Overexpression of the ABCB1 drug transporter in acute myeloid leukemia cells is associated with downregulation of latrophilin-1

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Abstract. Finding new markers with appropriate prognostic levels for the differential diagnosis of neoplastic diseases represents an important issue for biomedical research. Recently, latrophilin-1 (LPHN1) was reported to be expressed in human monocytic leukemia cell lines and in primary human acute myeloid leukemia (AML) cells. However, this expression was found to be absent in healthy leukocytes. LPHN1 was therefore considered a novel biomarker of human AML. In previous papers, we established two P-gp-positive variants (SKM-1/VCR and MOLM-13/VCR) of AML cell lines derived from parental human AML cells SKM-1 and MOLM-13 by selection with VCR. The present paper addresses the measurement of LPHN1 expression in SKM-1 and MOLM-13 cells and their P-gp-positive variants. Both parental AML lines were positive for LPHN1 expression at the mRNA and protein levels. However, the expression of LPHN1 at both the mRNA and protein levels was reduced in both P-gp-positive SKM-1/VCR and MOLM-13/VCR variants of AML cells. Interestingly, we observed an elevation of the latrophilin-3 transcript in P-gp-positive variants of AML cell lines. The combined results suggest that alterations in latrophilin expression occur in AML cells expressing P-gp.

Key words: AML — AML cell lines — P-glycoprotein — Latrophilins — Vincristine

Abbreviations: ABC, ATP binding cassette; ACTB, gene coding ß-actin; AML, acute myeloid leukemia; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LPHN1, latrophilin-1; LPHN2, latrophilin-2; LPHN3, latrophilin-3; MDR, multidrug resistance; P-gp (ABCB1), P-glycoprotein; VCR, vincristine.
AML cells and represents a real obstacle against effective chemotherapy of this type of leukemia (Broxterman et al. 2000; Gao et al. 2015). Recently, we described the establishment of a P-gp-positive variant of human AML cells (SKM-1/VCR and MOLM-13/VCR) by selection in medium with stepwise-increasing concentrations of vincristine (Imrichova et al. 2014, 2015; Coculova et al. 2016). The expression of P-gp in AML cells was associated with the coexpression of class 6 filament protein nestin, whose expression is typical for neural stem cells and neural progenitor cells (Imrichova et al. 2014; Coculova et al. 2016), and the downregulation of the plasma membrane expression of AML marker protein CD33 (Imrichova et al. 2015). This fact indicated significant protein remodeling in AML cells, which express P-gp.

In the current paper, we report a study of LPHN1 expression in AML cells SKM-1 and MOLM-13 and their P-gp-positive variants SKM-1/VCR and MOLM-13/VCR.

### Cell culture conditions

The following cell variants were used in this study:

1. P-gp-negative cells obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ Germany):
   - Human acute myeloid leukemia SKM-1 (ACC 547);
   - Human acute myeloid leukemia MOLM-13 (ACC 554).

2. P-gp-positive cell variants:
   - SKM-1/VCR, obtained by culturing in media with stepwise-increasing concentrations of VCR (Merck s.r.o. Slovakia) (Imrichova et al. 2014; Imrichova et al. 2015), which are able to grow in media containing 50 nmol/l of VCR;
   - MOLM-13/VCR, obtained by culturing in media with stepwise-increasing concentrations of VCR (Imrichova et al. 2014, 2015), which are able to grow in media containing 20 nmol/l of vincristine.

All variants of SKM-1 and MOLM-13 cells were grown in RPMI 1640 medium containing 12% fetal bovine serum, 100,000 units/l penicillin and 50 mg/l streptomycin (both purchased from Merck s.r.o.) in a humidified atmosphere containing 5% CO₂ at 37°C for two days.

### RT-PCR

Cells after the cultivation period were harvested by centrifugation (664 × g at 20°C) and washed twice in phosphate buffered saline (PBS, Merck s.r.o., Slovakia). The total RNA from susceptible and drug-resistant variants of SKM-1 and MOLM-13 cells was isolated using TRI REAGENT® (Merck s.r.o.) according to the manufacturer’s instructions. Reverse transcription was performed with 2 µg of DNase I (Thermo Scientific, Germany)-treated RNA and a RevertAid™ H Minus First-Strand cDNA Synthesis Kit (Thermo Scientific) according to the manufacturer’s protocol. PCR was performed in a total volume of 25 µl using a PCR kit according to the manufacturer’s protocol (Thermo Scientific). Expression of β-actin (from the ACTB gene) was used as an internal standard. The PCR products were separated on a 1.5% agarose gel (Life Technology, Slovakia) and visualized using GelRed™ nucleic acid gel stain (Thermo Scientific). The primer sequence and PCR conditions are documented in Table 1.

### qRT-PCR

Total RNA isolation, reverse transcription and the PCR primers were the same as described for RT-PCR. qPCR was run on a 96-well microtitration plate using a CFX96 Touch™ real-time PCR Detection System (Bio Rad, USA). PCR was run in a 10 µl solution containing 500 ng of cDNA, 5 µl of 2x iTaq Universal SYBR® Green Supermix (Bio-Rad), 1 µl of primer solution at a concentration of 5 μmol/l and 2.5 µl of RNase-free UltraPure™ DEPC-treated water for 39 cycles at 57°C. The samples were measured in triplicate.

### Western blotting

The proteins (P-gp, latrophilin-1 and GAPDH as an internal standard) were detected by Western blotting using a spe-

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**Table 1. RT-PCR conditions and primer sequences**

| Gene  | Primer sequences          | T_A (°C) | PCR products (bp) | Supplier                  |
|-------|--------------------------|----------|-------------------|---------------------------|
| ABCB1 | F: 5’-AAGTTGTATATGGTGGGAACCT-3’
R: 5’-ATTTTGGTACACCCAATTCITCATT-3’ | 57       | 429               | Merck s.r.o., Slovakia    |
| ACTB  | F: 5’-CTGGGACGACATGGGAGAAA-3’
R: 5’-AAGGAAAGCTTGGAGAGTCC-3’ | 54.4     | 564               | Microsynth AG, Switzerland|
| LPHN1 | F: 5’-ACCTCGACACAGGAGTCCAG-3’
R: 5’-GATCCAGGCGGATCAGCTAGA-3’ | 56.9     | 90                | Merck s.r.o., Slovakia    |
| LPHN3 | F: 5’-TGAGTCCGACACCAATCTG-3’
R: 5’-TCATACTAGAAATCTCGGCC-3’ | 60.0     | 198               | Merck s.r.o., Slovakia    |
Expression of P-gp is associated with LPHN1 downregulation

cific primary antibody in whole-cell lysates isolated from P-gp-negative and P-gp-positive SKM-1 and MOLM-13 cell variants. Cells were harvested by centrifugation (664 × g at 20°C) and washed twice in PBS, and then total cell proteins were obtained using a protein extraction kit (Merck s.r.o) according to the manufacturer's protocol. The proteins (30 μg per line) were separated via sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) on 8–16% polyacrylamide gradient gels (GeneScript, USA) using the Laemmli protocol (Laemmli 1970). The proteins were subsequently transferred by electroblotting onto nitrocellulose membranes (GE Healthcare Europe GmbH, Austria) using the Towbin protocol (Towbin et al. 1992). The rabbit polyclonal antibodies against GAPDH (FL-335 sc-25778 and 7947, goat polyclonal antibody against latrophilin-1 (D-20) sc-34484 (both from Santa Cruz Biotechnology, Inc, USA) and mouse monoclonal antibody c219 against P-gp (ENZO Life Sciences, USA) were used as primary antibodies at a 1:200 dilution. Goat anti-mouse, goat anti-rabbit and rabbit anti-goat polyclonal antibodies linked to horse radish peroxidase (all from Santa Cruz Biotechnology) were used as secondary antibodies at a 1:500 dilution. Bands were visualized with the aid of both an ECL detection system (GE Healthcare) and an Amersham Imager 600 (GE Healthcare) and quantified by densitometry.

Data processing

The qRT-PCR and Western blotting results were processed in triplicate and further analyzed by Student's t-test. The significance was then estimated at the level $p < 0.02$.

Results and Discussion

SKM-1/VCR and MOLM-13/VCR cells represent MDR variants of SKM-1 and MOLM-13 AML cells that express P-gp and manifest typical P-gp efflux activity measured in whole cells by the calcein retention assay (Imrichova et al. 2015). Both P-gp-positive cell variants were typical for P-gp-mediated MDR cells with cell resistance to P-gp substrates such as doxorubicin, vincristine and mitoxantrone (Pavlíková et al. 2016).

Parental SKM-1 and MOLM-13 did not express P-gp at the mRNA (Fig. 1) or protein level (Fig. 2). In contrast, P-gp-positive SKM1/VCR and MOLM-13/VCR cells express massive amounts of the \textit{ABCB1} transcript and P-gp protein.

Consistent with recently published findings (Sumbayev et al. 2016), SKM-1 and MOLM-13 cells, as typical AML cell models, express LPHN1 at the mRNA (Fig. 1) or protein levels (Fig. 2). However, the LPHN1 transcript and protein

![Figure 1](image-url). Cellular levels of \textit{ABCB1} and \textit{LPHN1} gene transcripts in SKM-1 (S), MOLM-13 (M), SKM-1/VCR (S\textsubscript{VCR}) and MOLM-13/ VCR (M\textsubscript{VCR}) cells estimated by RT-PCR. Left: Documentation of the respective gel with PCR products. The transcript for \textit{β-actin} gene \textit{ACTB} was used as the internal standard. The results were generated in triplicate independent measurements. Right: Quantification of the mRNA content by qRT-PCR. The data represent the mean ± S.E.M. from three independent measurements. The data for the SKM-1/VCR and MOLM-13/VCR cell variants differ significantly from the corresponding data for the SKM-1 and MOLM-13 cells at the level $p < 0.02$. 
levels are strongly downregulated in P-gp-positive SKM-1/VCR and MOLM-13/VCR. Three latrophilin proteins (latrophilin-1, -2, and -3) products of the LPHN1, LPHN2 and LPHN3 genes are expressed in the brain; of these, latrophilin-2 is also widely expressed in non-neuronal tissues (Boucard et al. 2014). Interestingly, depression of the LPHN1 transcript level in both P-gp-positive AML cell variants (Fig. 1) is associated with upregulation of the LPHN3 transcript level (Fig. 3).

The importance and function of LPHN1 expression in AML cells is not fully understood. All three latrophilins are adhesion-type G-protein-coupled receptors that are auxiliary α-latrotoxin receptors, suggesting that they may have a synaptic function (Lelianova et al. 1997; Sugita et al. 1998).

Conclusions
Both AML cell lines (SKM-1 and MOLM-13) express LPHN1 at the mRNA and protein levels, which is consistent with a recently published paper regarding the expression of this G-coupled receptor in AML cells (Sumbayev et al. 2016). However, after P-gp induction in these two cell lines by VCR, the levels of the LPHN1 transcript and protein were strongly downregulated. In contrast, the LPHN3 transcript level was elevated in the P-gp-positive AML cell variant used in the current study. Therefore, estimating the latrophilin
levels in AML cells may yield additional useful information for characterizing the AML cell status.

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