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Expression of the Stem Cell Marker ABCB5 in Normal and Tumor Tissues

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Abstract. *Background/Aim: The ATP-binding cassette subfamily B member 5 (ABCB5) transporter plays a pivotal role in melanocyte progenitor cell fusion and has been identified as a tumor-initiating cell marker. In this study, we determined ABCB5 expression in normal tissues among various species, i.e., Homo sapiens, Mus musculus (mouse), Rattus norvegicus (rat), Sus scrofa domesticus (pig), Gallus gallus (chicken), Anser anser (goose), Poecilia reticulata (Guppy fish), and Lumbricus terrestris (earthworm), as well as 426 biopsies of different human tumor types (colorectal, cervical, endometrium, vaginal, nasopharyngeal, kidney, breast, colon, prostate, pancreas, lung, gallbladder, bladder, brain, liver, skin, small intestine, testis, tonsil, uterus, thyroid, stomach, esophagus, fallopian, parotid, and ovary). Materials and Methods: Using immunohistochemical staining, ABCB5 expression was detected and evaluated in formalin-fixed, paraffin-embedded sections. Results: High ABCB5 expression was found in normal tissues in specialized cells with secretory and excretory functions, chorionic villi of the placenta, hepatocytes, and blood-tissue barrier sites in the brain and testis. Besides, heterogeneous expression of ABCB5 was also observed in many different tumor types derived from breast, endometrium, ovary, uterus, cervix, prostate, lung, brain, colon, liver, nasopharynx, and others. Conclusion: The localization of ABCB5 in different normal tissues suggests that this protein has an excretory pumping role for physiological metabolites and xenobiotics. This physiological role highlighted its possible impact on the development of multidrug resistance in tumors. Further studies are required to establish the possible clinical significance of ABCB5 as a predictive marker for drug resistance and as a prognostic marker for patient survival.*

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Detoxification of harmful substances belongs to the basic mechanisms of every organism to maintain essential life functions. ATP-binding cassette (ABC) transporters are a family of 49 transporters in the human genome with diverse transport functions of endogenous toxic and non-toxic substrates (*e.g.*, cholesterol, organic anions, phosphatidylcholine, steroids, bile salts, and others) as well as of exogenous xenobiotic compounds (1, 2). The ABC transporter genes are evolutionary highly conserved and can be found in pro- and eukaryotes (3, 4). Several of these transporters are involved in the transport of xenobiotic compounds in normal tissues (5, 6). ABC transporters also contribute to the failure of cancer chemotherapy by a phenomenon termed multidrug resistance (MDR). Over-expression of ABC-transporters enhances the efflux of anticancer drugs out of cancer cells resulting in sublethal intracellular concentrations of chemotherapeutic agents and subsequently therapy failure. MDR is characterized by the simultaneous cross-resistance among a wide range of functionally and structurally unrelated chemotherapeutic drugs (7). In addition to the well-known P-glycoprotein, other ABC-transporters such as MRP1 and BCRP also reveal typical cross-resistance profiles to anticancer drugs (8, 9). ABC transporters also contribute to the detoxification of carcinogenic compounds from the body and thereby prevent carcinogenesis (5, 10-12). In addition to the human body, environmental toxins are extruded from vertebrate and invertebrate aquatic organisms by ABC transporters (13-16). In analogy to MDR, ABC transporter-mediated detoxification in aquatic mechanisms has been termed multi-xenobiotic resistance (MXR) (5, 17-19). As ABC transporters extrude

harmful xenobiotics throughout the animal kingdom, it can be assumed that MXR is of tremendous importance not only in aquatic organisms but in many other – if not all – animals. Although MDR has been first described, the general biological relevance of MXR may exceed the one of MDR by far. Apart from P-glycoprotein (ABCB1/MDR1), MRP1 (ABCC1), and BCRP (ABCG2), ABCB5 represents another still incompletely understood member of human ABC transporters that also mediates MDR of tumors (20). The human *ABCB5* gene is located on chromosome 7p15, consists of 16 exons, spans over 108 kb, and encodes a protein of 812 amino acids (92 kDa). ABCB5 was firstly detected in tissues derived from the neuroectodermal lineage including melanocyte progenitors. It controls progenitor cell fusion by altering the membrane potential in normal melanocytes that express the stem cell marker CD133 (21). ABCB5 is also expressed in some other tissue types (22, 23) and tumor types originated from these tissues (24, 25). ABCB5-expressing melanoma cells possess the ability for self-renewal and differentiation (26, 27). In clinical settings, melanoma specimens that over-expressed ABCB5 were directly correlated with tumor progression suggesting that ABCB5 expression is linked with tumor aggressiveness. Furthermore, the growth of melanoma xenografts in mice was retarded upon treatment with a monoclonal anti-ABCB5 antibody (26). ABCB5 is functionally related to P-glycoprotein. It acts as an energy-dependent drug efflux transporter for the fluorescent probe rhodamine-123 (21). Moreover, high ABCB5 expression in melanoma cells mediated resistance to doxorubicin, camptothecin, 5-FU, and mitoxantrone. Silencing of *ABCB5* using siRNA re-sensitized melanoma cells (28). ABCB5 expression in oral squamous cell carcinoma (OSCC) was associated with tumor formation, metastasis and considered as putative compartment of cancer stem cells (29). In melanoma WM266-4 xenograft tumors, ABCB5-expressing cells survived after temozolomide treatment, although tumor regression was achieved. Moreover, treatment of metastatic melanoma cells *in vitro* with dacarbazine and vemurafenib showed an increased survival of ABCB5-expressing cell populations at doses that were cytotoxic for ABCB5-negative cells (30).

Given a general role of ABCB5 for detoxification of harmful xenobiotic compounds in normal tissues and of anticancer drugs in tumors, we were interested in exploring the expression of ABCB5 in normal tissues and a panel of different tumor types. In this context, we evaluated the expression of ABCB5 not only in normal human tissues but also among various other species, *i.e., Mus musculus* (mouse), *Rattus norvegicus* (rat), *Sus scrofa domesticus* (pig), *Gallus gallus* (chicken), *Anser anser* (goose), *Lumbricus terrestris* (earthworm), and *Poecilia reticulata* (Guppy fish) and 426 biopsies of tumors derived from different organs (kidney, breast, colon, prostate, pancreas, lung, throat, bladder, brain, liver, tonsil, uterus, thyroid, stomach, esophagus, thymus, cervix, nasopharyngeal carcinoma (NPC), and ovary) using immunohistochemistry.

Materials and Methods

Normal and tumor tissues specimens. Human tissues from normal organs from anonymized donors obtained as well as additional tissues from animals (mouse, rat, pig, goose, chicken, fish, earthworm) were obtained from the Department of Pathology, (University Hospital, Mainz, Germany). The tissues were chosen to represent the major organ systems. Routine hematoxylin-eosin staining was performed to verify morphology. Ethical approvals were obtained from the Ethics Committee of the State Authorization Association for Medical Issues (*Landesärztekammer*) Rheinland Pfalz to Prof. W. Roth (October 02, 2015; Ref. 837.031 9799) and to Prof. T. Efferth (March 22, 2018; Ref. 2018-13179). The human donors gave approval for evaluation of tissues and publication of data generated from these investigations prior to participation.

Immunohistochemistry staining. Commercially available ABCB5 monoclonal antibody clone 5H3C6 (Catalog no. MA5-17026, Thermo Fisher Scientific, Inc.) was applied on paraffin-embedded tissue slides by using a polymeric labeling technique. The mouse ABCB5 monoclonal antibody specifically detects an epitope covering the amino acids: 481-674 at the endoplasmic domain of the membrane. The specificity of this antibody for ABCB5 was recognized by western blotting according to the manufacturer's investigations and independent analyses by other authors (31-33). The slides were washed twice with xylene (98.5% xylene for 5 min each at room temperature) to remove paraffin. Then, sample tissues were rehydrated through graded washes with isopropanol in water. Heat-induced epitope retrieval was performed using a pressure cooker as heating device. Ultra-vision protein block and UltraVision Hydrogen Peroxide Block (catalog nos. TA-060-UB and TA-060-H2O2Q, respectively; Thermo Fisher Scientific, Inc.) were added to block endogenous proteins and endogenous peroxidase activity, respectively, to avoid non-specific background staining. Overnight incubation at 4˚C was performed following the addition of monoclonal primary antibodies. For the detection of ABCB5, 1:200 dilution was used as stated by the manufacturer's protocol. Subsequently, horseradish peroxidase-labeled polymers conjugated with secondary antibodies specific for mouse primary antibodies was added (catalog nos. TL-060-QPH and TL-060-QPB; Thermo Fisher Scientific, Inc.) at room temperature for 1 h, according to the manufacturer's protocol. The final staining reaction was performed with diaminobenzidine and slides were counterstained with hematoxylin for 3 min at room temperature.

Immunohistochemistry evaluation. The immuno-stained slides were scanned by Panoramic Desk (3DHISTECH, Budapest, Hungary) and evaluated by Panoramic Viewer software. The scoring system was based on the quantification of the positive immunolabeled cells over the total cells in each selected six independently annotated tumor areas (34). The staining intensities were represented in a box-plot graph. The box plot was used as a descriptive statistic to show the five-number set of data: including the minimum score, lower quartile, median, upper quartile, and maximum score. The distribution of intensity percentages reflects the heterogeneity of ABCB5 expression among different tumors, large difference

between minimum and higher scores indicates the heterogenic expression of the corresponding protein.

Epitope alignment and protein modelling. The multiple sequence alignment program, ClustalW2, was used to align protein sequences (35). The sequences were obtained form from UniProt (36). The epitope sequence that covers the amino acids at the endoplasmic domain of human ABCB5 was aligned with correspondent ABCB5 sequences of different species (mouse, rat, pig, chicken, and fish). Furthermore, the ABCB5 epitope was aligned to human myosin to examine whether the ABCB5 epitope has sequence similarity to myosin, elucidating the non-specific binding to myosin of the monoclonal anti-ABCB5 antibody 5H3C6. The structure of human P-glycoprotein (PDB ID: 7A65) was used as the template structure to build the ABCB5 models of different species. The Modeller 9.23 algorithm embedded in UCSF Chimera software was used as previously described (37).

SDS-PAGE and immunoblotting of normal human tissues. Total proteins were extracted from normal human tissues (colon, liver, and thyroid) using T-PER™ tissue protein extraction buffer (Thermo Fisher Scientific, Dreieich, Germany) mixed with 1% Halt™ protease inhibitor cocktail (Thermo Fisher Scientific). Briefly, 1 g tissue was homogenized in 20 ml T-PER reagent. Afterwards, the mixture was centrifuged at 10,000×g for 5 min to pellet tissue debris. The supernatant was collected to measure the protein concentration using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). Protein samples (20 ml equivalent to 30 μg per lane) were loaded to 10% SDS-PAGE to be separated and transferred to Ruti®191-PVDF membranes (Thermo Fisher Scientific). Then, 5% BSA (Carl Roth, Karlsruhe, Germany) was used to block the membranes for 1 h. Blots were incubated overnight at 4˚C with the following antibodies diluted in 5% BSA/TBS-Tween: ABCB5 monoclonal antibody clone 5H3C6 (catalog no. MA5-17026, Thermo Fisher Scientific, dilution 1:1,000), which detects an epitope within residues 481-674 and rabbit monoclonal antibody against β-actin (clone 13E5, Cell Signaling, Frankfurt, Germany, dilution 1:1,000). Afterwards, the membranes were incubated for 1 h at room temperature with the appropriate secondary antibody (IgG-HRP-linked) (Cell Signaling Technology, dilution 1:2,000). Finally, protein bands were visualized using Luminata™ Classico Western HRP substrate (Merck Millipore, Darmstadt, Germany). Images were captured by Alpha Innotech FluorChem Q system (Biozym, Oldendorf, Germany) and analyzed using ImageJ software.

Results

Expression of ABCB5 in normal human tissues. Assuming that ABCB5 exerts specific transport functions for a wide array of xenobiotic substances, we were interested in systematically investigating the localization of this ABC-transporter in normal organs of the human body and beyond. Therefore, we first analyzed ABCB5 expression in paraffin-embedded tissues of normal human organs by using immunohistochemistry followed by tissues derived from mammals (mouse, rat, pig), birds (goose, chicken), fish, and invertebrates (earthworm). The expression of ABCB5 was found in a wide variety of human tissues variated at specific morphological sites from intense to weak staining as shown in Table I. Representative immunohistochemical staining images of ABC expression in normal human tissues are shown in Figure 1.

Table I. *Immunohistochemical expression of ABCB5 in normal human tissues*.

Organ	Histology	ABCB5 Expression
Colon	Mucosa	+
	Submucosa	(+)
	Muscularis externa	+
	Mucosal Lieberkühn glands	+
	Stalk polyps	++
Duodenum	Mucosal villi	+
	Mucosal Lieberkühn glands	+
	Submucosal Brunner glands	(+)
	Muscularis externa	+
Gallbladder	Mucosa	+
	Gallbladder muscularis	+
	Gallbladder adventitia	–
	Gallbladder adipose tissues	–
Skin	Sweat glands	(+)
	Startified squamous epithilium	(+)
	Sepacious glands	(+)
	Hair follicles	+
	Arrector pilli muscle	+
	Collagen fibers	-
Liver	Hepatocytes	++
	Portal veins	++
	Bile ducts	++
Lung	Alveoli	(+)
	Bronchi	+
Placenta	Chorionic villi	+
	Maternal decidua	++
Thyroid	Thyroid follicles	++
	Thyroid parafollicular cells	++
Tonsils	Stratified squamous epithelium	(+)
	Pseudostratified columnar epithelium	(+)
	Mantle zone	(+)
	Germinal centers	(+)
Uterus	Endometrial stroma	(+)
	Uterinal glands in endometrium	+
	Uterine myometrium	+

Gastrointestinal tract. The submucosa layers of the colon and duodenum were weakly stained. Both the colon and duodenum mucosa, Lieberkühn glands, and muscularis externa showed moderate staining of ABCB5, whereas the colon stalk polyps were strongly stained. The ABCB5 expression was moderately detected in the duodenum villi. The gallbladder mucosa and muscularis expressed ABCB5, whereas it was entirely absent in the gallbladder adventitia and gallbladder adipose tissues. ABCB5 was strongly expressed in the liver, hepatocytes, portal veins and bile ducts.

Skin. Immunohistochemical staining showed weak reactivity of the sweat glands, stratified squamous epithelium, and sebaceous gland. The epithelial cells surrounding the hair

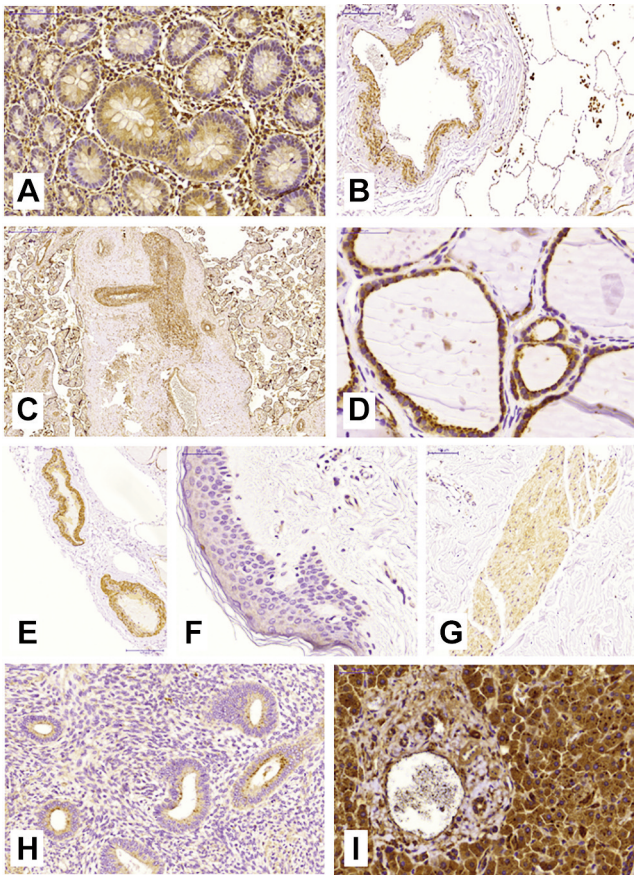

Figure 1. *Immunohistochemical detection of ABCB5 in normal human tissues. (A) Lieberkühn glands in the mucosa of colon; (B) lung bronchus; (C) maternal decidua of placenta; (D) thyroid follicles; (E) thyroid blood vessels; (F) stratified squamous cells of skin; (G) arrector pili of skin; (H) uterine glands in the endometrium; (I) portal vein and bile duct in the liver. Magnifications: 10× (B, C, E), 20× (A, G), 40× (D, F, H, I).*

Table II. *Correlation analysis of ABCB5 protein and mRNA expression in normal human tissues.*

	Immunostaining	
mRNA	Negative/Weak	Intermediate/Strong
0-100	4	1
>100	0	6

p=0.015; Fisher exact test. Protein expression was determined in the samples of the present investigation and the mRNA expression data taken from The Cancer Genome Atlas (www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga). The immunohistochemical protein expression results were categorized as negative or weakly positive *versus* intermediate or strongly positive. The mRNA expression values were categorized in two groups (0-100 *versus* >100 fragments per kilobase per million mapped fragments (FPKM).

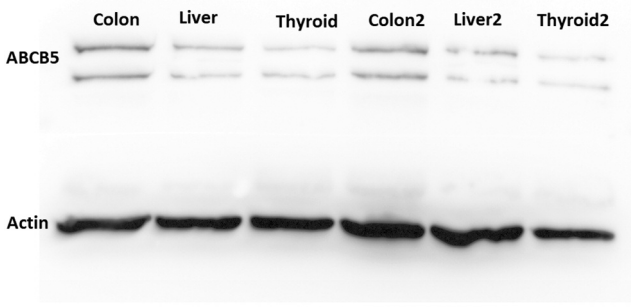

Figure 2. *Western blot analysis of ABCB5 in each two healthy tissue samples of the colon, liver, and thyroid using monoclonal antibody 5H3C6 against ABCB5. β-Actin was used to show equal loading and transfer in each lane.*

follicles and arrector pili muscle were moderately stained. ABCB5 was not expressed in collagen fibers.

Respiratory tract. The epithelial cells lining in the bronchi were strongly stained. Pneumocytes in alveoli were weakly stained by the ABCB5 antibody.

Reproductive system. The placental chorionic villi were moderately stained, whereas intense staining was observed in the maternal decidua. The endometrium uterine glands and myometrium expressed ABCB5.

Correlation between ABCB5 protein and mRNA expression. In order to exemplarily verify the ABCB5 protein expression determined by immunohistochemistry with ABCB5 mRNA expression, we compared the protein expression in our normal human tissue samples with the mRNA expression of normal human tissues deposited in The Cancer Genome Atlas (38). As shown in Table II, the comparison between our protein expression results and the mRNA expression of TCGA showed a significant relationship (p=0.015).

Western blotting. In order to confirm the specificity of the monoclonal antibody to detect ABCB5, protein lysates of each two colon, liver, and thyroid tissues were subjected to western blotting. As shown in Figure 2, all samples showed a specific double band at around 90 kD, indicating two isoforms or differences in the glycosylation pattern. β-Actin was used as loading control (40 kD). Non-specific bands did not appear.

Expression of ABCB5 in normal mouse tissues. The ABCB5 expression in murine tissues at specific morphological sites is shown in Table III.

Gastrointestinal tract. The epithelial cells of Lieberkühn glands of the colon were strongly stained. The muscularis externa was moderately stained. A similar staining was observed in the stomach. Hepatocytes intensely expressed ABCB5. Moderate staining was detected in the portal veins and bile ducts.

Table III. *Immunohistochemical expression of ABCB5 in normal murine tissues.*

Organ	Histology	ABCB5 Expression
Colon	Mucosa	++
	Muscularis externa	+
Brain	Grey matter of cerebellum	+
	White matter of cerebellum	++
	Grey matter of spinal cord	+
	White matter of spinal cord	++
	Central canal of spinal cord	+
	Granular layer of cerebellum	++
Heart	Myocardium	+
	Endocardium Purkinje fibers	+
Testis	Epithelium seminiferous tubules	++
	Seminiferous tubules	++
Liver	Hepatocytes	++
	Portal vein and bile ducts	+
Lung	Alveoli	+
	Bronchi	+
Stomach	Mucosa	+
	Submucosa	(+)
	Muscularis mucosa	+
Spleen	Spleen lymphocytes cells	–
	Spleen macrophages	++
	Trabeculae	+
Kidney	Medulla	++
	Cortex	++
	Glomeruli	–
Pancreas	Pancreatic acini	++
	Langerhans islets	+

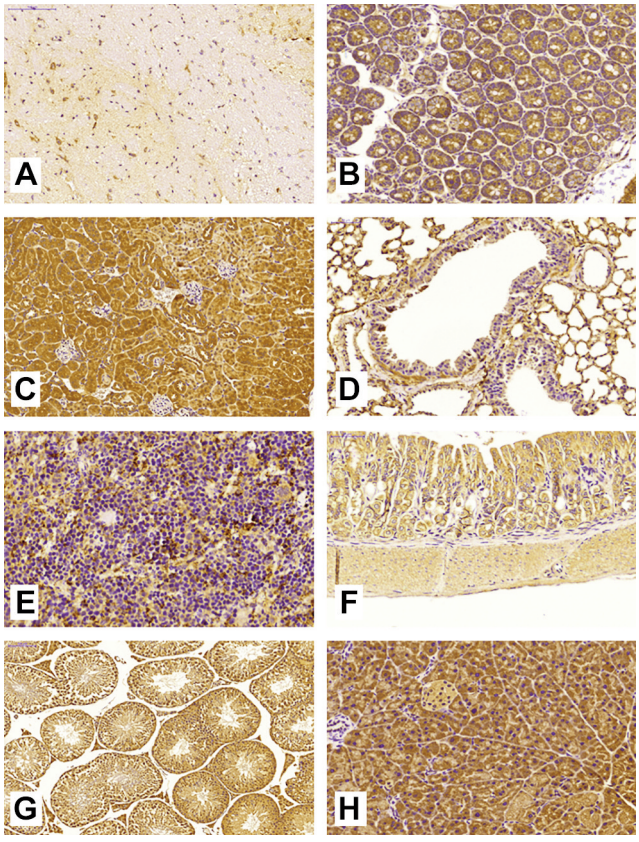

Figure 3. *Immunohistochemical detection of ABCB5 in normal murine tissues. (A) Grey matter of the spinal cord; (B) Lieberkühn glands in the colon; (C) Kidney cortex; (D) Bronchus and alveoli of the lung; (E) spleen; (F) mucosa (top), muscularis mucosa (middle and submucosa (bottom) of the stomach; (G) seminiferous tubules of the testis; (H) Langerhans islet surrounded by acini of the pancreas. Magnifications: 20× (A, C, G), 40× (B, D, E, F, H).*

Respiratory tract. The epithelial cells lining in the bronchi and pneumocytes in alveoli were moderately stained by the ABCB5 antibody.

Brain. Strong expression of ABCB5 was found in the glial cells of the white matter in both the cerebellum and spinal cord, and the granular layer of the cerebellum. The central canal of the spinal cord and glia cell of the grey matter in the cerebellum and spinal cord were moderately stained.

Heart. The myocardium cells and endocardium Purkinje fibers were moderately stained by the ABCB5 antibody.

Pancreas. The pancreatic acini highly expressed ABCB5. The Langerhans islets were moderately stained.

Spleen. ABCB5 was expressed on trabeculae and macrophages. However, splenic lymphocytes were negative.

Kidney. High expression was shown in the cortex and medulla, whereas the glomeruli did not show ABCB5 expression.

Testis. The epithelium seminiferous tubules were strongly positive for ABCB5.

Representative immunohistochemistry images of ABC expression in normal murine tissues are shown in Figure 3.

Expression of ABCB5 in normal rat tissues. The rat tissue evaluation of ABCB5 expression is depicted in Table IV.

Brain. Weak expression of ABCB5 was found in the central canal of the spinal cord, grey matter and granular layer of the cerebellum, whereas the grey matter and white matter of the spinal cord were moderately stained with ABCB5. ABCB5 expression was not detected on the white matter of the cerebellum.

Heart. The myocardium cells and endocardium Purkinje fibers were moderately stained by the ABCB5 antibody.

Liver. Hepatocytes intensely expressed ABCB5. The portal veins and bile ducts were moderately stained.

Lung. Both the epithelial cells lining in the bronchi and pneumocytes in alveoli were moderately stained by the ABCB5 antibody.

Spleen. The red pulp was weakly stained with ABCB5, whereas the while pulp and Trabeculae did not express ABCB5.

Table IV. *Immunohistochemical expression of ABCB5 in normal rat tissues.*

Organ	Histology	ABCB5 Expression
Brain	Grey matter of cerebellum	(+)
	White matter of cerebellum	–
	Grey matter of spinal cord	+
	White matter of spinal cord	+
	Central canal of spinal cord	(+)
	Granular layer of cerebellum	(+)
Heart	Myocardium	+
	Endocardium Purkinje fibers	+
Liver	Hepatocytes	++
	Portal vein and bile ducts	+
Lung	Alveoli	(+)
	Bronchi	(+)
Lymphnodes	Mandibular lymph nodes	(+)
Spleen	Red pulp	(+)
	White pulp	–
	Trabeculae	–
Kidney	Cortex	+
	Medulla	+
	Glomeruli	–
Pancreas	Pancreatic acini	(+)
	Langerhans islets	+
Thyroid	Thyroid follicles	++
	Parafollicular cells	++
Tongue	Mucosa	(+)
	Muscle fibres	–
	von Ebner glands	+

Kidney. Moderate expression was shown in the cortex and medulla. ABCB5 was not detected in the glomeruli.

Pancreas. The pancreatic acini weakly expressed ABCB5. The Langerhans islets were stained moderately.

Thyroid. The thyroid follicles and parafollicular cells were strongly stained by the ABCB5 antibody.

Tongue. The tongue mucosa cells were weakly stained for ABCB5. The von Ebner glands splenic lymphocytes were moderately stained.

Representative immunohistochemical stains of ABC expression in normal rat tissues are shown in Figure 4.

Expression of ABCB5 in normal pig tissues. The pig tissue evaluation of ABCB5 expression is depicted in Table V.

Duodenum. Intense staining was reported in the mucosa villi, muscularis externa, and Lieberkühn glands. The submucosa Brunner glands were moderately stained.

Heart. The myocardium cells and endocardium Purkinje fibers were moderately stained by the ABCB5 antibody.

Liver. Hepatocytes, portal vein, and bile ducts intensely expressed ABCB5.

Lung. Both the epithelial cells lining in the bronchi and pneumocytes in alveoli were strongly stained by the ABCB5 antibody.

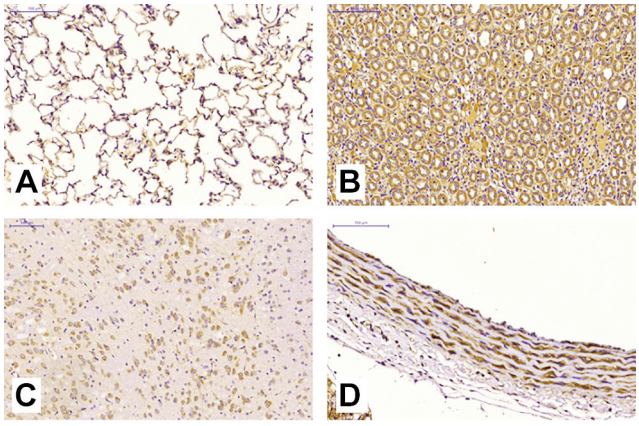

Figure 4. *Immunohistochemical detection of ABCB5 in normal rat tissues. (A) Alveoli of the lung; (B) collecting ducts of the kidney; (C) grey matter of the spinal cord; (D) tunica media of the aorta. Magnification: 20×.*

Kidney. High expression was shown in the cortex and medulla, whereas ABCB5 expression was not detected in the glomeruli.

Pancreas. The pancreatic acini and Langerhans islets weakly expressed ABCB5.

Lymphatic system. The thymus medulla was moderately stained for ABCB5. The pseudostratified columnar epithelium cells in the tonsils were strongly stained, and moderate staining was also reported in the stratified squamous epithelium and germinal centers of the tonsils.

Tongue. The von Ebner glands were weakly stained.

Representative immunohistochemistry images of ABC expression in normal pig tissues are shown in Figure 5.

Expression of ABCB5 in normal chicken tissues. The chicken tissue evaluation of ABCB5 expression is depicted in Table VI.

Colon. High ABCB5 expression was reported in the mucosa and Lieberkühn glands. The submucosa and muscularis externa layers were moderately stained by the ABCB5 antibody.

Liver. The hepatocytes strongly expressed ABCB5, whereas the portal vein and bile ducts showed weak ABCB5 staining.

Spleen. The red pulp, white pulp, and trabeculae were moderately stained with ABCB5.

Testis. The seminiferous tubules and interstitial connective tissue were moderately stained by the ABCB5 antibody.

Representative immunohistochemistry images of ABC expression in normal chicken tissues are shown in Figure 6.

Expression of ABCB5 in normal goose tissues. The goose tissue evaluation of ABCB5 expression is depicted in Table VII.

Table V. *Immunohistochemical expression of ABCB5 in normal pig tissues.*

Organ	Histology	ABCB5 Expression
Duodenum	Mucosa villi	++
	Submucosa Brunner glands	+
	Muscularis externa	++
	Mucosal Lieberkühn glands	++
Heart	Myocardium	+
	Endocardium Purkinje fibers	+
Liver	Hepatocytes	++
	Portal vein and bile ducts	++
Lung	Alveoli	++
	Bronchi	++
Kidney	Cortex	++
	Medulla	++
	Glomeruli	-
Pancreas	Pancreatic acini	(+)
	Langerhans islets	(+)
Thymus	Cortex	(+)
	Medulla	+
Tonsills	Stratified squamous epithelium	+
	Pseudostratified columnar epithelium	++
	Germinal centers	+
Tongue	Muscle fibres	-
	von Ebner glands	(+)

Table VI. *Immunohistochemical expression of ABCB5 in normal chicken tissues.*

Organ	Histology	ABCB5 Expression
Testis	Seminiferous tubules and interstitial connective tissue	+
	Seminiferous tubules	+
Liver	Portal vein and bile duct	(+)
	Hepatocytes	++
Lung	Parabronchus	+
	Alveoli	(+)
Spleen	Red pulp	+
	White pulp	+
	Trabeculae	+
Colon	Mucosa	++
	Submucosa	+
	Muscularis externa	+
	Mucosal Lieberkühn glands	++

Colon. Moderate staining was reported in the mucosa and Lieberkühn glands. The submucosa and muscularis externa were weakly stained by the ABCB5 antibody.

Liver. Hepatocytes moderately expressed ABCB5, whereas the portal vein and bile ducts showed only low ABCB5 expression.

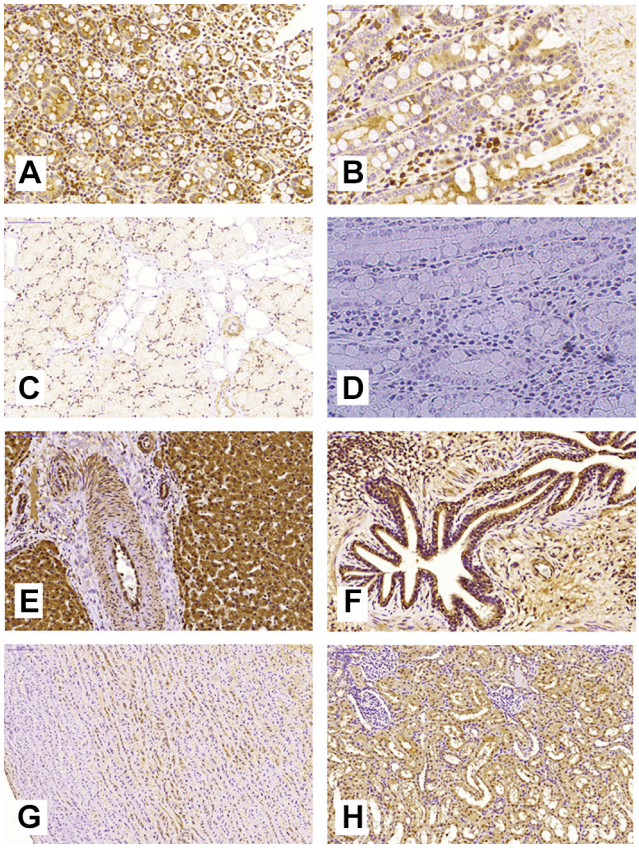

Figure 5. *Immunohistochemical detection of ABCB5 in normal pig tissues. (A) Lieberkühn glands in the mucosa of the duodenum; (B) mucosa villi in the duodenum; (C) Brunner glands in the submucosa of the duodenum; (D) negative control staining of the duodenum (first antibody omitted); (E) portal vein and bile duct of the liver; (F) bronchus in the lung; (G) tunica intima (left) and tunica media of the aorta (right); (H) cortex and glomeruli of the kidney. Magnifications: 20× (A, CE, G, H), 40× (B, D).*

Pancreas. The pancreatic acinar cells and Langerhans islets were moderately stained for ABCB5 (Figure 6F).

Spleen. The red pulp and white pulp were moderately stained with ABCB5.

Heart. The myocardium cells and endocardium Purkinje fibers were moderately stained by the ABCB5 antibody.

Expression of ABCB5 in normal tissues of Guppy fish. The intestine, ovary, and esophagus showed weak ABCB5 expression. The liver was moderately stained, whereas the kidney showed robust expression of ABCB5 (Table VIII). Representative immunohistochemistry images of ABC expression in normal fish tissues are shown in Figure 7A-C.

Expression of ABCB5 in normal tissues of earthworm. The intestine and dorsal blood vessels were strongly stained by the ABCB5 antibody. The epidermis showed moderate

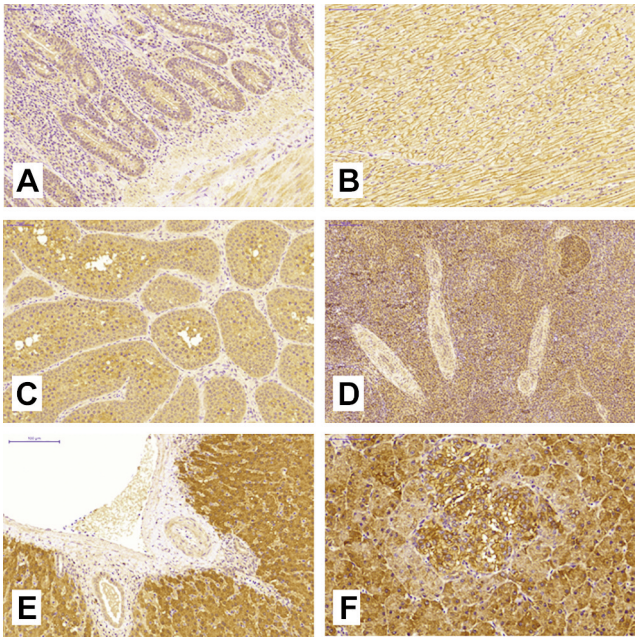

Figure 6. *Immunohistochemical detection of ABCB5 in normal chicken and goose tissues. (A) Lieberkühn glands in the colon; (B) heart; (C) seminiferous tubules in the testis; (D) trabeculae in the spleen; (E) portal vein and bile duct in the liver; (F) Langerhans islets in the pancreas. Magnifications: 20× (B, D, E), 40× (A, C, F).*

Table VII. *Immunohistochemical expression of ABCB5 in normal goose tissues.*

Organ	Histology	ABCB5 Expression
Pancreas	Langerhans islets	+
	Pancreatic acinar cells	+
Colon	Mucosa	+
	Submucosa	(+)
	Muscularis externa	(+)
	Mucosa Lieberkühn glands	+
Liver	Portal vein and bile duct	(+)
	Hepatocytes	+
Lung	Parabronchi	(+)
Spleen	Red pulp	+
	White pulp	+
Heart	Cardiac myocyte	+
	Purkinje cells	+

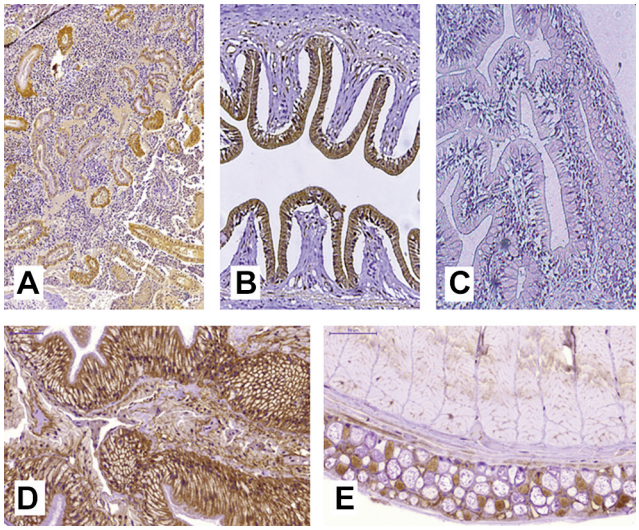

Figure 7. *Immunohistochemical detection of ABCB5 in normal fish and earthworm tissues. (A) kidney of fish; (B) colon of fish; (C) negative control staining of fish colon (first antibody omitted); (D) intestine of earthworm; (E) epidermis and circular muscles of earthworm. Magnifications: 20× (A), 40× (B, C, D).*

Table VIII. *Immunohistochemical expression of ABCB5 in Guppy fish tissues.*

Histology	ABCB5 Expression
Intestine	(+)
Esophagus	(+)
Kidney	++
Liver	+
Ovary	(+)

Table IX. *Immunohistochemical expression of ABCB5 in Guppy fish tissues.*

Histology	ABCB5 Expression
Circular muscles	(+)
Longitudinal muscles	(+)
Dorsal blood vessel	++
Epidermis	+
Intestine	++

staining, and weak expression was reported in the circular and longitudinal muscles (Table IX). Representative immunohistochemistry images of ABC expression in normal earthworm tissues are shown in Figure 7D-E.

Expression of ABCB5 in human tumors. ABCB5 is not only expressed in normal tissues but also in tumors, where it presumably extrudes anticancer drugs out of tumor cells rendering them resistant to chemotherapy. Therefore, we also investigated ABCB5 expression in human tumors. Immunohistochemical staining was carried out on a wide variety of human tumors, including, colorectal, cervical, endometrial, vaginal, nasopharyngeal carcinomas and others. In addition to single tumor sections, we also evaluated tumor

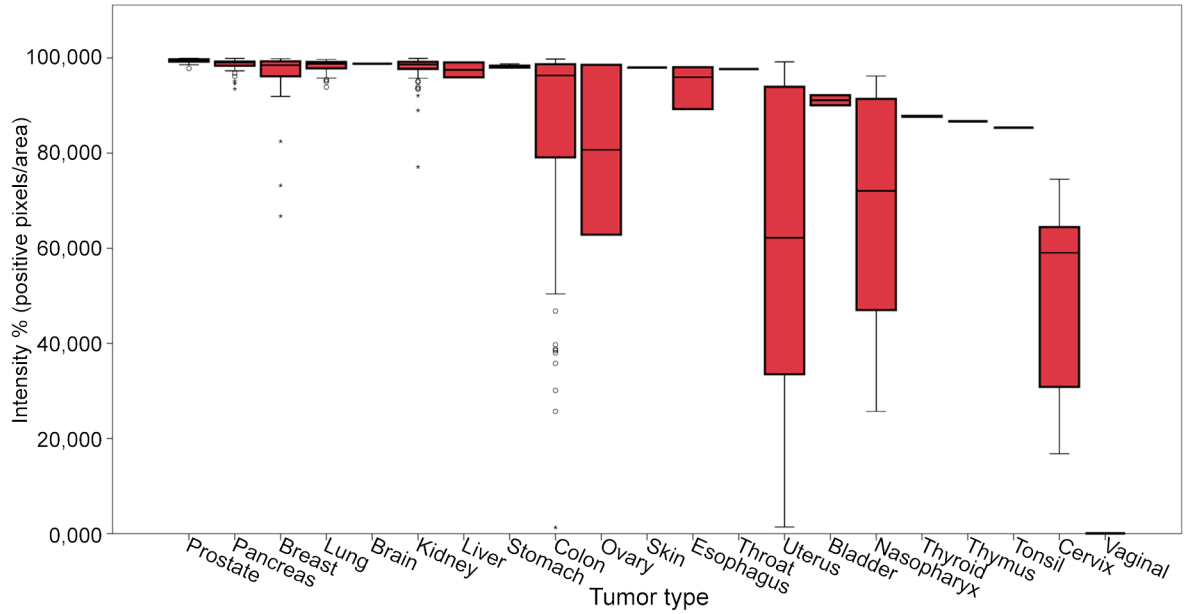

Figure 8. *Waterfall box plot of the intensity of ABCB5 immunostaining in 426 tumors of different tumor entities. The box plot was used as a descriptive statistics to show the five-number set of data including the minimum score, lower quartile, median, upper quartile, and maximum score.*

tissue arrays with a total of 426 tumors from different organs including kidney (110 samples), breast (42 samples), colon (65 samples), prostate (40 samples), pancreas (41 samples), lung (44 samples), skin (1 sample), throat (1 sample), bladder (2 samples), brain (1 sample), liver (2 samples), tonsil (1 sample), uterus (46 samples), thyroid (2 samples), stomach (3 samples), esophagus (3 samples), thymus (1 sample), cervix (8 samples), vaginal (1 sample), nasopharyngeal (10 samples), and ovarian tumors (2 samples).

Importantly, heterogeneous expression of ABCB5 was observed in different tumor types derived from the breast, colon, uterus, nasopharynx, and cervix. High expression of ABCB5 was detected in the prostate, pancreas, brain, kidney, liver, stomach, ovary, esophagus, throat, bladder, and thyroid, whereas the thymus and tonsil revealed moderate expression. In general, strong ABCB5 staining was observed in tumors, although the expression was heterogeneous – not only between different tumor entities but also within the same tumor type. The staining intensity of ABCB5 expression in all 426 individual tumors was quantified and represented as waterfall box plot (Figure 8).

Some tumor types generally showed a strong ABCB5 expression (*e.g.,* carcinoma of the prostate, pancreas breast, lung, kidney, liver or stomach), while other tumor types revealed large interindividual variation in ABCB5 expression (*e.g.,* carcinoma of colon, ovary, uterus, nasopharynx or cervix). Representative immunohistochemistry images of ABC expression in tumors are shown in Figure 9.

Epitope alignment identity and homology models. In order to have an insight regarding epitope similarity among different species, we carried out homology modeling and sequence alignment analysis. Interestingly, high percentage identities (PIDs) were observed in the pig, mouse, and rat with 81%, 74.9%, and 73.8%, respectively. The PIDs for chicken and Guppy fish were 69.2% and 62.5%, respectively. Importantly, the homology modelling for the ABCB5 of different species revealed high structural similarities with the human ABCB5 and the epitope sequence was located at the endoplasmic domain in all modelled structures (Figure 10), indicating that monoclonal antibody 5H3C6 was specifically bound to ABCB5 in these species.

In the past, there was a discussion that another antibody (C219) against another ABC-transporter (ABCB1, P-glycoprotein) may non-specifically cross-react with myosin (39). Therefore, we aligned the ABCB5 epitope amino acid sequence of monoclonal antibody 5H3C6 to myosin (Figure 11). The epitope of 5H3C6 on ABCB5 has a size of 193 amino acids (residues 481-674). Of them, 142 amino acids aligned as fragmentary pieces with large gaps to myosin. Even at positions where the epitope fragments were aligned to myosin, only 26 of 142 amino acids were identical between the ABCB5 epitope and myosin (25%). Hence, it is highly improbable that the monoclonal antibody 5H3C6 would non-specifically cross-react with myosin.

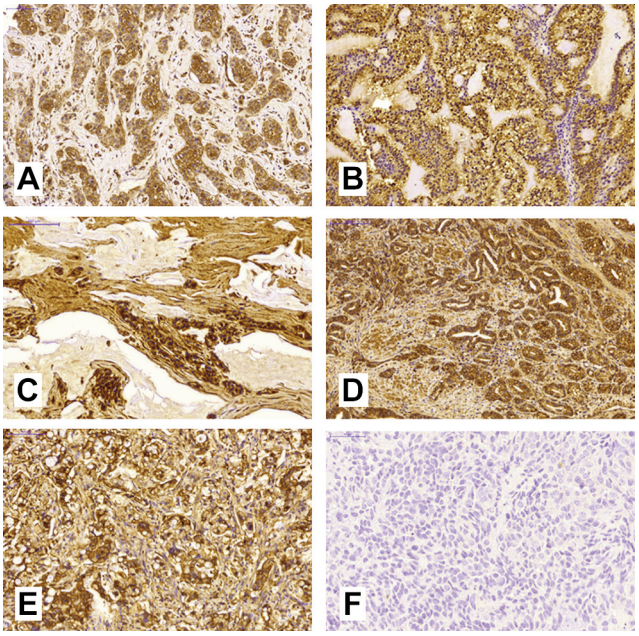

Figure 9. *Immunohistochemical detection of ABCB5 in human tumors. (A) Breast carcinoma; (B) endometrium carcinoma; (C) ovary carcinoma; (D) prostate carcinoma; (E) pancreas carcinoma; (F) vagina carcinoma. Magnifications: 20× (A-F).*

Discussion

In the present study, we showed the histological localization of ABCB5 in normal tissues among different species and reported its expression in human tumors using immunohistochemistry. The close functional relation of ABCB5 to P-glycoprotein (ABCB1/MDR1) urges further understanding of its abundance in various tissues to predict its possible role in the transport of xenobiotic and chemotherapeutic substances.

Investigating the expression pattern and localization of ABC transporters in normal organs helps to define their physiological function. For instance, P-gp (ABCB1) was reported to be highly expressed in normal tissues including the stomach, biliary tract of the liver, proximal tubules of the kidney, colon, small bowel, adrenal glands, blood-brain and blood-testis barriers, and hematopoietic stem cells, where it plays an important role in the extrusion of xenobiotic compounds from cells (40-43). ABCA1 has a broad tissue distribution (adipose tissues, lung, placenta, and stomach) and was linked to Tangier disease (44). ABCA1 plays a crucial role in active apolipoprotein A-I (apoA-I) lipidation, a key step in reverse cholesterol transport (45). ABCA4 is associated with Stargardt disease, and exclusively expressed at high levels in the retina, where it transports phosphatidylethanolamine from the outer segment of the photoreceptor disks into the cytoplasm (46). ABCB4 and ABCB11 are involved in phosphatidylcholine translocation and bile salts secretion, respectively (5, 47). Both transporters are highly expressed in the bile canalicular membranes of the liver.

ABCB5 is expressed in normal melanocytes and retinal pigment epithelial cells, suggesting that it plays a role in melanogenesis - the steps needed to produce melanin within melanosomes (48). In our study, we investigated ABCB5 expression in a systematic manner where we evaluated its expression in various tissues among different species. In the gastrointestinal (GIT) system, ABCB5 was present in the epithelial cells of mucosa, submucosa, and Lieberkühn glands in the colon, duodenum and stomach of all mammal species investigated. Furthermore, high ABCB5 expression was observed in all histological compartments of the liver (hepatocytes, portal veins, and bile ducts) of the different species. The presence of ABCB5 in these organs indicates its function to extrude xenobiotics and thereby limiting the cellular uptake of toxic substances along the GIT. Moreover, the pharmacological ADME process (absorption, distribution, metabolism, and excretion) is mediated by drug-metabolizing enzymes, ABC transporters, and solute carrier transporters (49). The interplay between enzymes/transporters in determining pharmacokinetics and pharmacodynamics of xenobiotic compounds may explain the abundant expression of ABCB5 in GIT organs. ABC transporters are often co-expressed on the cellular membrane and possess overlapped specificity for a wide variety of diverse substrates (50). This observation was repeatedly affirmed by experiments showing that selective inhibition of one ABC transporter results in the compensation of the efflux function by the remaining other ABC transporters (51).

ABCB5 was expressed in the chorionic villi and maternal decidua of the placenta. This might be attributed to the presence of the cytotrophoblast stem cells which are defined as precursors of differentiated cells in the placenta and are believed to be most abundant during early stages (52). ABCB5 regulates progenitor cell fusion by altering the membrane potential in normal melanocytes that expressed the stem cell marker CD133 (53). Therefore, it is well conceivable that ABCB5 might regulate the cellular membrane potential during cytotrophoblast fusion to form syncytiotrophoblasts in the placenta.

High ABCB5 expression was observed at the blood-tissue barriers sites, as detected in the brain of mice and rats and the testis of chicken. Moreover, the dorsal blood vessels of earthworm highly expressed ABCB5. These vessels are responsible for carrying blood to the front of the earthworm's body. These findings concur with the fact that the presence of ABC transporters at the blood tissue capillaries prevents tissues, such as the brain from the uptake of xenobiotics, and toxic substances (54). Importantly, the cortex and medulla of the kidney highly express ABCB5. Indeed, the presence of

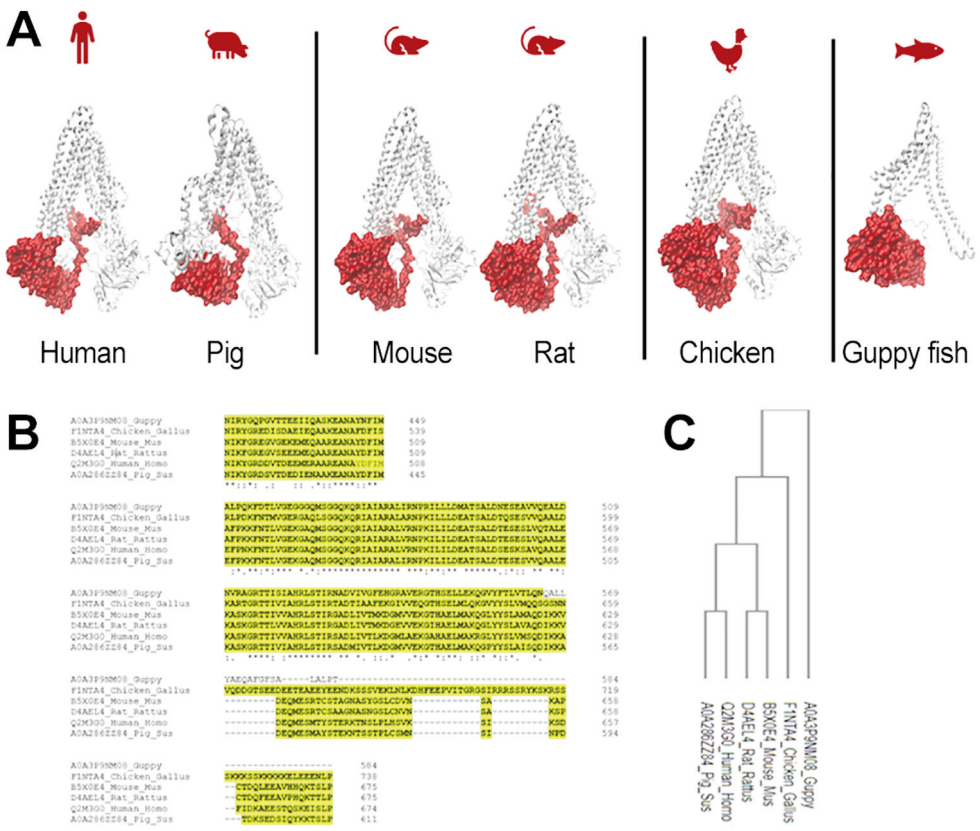

Figure 10. *Homology modelling of ABCB5 in different animal species and epitope identification. (A) Localization of the epitope of monoclonal antibody 5H3C6 in ABCB5 of different animal species; (B) Sequence alignment analysis of the epitope domains of ABCB5 of different animal species; (C) Phylogram of ABCB5 epitope sequences obtained by hierarchical cluster analysis indicating the degree of relatedness.*

ABCB5 and other ABC transporters in the kidney could support the renal clearance of toxic substances absorbed with food and carried on in the blood stream.

Certain patterns can be recognized in the expression of ABCB5 in normal organs of the various species investigated, which allow conclusions about the function of this ABC transporter: 1. ABCB5 was frequently in excretory organs of the gastrointestinal tract, *e.g.,* esophagus, stomach, colon, liver, and kidney. It can be assumed that exogenous xenobiotic and toxic compounds absorbed by food may be excreted by these organs. 2. ABCB5 expression in respiratory organs (bronchi, alveoli of lungs) indicates the detoxification function of xenobiotics inhaled with breath. Moreover, ABCB5 in the skin may protect organisms from harmful substances in the air. 3. ABCB5 may not only be involved in the translocation of exogenous but also endogenous substances. ABCB5 expression in the pancreas and gallbladder may indicate the secretion of compounds into bile and other substances supporting digestion. 4. Secretion of hormones can be hypothesized from the expression of ABCB5 in the placenta, uterus, testis, and thyroid. 5. ABCB5 expression was also observed in the central nervous system but not in brain blood vessels as reported for ABCB1 and ABCG2 (55-57). Instead, ABCB5 was found in the neurons of the spinal cord. A possible role of ABCB5 in the neuronal transport of neurotransmitters warrants further analyses. 6. ABCB5 might have specific functions in the immune system as indicated by its expression in the spleen, tonsils, lymph nodes, and thymus. Whether ABCB5 is involved in cytokine and chemokine secretion and antigen presentation is worth further investigation. 7. ABCB5 immunostaining was also observed in organs with smooth musculature (heart, blood vessels) and striated musculature (tongue, circular and longitudinal muscle strands).

We showed whether muscle staining might be non-specific, as previously shown for the antibody C219 recognizing not only ABCB1 but also myosin in a non-specific manner (39). Therefore, we analyzed whether the epitope of our ABCB5 antibody might share sequence homology with myosin. This was not the case. Hence, we concluded that ABCB5 may indeed be expressed in muscle tissues.

Figure 11. *Sequence alignment of the ABCB5 epitope amino acid sequence (residues 481-674) of monoclonal antibody 5H3C6 to human myosin.*

The hypothetical transport functions outlined above are not only based on ABCB5 expression in organs with specific transport tasks in the organisms but also on the excretory function of other ABC-transporters such as P-glycoprotein, MRP1, MRP2, BCRP for xenobiotic substances in gastrointestinal organs (58-60). Endogenous substance transport is also known for ABC-transporters (*e.g.,* cholesterol, phosphatidylcholine, hormones, biliary organic anions, and others) (1, 61, 62). The interaction of ABC-transporters with the immune system has been addressed in numerous investigations (63-66). Cyto- and chemokines (γ-interferon, tumor necrosis factor α) influence ABCB1 expression and function (67-69). TAP1 (ABCB2) and TAP2 (ABCB3) translocate peptide fragments from the cytosol to the cell surface and thereby contribute to antigen presentation of immune cells (70, 71).

The categorization of ABCB5-expressing organs into the above-mentioned seven functional groups was not only apparent in human tissues, but also in the other species of mammals, birds, fish, and helminths. The sequence homology of the antibody epitope was very high (62.5% to 81%). This indicates not only the cross-reactivity of the antibody across the borders of different species but also the high sequence conservation of ABCB5 that has been also previously reported for other ABC-transporters. Based on an evolutionary perspective, the functional convergence might explain the expression pattern similarity of ABCB5 among different mammalian species. The protein's conformational structure is conserved during evolution much better than the protein sequence (72). Evolutionary analysis on ABCB5 showed that ABCB5, P-gp, ABCB4, and ABCB11 share a common ancestor, which began duplicating early in the evolutionary history of chordates (73). This observation concurs with our previous study, in which we reported a 100% structural identity in the nucleotide binding domains of two ABC transporters (P-gp and ABCB5) in mouse and human (51). The phylogenetic analyses indicated that ABCB5 is a half transporter that derived from an ancestral full transporter throughout its evolutionary history with an absence of any major shifts in selection between the various lineages suggesting that the function of ABCB5 has been maintained during mammalian evolution. Furthermore, the coding single nucleotide polymorphisms (cSNP) and topological structures of the human ABCB5 analyses perceived a large number of non-synonymous cSNPs mapped to important functional regions of the protein, such as the substrate binding site (73).

The over-expression of several ABC transporters, including ABCB1 (MDR1), ABCC1 (MRP1), ABCG2 (BCRP/MXR), and ABCB5 confer MDR to cancer cells (5,

51). In this study, high ABCB5 expression was found in a large variety of tumor types. Previously, ABCB5 was identified in a subpopulation of malignant melanoma-initiating cells (MMICs), in addition to the human epidermal melanocytes progenitors that are considered as cancer stem cells (CSCs) (26). *In vivo*, xenotransplantation of patient-derived ABCB5$^+$ human melanoma cells to primary non-obese diabetic/severe combined immunodeficiency (NOD/SCID) murine recipients revealed tumor initiation in 60.9% of animals compared to tumor formation in 4.3% of animals with ABCB5$^-$ tumor xenografts (26). Down-regulation of ABCB5 was associated with induced terminal differentiation in human melanoma cells and resulted in the concurrent loss of chemoresistance and growth potential (74). Moreover, the expression of ABCB5 in liver stem cancer cells was associated with tumor progression, chemoresistance and reduced survival times in hepatocellular carcinoma patients (24). Intriguingly, ABCB5 was a novel molecular marker of therapy-refractory tumor cell population in colorectal cancer patients (25). ABCB5 was reported to be highly expressed in a small subpopulation of fused MCF-7 breast cells (74). The fused cells gained acquired resistance and subsequently became a stably doxorubicin-resistant cell line. Furthermore, ABCB5 is a fusogenic factor that might be responsible for increasing heterogeneity in the cancer cell population (75). Genomes derived from fused cells may provide opportunities for genomic reprogramming allowing the fused tumor cells to escape from the detrimental effects of chemotherapy. This suggests that cell fusion and clonal selection may represent a novel mechanism of acquired drug resistance (75). The evidence from the beforementioned findings emphasizes the hypothesis that ABCB5 might not only be involved in the transport of xenobiotics in normal tissues and the development of MDR in tumors but also in the process of tumorigenesis and neoplastic progression. This was further supported by a recent study that demonstrated the functional blockade of ABCB5 induced G_2/M arrest and re-sensitized glioblastoma cells to temozolomide-induced apoptosis *in vitro* and *in vivo* (76).

Importantly, we observed high expression of ABCB5 in normal and tumor tissues of the colon, kidney, breast, pancreas, lung, brain, liver, skin, small intestine, testis, tonsil, uterus, and ovary. The most conspicuous observation emerging from the comparable ABCB5 expression in normal and tumor tissues might be attributed to the presence of a small subpopulation of cancer stem cells. A trait of stem cells is their potential for multilineage differentiation. ABCB5 was firstly identified as marker of melanocyte progenitor cells and melanoma stem cells, where it plays an important role in the regulation of cellular differentiation. Furthermore, ABCB5-positive limbal stem cells (LSCs) have been isolated, implying that ABCB5 is required for LSC maintenance, corneal development, and repair (77). Recently, newly defined ABCB5$^+$ dermal mesenchymal stem cells were found to be involved in the wound healing process by maintaining the capacity of clonal self-renewal and clonal tri-lineage differentiation (78).

Taken together, the findings of the present investigation indicate that the physiological function of ABCB5 as a xenobiotic transporter justify the conserved pattern of expression in the different species. Furthermore, ABCB5 might be a suitable therapeutic, diagnostic, and prognostic target for cancer patients. Further studies are still needed to establish the possible clinical significance of ABCB5.

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Conflicts of Interest

All Authors declare that they have no competing interests in relation to this study.

Authors' Contributions

TE designed the study. WR provided the tissue material. MEMS performed the immunostaining. KM, NA, JCB, and TM carried out the quantification of stained slides. TE supervised the work and provided the facilities for the study. TE and MEMS wrote the manuscript. All Authors read the manuscript and approved the final version.

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