at 605-bp for the wild-type (W) and at 350-bp for the knockout -type mice (K), while heterozygous -type mice have both PCR product size positions (H)

AQP9$^{+/+}$, while in the AQP9$^{-/-}$ mice was 350-bp-PCR product. Heterogeneous- type mice (AQP9$^{+/-}$) have both PCR product sizes (Fig. 8). We examined the expression of AQP9 in a range of mice organs using mRNA encoding AQP9 of these tissues and AQP9-specific primer set. A house-keeping gene, GAPDH, was used as a positive

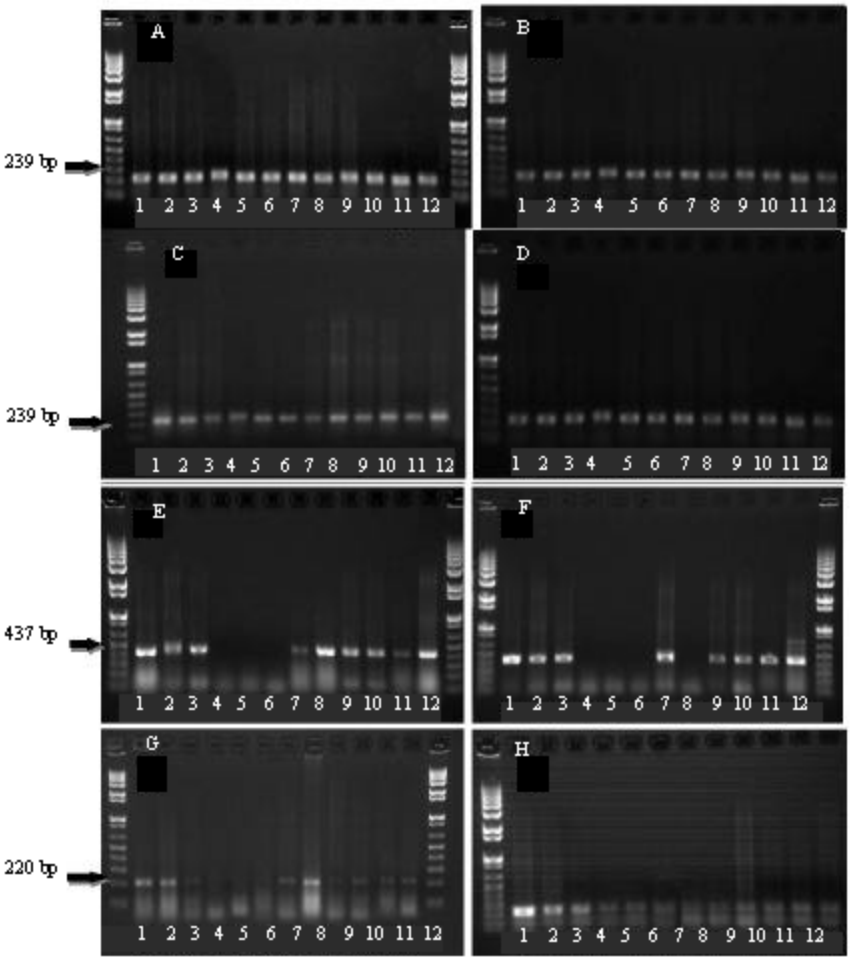

Fig. 9: AQP9 transcript analysis by RT-PCR of RNA isolated from different organs of mice. A house keeping gene, GAPDH, was used as a control positive with PCR product at 239-bp for male (A) and female (B) wild-type mice and for KO- mice respectively (C and D). Primer pair "sense" and "antisense" produced the correct wild-type 437-bp-long product in the AQP9$^{+/+}$ for male (E) and female (F) mice which is absent in the AQP9$^{-/-}$ mice. However, in AQP9$^{-/-}$ male (G) and female (H) mice, these primers amplified 220-bp-long PCR product, because whole exon5 containing the neo expression cassette is lost. Lane1- liver, 2- kidney, 3- spleen, 4- pancreas, 5- stomach, 6- intestine, 7- testis or uterus, 8- epididymis or ovary, 9- heart, 10-lung, 11- skin, 12-brain

Table 1: Serum values for some biochemical analyses in AQP9$^{+/+}$ and AQP9$^{-/-}$ mice

		Wild- type mice (AQP9$^{+/+}$)		KO- type mice (AQP9$^{-/-}$)	
Parameter		Male (N = 5)	Female (N = 5)	Male (N = 5)	Female (N = 5)
Glycerol, μmol L^{-1}		322±18.95	319.55±19	479.88±22.5*	481±24.14*
Triglycerides, mg 100 mL		75±4.0	80.55±6.13	98.77±7.4*	110±6.95*
Total cholesterol, mg 100 mL^{-1}		180±8.10	174.18±9	199±11.54	187.55±9.75
Glucose mg 100 mL^{-1}	Random	120±4.88	118±5.11	117.55±4.16	118.22±3.75
	9hs afterfasting	115±4.13	113±4.0	80±3.5*	85±4.05*
Urea, mg 100 mL^{-1}		76.42±4.19	73±4.5	77±3	75.22±3.55
ALT, units L^{-1}		85.42±5.22	84±4.05	87.66±4.14	85.53±6.17
ALP, units L^{-1}		144.33±8.11	145±10	142±9.34	141±8.5

Values are means ± SE; N, number of mice; *p<0.05, AQP$^{-/-}$ compared with the control (AQP$^{+/+}$)

control (Fig. 9a-d). In AQP9$^{+/+}$ mice of both sexes, all organs showed AQP9 expression by RT-PCR, except pancreas, stomach, intestine and ovary (Fig. 9). The already reported tissues containing AQP9 in wild-type

mice were found as amplified bands at the expected 437-bp position. AQP9$^{-/-}$ mice showed small PCR product size at 220 bp (Fig.9 g, H).

Biochemical analysis: To examine whether AQP9$^{-/-}$ mice exhibit defects in the metabolism of various solutes, the concentration of glycerol, triglycerides, total cholesterol, glucose, urea, alanine aminotransferase (ALT) and Alkaline Phosphatase (ALP) in serum samples from AQP9$^{+/+}$ and AQP9 $^{-/-}$mice were determined (Table 1). AQP9$^{-/-}$ mice exhibited a marked increase in serum glycerol and triglyceride levels with a decrease in serum glucose (only after fasting) compared with AQP9$^{-/-}$ mice. There were no significant differences in the other measured parameters. Moreover, there were no statistical changes between male and female, wild and/or KO-type mice.

DISCUSSION

The goal of this study was to identify the main expression sites of AQP9 in wild and AQP9 knockout mice of both sexes and to determine whether knockout for AQP9 plays a role or affect the biochemical analyses in mice that thus far have remained undefined.

Our data determined that AQP9 is highly expressed in liver, which represents its main expression site, then epididymis, kidney and spleen. The major finding reported here relate to the expression and subcellular localization of AQP9. Our results demonstrated that AQP9 expression is confined to the mice hepatocyte basolateral membrane, the plasma membrane facing the sinusoids. This pattern of staining may be consistent with previous observations of Caperna *et al.* (2007), Carbrey *et al.* (2003), Rojek *et al.* (2007), Kuriyama *et al.* (2002), Elkjaer *et al.* (2000) and Neil *et al.* (2003). A new and interesting finding of the present study is the different expression observed between male and female AQP9$^{+/+}$ mice. In liver from female mice the Immunohistochemistry (IHC) signal was progressively more intense in hepatocytes close to the central vein (perivenous hepatocytes), whereas in the liver from male mice the label appears to be more uniformly distributed in all the hepatocytes. This indicates that differences in the liver may be gender-dependent (Pastor-Soler *et al.*, 2002). Moreover, our immunofluorescence signal confirmed the basolateral localization of AQP9 with IHC results and this agree with Huebert *et al.* (2002). It is well established that many hepatic functions are expressed in a sexually dimorphic fashion (Grazia *et al.*, 2001). A number of hepatic enzymes, plasma membrane receptors and transcription factors have been shown to be sexually dimorphic (Simon *et al.*, 1996). On contrary, AQP9$^{-/-}$ mice did not show any immunostaining.

Regarding IHC results for the kidney, a recently identified member of the AQP family, AQP9, was expressed specifically in the distal and collecting tubules (not previously identified expression site). This indicates a key role of AQP9 function in kidney as a water channel protein. Moreover, renal principal cells in some parts of the collecting duct have high levels of both AQP3 and AQP4 on their basolateral plasma membranes (Ishibash *et al.*, 1997). In this case also, one of the expressed channels (AQP4) is conductive only to water, while the others (AQP3, AQP9) are a promiscuous water channel with a high urea and glycerol permeability.

At high concentration, the AQP9 antibody also stained different structures in spleen and pancreas but these results may be attributed to non specific binding of the antibody especially for pancreas which showed no band corresponds to a specific AQP9 fragment with RT-PCR. Our results were in coincidence with Ishibashi *et al.* (1998), Elkjaer *et al.* (2000) and partly agree for pancreas with Rojek *et al.* (2007).

Concerning IHC for male reproductive tract, AQP9 is abundantly expressed in different segments of AQP9$^{+/+}$ mice, where it could represent an apical pathway for transepithelial water flow. In epididymis, AQP9 was localized on the microvilli of the principal cells of all regions (Huang *et al.*, 2006; Pastor-Soler *et al.*, 2002; Elkjaer *et al.*, 2000; Ruz *et al.*, 2006). However, in the present study, AQP9 expression on the microvilli of the principal cells was noted to be region-specific, with the most intense reaction being noted in the initial segment and cauda regions. Our results agree with Badran and Hermo (2002). Therefore, in the proximal portion, AQP9 is the only AQP so far detected on principal cells, whereas in the distal portion it is coexpressed with AQP2 on principal cell apical membranes (Pastor-Soler *et al.*, 2001). The testis did not show any AQP9 labeling, although other authors observed it. The difference may be due to usage single anti-AQP9 antibody in this study and/or probably because its expression level from AQP9 is very low. These results are consistent with localization of AQP9 in testis by weak intensity than in epididymis which revealed strong PCR product band as shown by RT-PCR. Our data now show that AQP9 is an abundant apical membrane protein in the mice epididymis and other regions of the reproductive tract, it appears to be a constitutive apical membrane protein that may be responsible for apical membrane water and/or solute permeability of these epithelia. Nihei *et al.* (2001) have suggested that AQP9 in Leydig cells of the testes participates in the rapid cellular uptake and release of various small size lipid or cholesterol metabolites through their apical membrane. In female wild-type mice, AQP9,

which had not been identified by IHC analysis probably because its expression level is low, was found to be present in the uterus as shown by RT-PCR.

Using RT-PCR, we showed that most tissues of wild-type mice expressed AQP9-PCR product at 437 bp position. However, a smaller 220-bp product was amplified from cDNA from KO-mice because the whole exon5 containing the neoexpression cassette is lost. In KO-mice DNA sequencing showed that the smaller product is an alternatively spliced transcript, where exon4 is spliced directly to exon6. Our data agree with Caperna *et al.* (2007) and Warth *et al.* (2007).

With regarding to the biochemical study, serum levels of glycerol and triglycerides were markedly increased, in association with hypoglycemia in AQP9$^{-/-}$ mice, revealing a role of AQP9 in glycerol metabolism. Glycerol, a product of adipose tissue lipolysis, is an important substrate for hepatic gluconeogenesis. As the substrate for hepatic glucose production, glycerol accounts for 90% in the prolonged fasting state and 50% in the postabsorptive state in rodents (Kuriyama *et al.*, 2002). The abundant expression in the liver of normal mice strongly suggests that the increased serum glycerol levels in AQP9$^{-/-}$ mice are caused by an absence of hepatic AQP9 and an impaired uptake of glycerol through the hepatocyte plasma membrane. Although the increased serum glycerol level in AQP9 null mice is likely to be mediated by absence of AQP9, it cannot be excluded that the uptake and release of glycerol by other organs expressing aquaglyceroporins, e.g., kidney cortex (AQP3 and AQP7), fat tissue (AQP7), or intestine (AQP3 and AQP10), may also influence the final plasma glycerol level (Rojek *et al.*, 2007). Interestingly, according to the model of metabolic zonation of the liver, gluconeogenesis predominantly takes place in the hepatocytes surrounding the portal vein (periportal) that express little AQP9, whereas relatively less gluconeogenesis takes place in the hepatocytes surrounding the central vein (perivenous), where highest AQP9 levels are detected (Junggerman and Kietamann, 1997). Thus, it cannot be excluded that AQP9 may have additional functions in the mouse liver other than glycerol transport. The hypoglycemia in our results suggesting that the absence of AQP9 in the hepatocyte membrane reduced the capacity for glycerol entrance for gluconeogenesis, leading to reduced serum glucose levels specially during fasting of AQP-null mice (Carbrey *et al.*, 2003; Rojek *et al.*, 2007; Kuriyama *et al.*, 2002). Therefore, our studies of AQP9 protein strongly support the role for AQP9 expression in liver as a molecular mechanism for maximizing glycerol influx during states requiring increased gluconeogenesis.

CONCLUSION

Our data showed that AQP9 is a major water channel protein that is expressed throughout different organs, with high expression in the liver of male mice. This AQP could represent an important apical pathway for transmembrane water movement and it could also serve as a conduit for other solutes. We have also demonstrated mRNA expression of AQP9 in most tissues of mice by RT-PCR analysis, although they were not detectable by immunohistochemistry. Although an important role of AQP9 in glycerol metabolism has been demonstrated by numerous studies, the underlying mechanisms have remained elusive. Based on our study, it can be concluded that AQP9 is important for hepatic glycerol metabolism and, via this and other mechanisms, importantly influences hepatic glucose production.

ACKNOWLEDGMENT

The author thanks Tadashi Yamamoto and Hidehiko Fujinaka for providing AQP9$^{-/-}$ mice and antibodies. We are grateful to Eishin Yaoita and Tasaki for their assistance.

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