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The human porphyrin transporter ABCB6 is dispensable for erythropoiesis but responsible for the new blood group system Langereis

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Abstract

The human ATP-binding cassette (ABC) transporter ABCB6 has been described as a mitochondrial porphyrin transporter essential for heme biosynthesis¹, but is also suspected to contribute to anticancer drug resistance^{2–4}, as do other ABC transporters located at the plasma membrane. We identified ABCB6 as the carrier of the blood group antigen Lan on red blood cells, but also at the plasma membrane of hepatocellular carcinoma (HCC) cells, and established that *ABCB6* actually encodes a new blood group system (Langereis, Lan). Targeted sequencing of *ABCB6* in 12 unrelated individuals of the blood type Lan– identified 10 different *ABCB6* null mutations. This is the first report of deficient alleles of this human ABC transporter gene. Surprisingly, Lan– (*ABCB6*^{−/−}) individuals do not suffer any clinical consequences, albeit their deficiency in ABCB6 may place them at risk when defining drug dosage.

Blood group antigens arise from genetic polymorphisms and define numerous inter-individual differences, which can have tragic consequences in blood transfusions or

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Contributions

V.H. performed flow cytometry and western blot analysis, made expression constructs and cell culture. C.Sa. performed immunoprecipitation and genomic DNA sequencing. B.A.B. performed mass spectrometry analysis. T.P. provided immunohematological information and provided most Lan– blood samples. J.T., H.T. and M.T. isolated and produced the monoclonal antibody OSK43. Y.T. conceived and supervised the study at the Japanese Red Cross Osaka Blood Center. H.P. performed porphyrin analysis. M.L.G. performed statistical analysis and provided human cell lines. C.Su. provided the HuH-7 cell line. B.-N.P. provided two Lan– blood samples. P.-Y.L.P. was a former chief operating officer of CNRGS. J.-P.C. initiated the study conducted by L.A. by contacting Y.T. for OSK43, contacted J.-C.D. for porphyrin analysis and continuously supported the study. L.A. conceived and supervised the study at the National Institute of Blood Transfusion, performed experiments, made the figures and wrote the manuscript which was reviewed by J.-P.C., T.P., M.L.G., H.P., C.Su. and B.A.B. All authors approved the submitted manuscript.

Competing financial interests

The authors declare no competing financial interests.

pregnancy. Although the genetic locus of most blood group antigens has been identified (for recent reviews, see^{5,6}) with direct implications in transfusion medicine and obstetrics, the genetic basis remains unknown for some blood group antigens, despite their clinical significance. This is the case for the blood group antigen Lan, which can cause severe transfusion reactions⁷ as well as hemolytic disease of the fetus and newborn (HDFN)^{8,9}. Lan is a high-frequency antigen, not linked to any known blood group system, strongly suggesting that it is encoded by a new genetic locus. The Lan⁻ blood type is very rare worldwide and seems inherited as a recessive trait⁷⁻¹⁰. Lan-individuals are usually identified during serologic testing in order to investigate feto-maternal incompatibility and/or to find them compatible blood units, as they have developed an anti-Lan, *i.e.* an alloantibody reacting with all red blood cells (RBCs) but Lan⁻. Transfusion support of patients with an anti-Lan is highly challenging due to the scarcity of compatible blood donors, but mainly due to the lack of reliable reagents for blood screening.

We have succeeded in generating the first monoclonal antibody with Lan specificity (OSK43, IgG1 κ) from immortalized lymphocytes of a healthy Japanese Lan⁻ woman who had developed an anti-Lan during pregnancy. OSK43 works in hemagglutination assays as well as flow cytometry and immunofluorescence analyses (Fig. 1a,b), reacting with all tested RBCs but Lan⁻. We conducted a high-throughput screen by hemagglutination assay in Japan, and identified 14 Lan⁻ persons in 713,384 blood donors, establishing a frequency of 0.002% for the Lan⁻ blood type in this population. The intra- and inter-individual variation in RBC expression of the Lan antigen appeared notably low when evaluated by flow cytometry with OSK43 (Fig. 1a,c). Nevertheless, we observed that OSK43 showed a higher reactivity with umbilical cord blood than adult RBCs (Fig. 1d) indicating that the Lan antigen expression can vary during development.

The specificity and affinity of OSK43 suggested that it could be an important reagent to elucidate the genetic basis of the Lan blood group antigen. Towards this goal, we decided to use OSK43 to purify the Lan antigen-carrier protein from RBCs. As shown in Figure 2a, OSK43 was able to immunoprecipitate a RBC membrane protein of approximately 80 kDa, which was unambiguously identified as ABCB6 by mass spectrometry (Fig. 2b). ABCB6 belongs to the large family of ABC transporters that use ATP to transport a wide variety of endogenous or xenobiotic substrates across cellular membranes (see¹¹). Based on phylogenetic analysis, ABCB6 has been classified in the sub-family B along with the multidrug resistance P-glycoprotein (P-gp aka ABCB1). ABCB6 has been described as a porphyrin transporter located in the outer mitochondrial membrane, highly expressed during erythroid differentiation and required for mitochondrial porphyrin uptake¹. Nevertheless, ABCB6 has also been found at the plasma membrane¹², consistent with its suspected role in multidrug resistance. Indeed, increased expression of *ABCB6* correlated with increased resistance of different cancer cell lines to drugs^{2,3}, and *ABCB6* copy number was increased in the camptothecin-resistant cell line A549/CPT⁴. Thus, ABCB6 could transport anticancer drugs as well as endogenous substrates, as does ABCB1 and other multidrug ABC transporters (see^{13,14}).

In order to validate *ABCB6* as a novel genetic locus encoding blood group antigens, we first confirmed by western blot analysis that ABCB6 was present in the membrane of Lan⁺ RBCs (Fig. 2c, top panel, lanes 3 and 6). In contrast, we detected no *ABCB6* products in Lan⁻ RBCs (Fig. 2c, top panel, all other lanes), suggesting that null allele(s) of *ABCB6* may be responsible for the Lan⁻ blood type. We then established *ABCB6*-expressing clones of the human cell line K-562 and analyzed them by flow cytometry with OSK43. We observed that exogenous expression of *ABCB6* in K-562 cells correlated with cell surface expression of the Lan antigen (Fig. 2d). Together, these results validate that the blood group antigen Lan is encoded by *ABCB6* (2q36), thus defining a novel blood group system. Of note, 30

blood group systems are currently recognized by the International Society for Blood Transfusion, and none of them is encoded by an ABC transporter gene (<http://ibgri.blood.co.uk/isbtpages/isbtterminologypages/tableofbloodgroupsystems.htm>).

To identify the *ABCB6* mutations responsible for the Lan⁻ blood type, we used the Lan⁻ blood samples cryopreserved in the rare blood collection of the National Reference Center for Blood Groups (Paris, France; the oldest sample dating to 1976). Of note, none of these Lan⁻ blood samples was reactive with OSK43 by flow cytometry analysis (Supplementary Fig. 1), confirming their original identification. We extracted genomic DNA from these blood samples and sequenced *ABCB6* (see Supplementary Fig. 2 for sequencing strategy). From a cohort of 11 unrelated Lan⁻ subjects, we identified 6 frameshift, 2 nonsense and 1 donor splice site mutations in *ABCB6* (Table 1, mutations n° 1 to 9, and Supplementary Fig. 3a–i). Nine of these Lan⁻ subjects were homozygous for a single mutation and two were double heterozygous (Supplementary Table 1). Analysis of the available pedigrees confirmed that the Lan⁻ blood type results from recessive inheritance of these *ABCB6* mutations (Supplementary Fig. 4). We also sequenced the genomic DNA of the Japanese Lan⁻ woman whose lymphocytes were used to generate OSK43, and found that she was homozygous for yet another frameshift mutation in *ABCB6* (Table 1, mutation n° 10 and Supplementary Fig. 3j), suggesting a wide variety of *ABCB6* null alleles. All ten *ABCB6* null mutations identified in this study were absent in control subjects (Lan⁺) and in NCBI dbSNP (build 132).

Surprisingly, although *ABCB6* was suspected to play an essential role in heme biosynthesis during erythroid differentiation by importing porphyrin into mitochondria¹, Lan⁻ (*ABCB6*^{-/-}) individuals did not exhibit anemia or abnormal erythropoiesis (Supplementary Fig. 5). This unexpected finding indicates either that no transporter is actually required for porphyrin import into the mitochondrial inter-membrane space or, more likely, that another porphyrin transporter, yet unknown, can compensate for the absence of *ABCB6* at the mitochondrial outer membrane. We show here that *ABCB6* is present at the plasma membrane of RBCs, which are devoid of mitochondria in mammals, suggesting that *ABCB6* could play a role in exporting porphyrin excess out of RBCs. Therefore, we measured blood levels of porphyrin in Lan⁻ (*ABCB6*^{-/-}) individuals. Their RBC levels of porphyrin were slightly increased ($2.1 \mu\text{mol/L} \pm 0.2$ (n=4), $0.1 < \text{normal range} < 1.9$) but more strikingly, their plasma levels of porphyrin were very low and actually below the detection threshold ($< 5.0 \text{ nmol/L}$ (n=4), $6.5 < \text{normal range} < 20.0$). While these findings were fully consistent with the absence of porphyria symptoms¹⁵ in Lan⁻ individuals, they confirmed that *ABCB6* is involved in porphyrin export from RBCs. However, the modest increase in RBC levels of porphyrin in *ABCB6*^{-/-} individuals suggests that another porphyrin transporter of the RBC membrane, such as the breast cancer resistance protein (BCRP aka *ABCG2*; Fig. 2c, bottom panel)¹⁶, may compensate for the absence of *ABCB6* at the plasma membrane of RBCs. In fact, we show in an accompanying paper (NG-LE30370, Saison *et al.*) that *ABCG2*^{-/-} individuals exhibit similarly impaired blood levels of porphyrin, indicating that *ABCG2* and *ABCB6* play a similar role in porphyrin export from RBCs. Yet, *ABCG2* does not fully replace *ABCB6* when the latter is absent, and *vice versa*, otherwise *ABCB6*^{-/-} or *ABCG2*^{-/-} individuals would not exhibit impaired blood levels of porphyrin.

Since the Lan antigen is carried by *ABCB6*, and OSK43 is suitable for flow cytometry analysis of native cells, we decided to use OSK43 to evaluate cell surface expression of *ABCB6* in different cell lines. As shown in Figure 3, we found the Lan antigen on HepG2 hepatocellular carcinoma (HCC) cells, but not on A-498 renal cell carcinoma (RCC), A-431 squamous cell carcinoma (SCC), MOLT-4 acute lymphoblastic leukemia (ALL) or HeLa cervical cancer (CC) cells. When we examined another HCC cell line, HuH-7, we also detected the Lan antigen (Fig. 3f). Expression of *ABCB6* at the plasma membrane of HepG2

and HuH-7 cells may be either related to their hepatic origin, or acquired during hepatocarcinogenesis. Future studies will need to dissect the role of *ABCB6* at the plasma membrane of human hepatocytes in health and disease. Nevertheless, it is worth mentioning that overexpression of *ABCB6* has been observed in HCC compared to surrounding non-malignant tissue, and may contribute to multidrug resistance in HCC¹⁷.

In summary, we show in this report that *ABCB6* is responsible for a novel blood group system, currently defined by the Lan antigen, which is present at the plasma membrane of RBCs but also HCC cells. A Lan-specific monoclonal antibody (OSK43) has been developed and will greatly facilitate the identification of Lan- blood donors as well as Lan-pregnant women, whose pregnancy is at risk of hemolytic disease of the fetus and newborn. By elucidating the genetic basis of the Lan- blood type, we have uncovered 10 null mutations of *ABCB6* (Table 1 and Fig. 2e). No deficient alleles of *ABCB6* have previously been reported and it was questionable whether complete deficiency of this porphyrin transporter was compatible with life. Not only is the complete deficiency of *ABCB6* viable, but it is asymptomatic. Nevertheless, it will be necessary to closely monitor Lan- (*ABCB6*^{-/-}) patients, especially those treated with drugs potentially transported by *ABCB6*, since its deficiency may alter pharmacokinetics of these drugs or result in adverse effects such as hepatotoxicity.

METHODS

Subjects

Subjects of the Lan- blood type were identified at the National Reference Center for Blood Groups (Paris, France) and blood samples were cryopreserved in its rare blood collection, in order to establish the Lan- blood type of other subjects and ultimately to identify the Lan-blood type-causing gene. Fresh blood samples were obtained after informed consent. The study was conducted according to the ethical standards of the Japanese Red Cross Osaka Blood Center (Osaka, Japan) and those of the National Institute for Blood Transfusion (Paris, France).

Identification and characterization of monoclonal antibody OSK43

Peripheral blood lymphocytes from a healthy Japanese woman (G4P2) whose serum contained an anti-Lan (titer 8; no known history of transfusion) were transformed with Epstein-Barr virus (EBV) then electrofused with the human myeloma cell line JMS-3. Hybridoma were selected by growth in hypoxanthine-aminopterin-thymidine (HAT) containing medium and cloned by limiting dilution. Anti-Lan-secreting hybridoma were identified by antiglobulin tests with Lan+ and Lan- RBCs. Clone OSK43 was expanded for antibody production. The reactivity titer of produced OSK43 was 2,560 with native or DTT-treated Lan+ RBCs, 5,120 with alphachymotrypsin-, pronase- or sialidase-treated Lan+ RBCs, and 10,240 with trypsin-, papain-, bromelin- or ficin-treated Lan+ RBCs as determined by antiglobulin test. OSK43 is also suitable for extracellular staining of *ABCB6* for flow cytometry and immunofluorescence studies, but not for western blot analysis.

Sequencing

The primers used to amplify and sequence *ABCB6* were designed from the NCBI reference sequence of human chromosome 2, NT_005403.17, using DNA Workbench software (CLC bio) and described in Supplementary Figure 1. Detailed PCR conditions are available upon request. PCR products were gel-purified with NucleoSpin Extract II kit (Macherey-Nagel) and sequenced with ABI BigDye terminator chemistry (GATC Biotech). Sequence analysis was performed using DNA Workbench software (CLC bio). Mutations were screened by unidirectional sequencing and confirmed by bidirectional sequencing.

Flow cytometry analysis

RBCs thawed from frozen blood samples and stored in stabilization solution (ID-CellStab, DiaMed) were washed in Dulbecco's phosphate-buffered saline solution (DPBS, Gibco) and then resuspended in low-ionic strength solution (LISS, Formule 735, B. Braun Medical) supplemented with 0.15 % bovine serum albumin (BSA) and incubated with OSK43 (1:100). Cancer cells were washed and resuspended in DPBS supplemented with 0.15 % BSA and then incubated with OSK43 (1:100) or the human monoclonal anti-K1 T27S (1:200; hybridoma supernatant) as control. Adherent cancer cells were harvested by treatment with 0.05 % trypsin and 0.02 % EDTA.4Na (Gibco). OSK43 labeling was revealed with goat F(ab')₂ anti-human IgG(H+L)-PE (1:100; Beckman Coulter) and immediately analyzed with a FACSCanto II flow cytometer (BD Bioscience) equipped with FACSDiva software (v. 6.1.2) (BD Bioscience). Ten thousand RBCs or viable cancer cells, gated on forward scatter (FSC) vs. side scatter (SSC), were collected for each sample. Data were analyzed with FlowJo software (v. 7.2.5) (TreeStar).

Immunofluorescence microscopy analysis

RBCs were first labeled with OSK43 as previously described for flow cytometry analysis (see above) then with the mouse monoclonal anti-glycophorin A R18 (1:100; hybridoma supernatant). OSK43 labeling was revealed with goat F(ab')₂ anti-human IgG(H+L)-biotin (1:100; Beckman Coulter) and Alexa Fluor 568-conjugated streptavidin (1:1,000; Molecular Probes), while R18 labeling was revealed with Alexa Fluor 488-conjugated goat anti-mouse IgG(H+L) (1:1,000; Molecular Probes). Images were acquired with an Eclipse TE300 inverted microscope (Nikon) equipped with a Plan Fluor 100 × / 1.30 oil immersion objective (Nikon) using a D-Eclipse C1 laser scanning confocal module (Nikon) and EZ-C1 (v. 3.50) software (Nikon). Red and green colors were digitally exchanged in Figure 1b to increase visibility.

Mass spectrometry analysis

Polyacrylamide gels were stained with the SilverQuest Silver Staining kit (Invitrogen). Excised bands were diced into small pieces, washed with water and destained with the Silver D-Stain kit (G-Biosciences). Gel pieces were subjected to two rounds of the following: washing with water for 5 min at room temperature and then incubation with 50 % acetonitrile, 50 mM ammonium bicarbonate for 30 min at 37 °C. Gel pieces were then completely dehydrated by adding 100 % acetonitrile. After removal of acetonitrile, gel pieces were dried in a speed vacuum centrifuge then placed on ice and allowed to swell with 12.5 ng/μl Sequencing Grade Modified Trypsin (Promega) in 50 mM ammonium bicarbonate for 30 min on ice. An equal volume of 50 mM ammonium bicarbonate was then added and the samples were incubated overnight at 37 °C. Peptides were collected and gel pieces were further extracted once with 50 % acetonitrile, 2.5 % formic acid then once with 100 % acetonitrile. Pooled peptide extractions were dried in a speed vacuum centrifuge, and peptides were resuspended in 2.5 % acetonitrile, 2.5 % formic acid, and loaded for nanoscale liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis in an LTQ Orbitrap mass spectrometer (ThermoElectron) as previously described¹⁸. Tandem mass spectra were searched against a concatenated forward and reverse human IPI database¹⁹ using SEQUEST software and requiring fully tryptic peptides, allowing for a precursor mass tolerance of 30 PPM, and dynamic modification of methionine (15.9949 for oxidation) and cysteine (71.0371 for acrylamidation). Requiring 3 unique peptide identifications, precursor measurements within 8 PPM of the theoretical mass, and XCorr values of 1.8, 2.2, 2.5 and 2.8 for 1+, 2+, 3+ and 4+ charge states respectively yielded only one protein hit and no reverse hits. The identified protein was not found in the control sample.

Western blot analysis

Lysates of RBC membranes were prepared from frozen blood samples that were previously thawed, resuspended in stabilization solution (ID-CellStab, DiaMed) and washed in 0.9 % NaCl (B. Braun Medical). RBC membranes were prepared at 0–4 °C by hypotonic lysis with 5P8 buffer (5 mM Na₂HPO₄ pH8.0 and 350 µM EDTA pH8.0) supplemented with 1 mM AEBSF, stripped by incubation with 10 mM NaOH and finally solubilized with an equal volume of 4X LDS Sample Buffer (Invitrogen). Equal amounts of RBC membrane lysates were reduced with 100 mM DTT without boiling, resolved by Tris-Glycine 8 % SDS-PAGE and transferred to PolyScreen PVDF Transfer Membrane (Perkin Elmer) by submarine transfer. The Mark12 Unstained Standard (Invitrogen) was used as reference of molecular weights. Membranes were blocked in 1X Blocking Buffer (Sigma) overnight at 4 °C and then incubated with affinity-purified rabbit anti-ABCB6 (1:1,000; Rockland) for 90 min at 21 °C. Anti-ABCB6 labeling was revealed with an anti-rabbit IgG(H+L) horseradish-peroxidase-linked goat antibody (1:1,000; P.A.R.I.S Biotech) for 45 min at 21 °C, the Amersham ECL Plus Western Blotting Detection System (GE Healthcare) and Kodak BioMax MR films (Eastman Kodak Company). Membranes were similarly probed with mouse monoclonal anti-ABCG2 BXP21 (1:1,000; Santa Cruz Biotechnology).

Plasmid construction

The coding sequence of human *ABCB6* cDNA was amplified from a Human Fetal Liver Marathon-Ready cDNA library (Clontech), cloned into pCR4Blunt-TOPO vector (Invitrogen), sequence-verified (identical to NM_005689.1 aside from the synonymous mutation c.546A>G in agreement with NT_005403.17) and subcloned into pEF1/Myc-HisB vector (Invitrogen) as an Acc65I/PmeI fragment, removing the multiple cloning site and the Myc-His₆ tag of the vector. Complete sequence of pEF1-ABCB6 plasmid is available upon request.

Cell culture and transfection

Human K-562 cells were grown in Iscove's modified Dulbecco's medium (IMDM + GlutaMAX-I, Gibco) supplemented with 10 % fetal bovine serum (Pan-Biotech) and 0.5 × antibiotic-antimycotic solution (Gibco) at 37 °C under a humidified atmosphere containing 5 % CO₂. Cells were periodically tested for mycoplasma contamination using a home-made PCR assay. To obtain K-562 transfectants, 10⁶ K-562 cells were first transfected with 2 µg of pEF1-ABCB6 plasmid by nucleofection using the Nucleofector II device and the Cell Line Nucleofector Kit V (Amaza) according to the manufacturer's protocol (program T-016). Stable clones of K-562 cells were obtained by growth in presence of neomycin (0.8 mg/ml, Invitrogen) after limiting dilution. Plasmid DNA used for transfection was purified with NucleoBond Xtra Midi Plus (Macherey-Nagel) and verified by restriction analysis before transfection. Human HuH-7 cells were grown in Williams' medium E (WME + GlutaMAX-I, Gibco) supplemented with 10 % fetalclone II (HyClone), 10 µg/ml gentamicin (Gibco) and 10 µg/ml fungin (InvivoGen).

Porphyrin analysis

Porphyrin levels in plasma and RBCs were measured on blood samples, taken on EDTA and kept less than 24 h at 4–8 °C in the dark, as previously described²⁰.

Statistical analysis

The Mann-Whitney *U* test was used to assess the difference between data sets, and box-and-whisker diagrams were generated to interpret the distribution of data within a group, by using StatEl software (ad Science).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Characterization of the Lan blood group antigen expression on RBCs with the monoclonal antibody OSK43

(a) Specificity of the monoclonal antibody OSK43 as shown by flow cytometry analysis of Lan+ and Lan- RBCs. OSK43 shows a strict Lan specificity so that the histogram profiles of Lan- RBCs labeled with and without OSK43 were superposable.

(b) Immunofluorescence analysis of Lan+ and Lan- RBCs co-labeled with the human monoclonal OSK43 (green) and a mouse monoclonal anti-glycophorin A, the most abundant glycoprotein on RBCs (red). Scale bars represent 5 μ m.

(c) Lan antigen expression on RBCs from 39 random blood donors from France (ABO type O) as measured by flow cytometry with OSK43 as in (a). The geometric means of fluorescence intensity (in arbitrary units of the flow cytometer) are shown as a box-and-whisker diagram: lower and upper boundaries of boxes represent the 25th and 75th percentiles, horizontal lines the medians, whiskers the minimum and maximum non-atypical values, and circles the atypical values.

(d) Lan antigen expression on RBCs from adult or cord blood as measured by flow cytometry with OSK43 as in (a). Bars represent the means of fluorescence intensity, and error bars the s.d. (n=4); ** $P < 0.01$ by Mann-Whitney U test.

Figure 2. Identification of ABCB6 as the carrier of the Lan blood group antigen

(a) Purification of the Lan antigen from RBCs by immunoprecipitation with OSK43. Lysates (lane 1: 1/300th, lane 2: 1/900th and lane 3: 1/2,700th) were prepared from membranes of Lan⁺ RBCs labeled with or without OSK43, and corresponding immune complexes (lanes 4 and 5) were analyzed by polyacrylamide gel electrophoresis and silver staining. Identity and position of heavy and light chains of OSK43 are indicated.

(b) ABCB6 peptides identified by mass spectrometry from the band of approximately 80 kDa that was immunoprecipitated by OSK43 from RBCs. In total, 27 tryptic peptides of ABCB6 were identified (green); the predicted trypsin cleavage sites are underlined.

(c) ABCB6 is present in the plasma membrane of RBCs from Lan⁺ but not Lan[−] individuals as shown by western blot analysis. RBC membranes were prepared from 2 Lan⁺ (lanes 3 and 6) and 6 Lan[−] (lanes 1, 2, 4, 5, 7 and 8) individuals and analyzed by western blot with a rabbit polyclonal anti-ABCB6 (top panel). Of note, ABCB6 runs as monomeric and multimeric forms even under reducing conditions (arrows, see Supplementary Figure 6 for details). The western blot membrane was reprobed with a mouse monoclonal anti-ABCG2, another ABC transporter able to transport porphyrin (bottom panel; only monomeric form of ABCG2 is shown, see Supplementary Figure 6).

(d) Exogenous expression of ABCB6 in K-562 cells results in cell surface expression of the Lan antigen. Live cells of representative K-562 clones stably transfected with an expression construct of *ABCB6* cDNA (green profile) or the corresponding empty vector (red profile) were analyzed by flow cytometry with OSK43.

(e) Diagram showing the positions of *ABCB6* null mutations identified in this study (see Table 1 for details). Green boxes represent exons and broken lines introns. Red diamonds at the top represent the number of unrelated Lan[−] subjects found with each mutation.

Figure 3. Expression of the Lan antigen on human cancer cell lines

Cell surface expression of the Lan antigen was analyzed on native HepG2 (a), A-498 (b), A-431 (c), MOLT-4 (d), HeLa (e) and HuH-7 (f) cells by flow cytometry with OSK43 (green profiles) or T27S (a non relevant human monoclonal antibody, grey profiles).

Table 1*ABCB6* null mutations causing the Lan– blood type

	Nucleotide change ^a	Predicted amino acid change ^b	Location
1	c.197_198insG	p.A66GfsX96	exon 1
2	c.953_956delGTGG	p.G318AfsX8	exon 4
3	c.1533_1543dupCGGCTCCCTGC	p.L515PfsX17	exon 9
4	c.1709_1710delAG	p.E570GfsX21	exon 11
5	c.1867delinsAACAGGTGA	p.G623NfsX3	exon 14
6	c.1985_1986delTC	p.L662PfsX15	exon 15
7	c.717G>A	p.W239X	exon 3
8	c.1942C>T	p.R648X	exon 14
9	c.2256+2T>G	(splicing defect)	intron 16
10	c.1690_1691delAT	p.M564VfsX2	exon 11

^aReference sequence NM_005689.1^bReference sequence NP_005680.1