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Short Communication

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.

Acute myeloid leukemia (AML) is a heterogeneous group of malignant hematological disorders characterized by the clonal expansion of myeloid blasts in peripheral blood, bone marrow and/or other tissues (O'Donnell et al. 2012, 2017). AML accounts for 80% of all acute leukemia cases in adults. Recently, neuronal receptor latrophilin-1 (LPHN1) was found to be expressed in human monocytic leukemia cell lines and in primary human AML cells (Sumbayev et al. 2016). However, this expression is absent in healthy leukocytes. Moreover, LPHN1 was assumed to be a novel biomarker of human AML, which offers potential new avenues for AML diagnosis and treatment (Sumbayev et al. 2016).

Overexpression of P-glycoprotein (P-gp, an ABCB1 member of the ABC transporter gene family) represents the most often observed molecular cause of multidrug resistance (MDR) of neoplastic cells against chemotherapy (Breier et al. 2013). P-gp expression also plays a role in

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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% CO_2 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-

Table 1. RT-PCR conditions and primer sequences

Gene	Primer sequences	T_A (°C)	PCR products (bp)	Supplier
ABCB1	F: 5′-AAGTTGTATATGGTGGGAACT-3′ R: 5′-ATTTTGTCACCAATTCCTTCATT-3′	57	429	Merck s.r.o., Slovakia
ACTB	F: 5′-CTGGGACGACATGGAGAAAA-3′ R: 5′-AAGGAAGGCTGGAGAGTCC-3′	54.4	564	Microsynth AG, Switzerland
LPHN1	F: 5′-ACCTCGACACACGAGTCAGA-3′ R: 5′-GATCCAGGGCATCACGTAGA-3′	56.9	90	Merck s.r.o., Slovakia
LPHN3	F: 5′-TGAGTCCGACCACCAATCTG-3′ R: 5′-TCATACACTAGAAATCCTGTGCC-3′	60.0	198	Merck s.r.o., Slovakia

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 (Pavlikova 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 *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

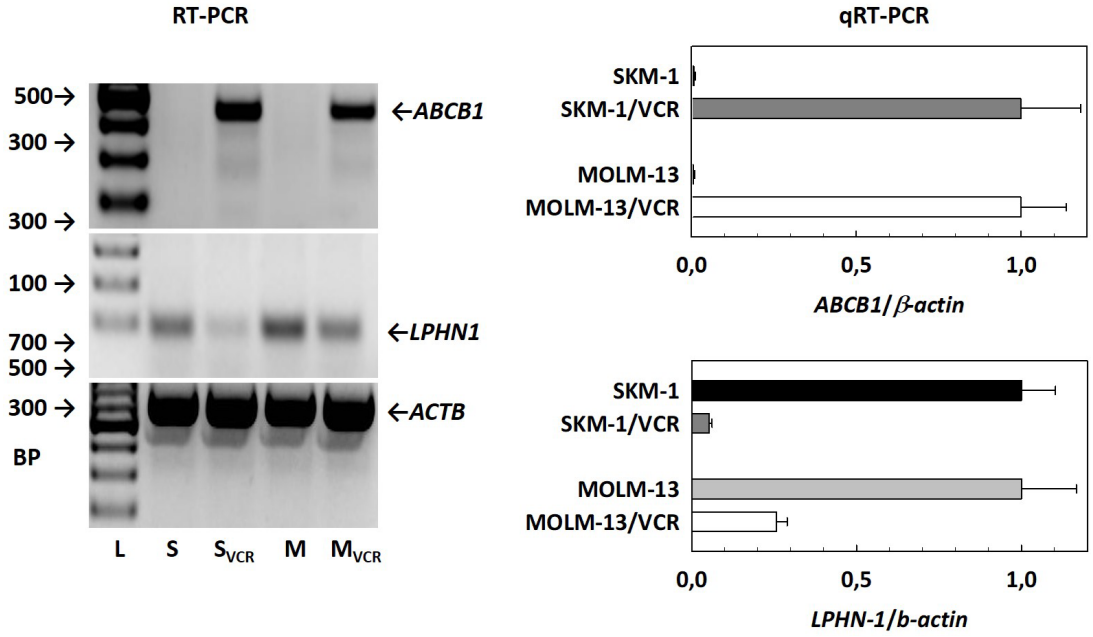

Figure 1. Cellular levels of *ABCB1* and *LPHN1* gene transcripts in SKM-1 (S), MOLM-13 (M), SKM-1/VCR (S_{VCR}) and MOLM-13/VCR (M_{VCR}) cells estimated by RT-PCR. Left: Documentation of the respective gel with PCR products. The transcript for β-actin gene *ACTB* was used as the internal standard. 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).

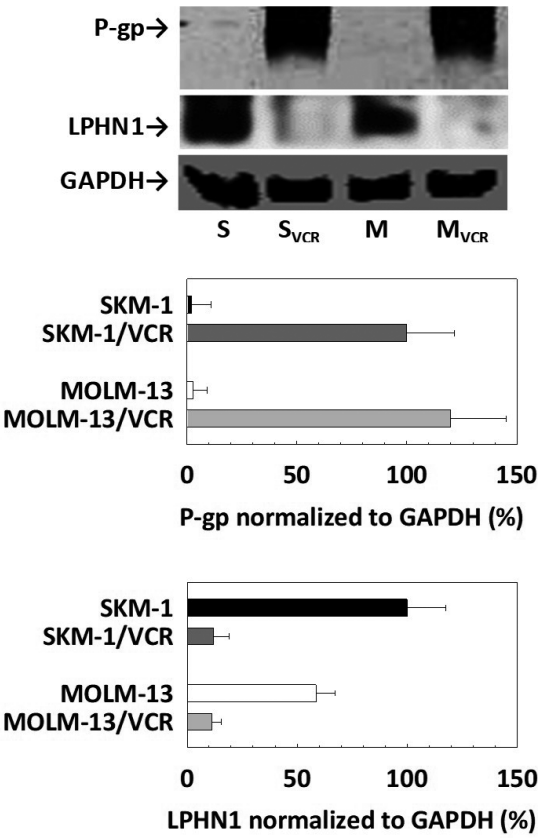

Figure 2. Cell contents of P-gp and latrophilin-1 in SKM-1 (S), MOLM-13 (M), SKM-1/VCR (S_{VCR}) and MOLM-13/VCR (M_{VCR}) cells estimated by Western blotting. Upper panel: Documentation of the respective blot with protein bands stained specifically with the respective antibodies (see the description of the Western blotting procedure). The results were generated in triplicate independent measurements. Middle and lower panels: Quantification of Western blotting by densitometry. 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 $p < 0.02$.

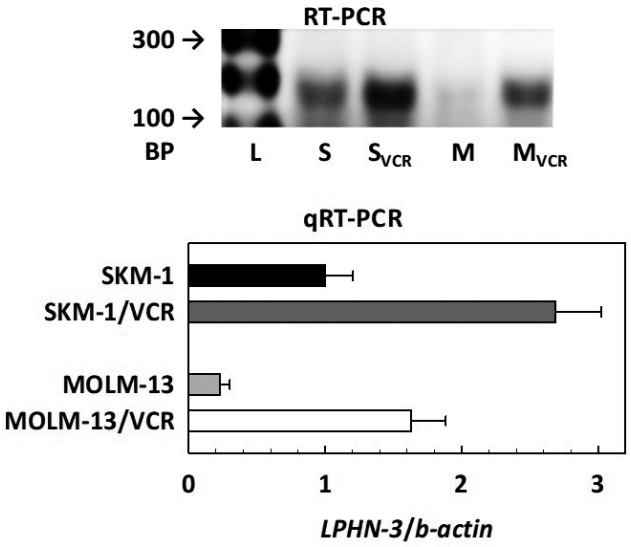

Figure 3. Cellular contents of the *LPHN3* gene transcripts in SKM-1 (S), MOLM-13 (M), SKM-1/VCR (S_{VCR}) and MOLM-13/VCR (M_{VCR}) cells estimated by RT-PCR. Upper panel: Documentation of the respective gel with PCR products. The β-actin gene *ACTB* transcript was used as the internal standard. The results were generated in triplicate independent measurements. Lower panel: 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 $p < 0.02$.

Only few and sometimes contradictory expression data on latrophilins in tumors and tumor cell lines are available (Aust 2010). However, interesting results regarding the function of LPHN1 in the etiopathogenesis of AML were published in 2017. Ectopically expressed LPHN1 in AML cells may secure their capability to escape the immune system by inactivating cytotoxic lymphoid cells *via* the ligand-dependent activation of latrophilin-1 and possibly other G-protein coupled receptors leading to increased translation and exocytosis of the immune receptor Tim-3 and its ligand galectin-9 (Goncalves Silva et al. 2017).

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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References

Aust G (2010): Adhesion-GPCRs in tumorigenesis. Adv. Exp. Med. Biol. **706,** 109–120 https://doi.org/10.1007/978-1-4419-7913-1_9

Boucard AA, Maxeiner S, Sudhof TC (2014): Latrophilins function as heterophilic cell-adhesion molecules by binding to teneurins: regulation by alternative splicing. J. Biol. Chem. **289,** 387–402 https://doi.org/10.1074/jbc.M113.504779

Breier A, Gibalova L, Seres M, Barancik M, Sulova Z (2013): New insight into p-glycoprotein as a drug target. Anticancer Agents Med. Chem. **13,** 159–170 https://doi.org/10.2174/187152013804487380

Broxterman HJ, Sonneveld P, van Putten WJ, Lankelma J, Eekman CA, Ossenkoppele GJ, Pinedo HM, Lowenberg B, Schuurhuis GJ (2000): P-glycoprotein in primary acute myeloid leukemia and treatment outcome of idarubicin/cytosine arabinoside-based induction therapy. Leukemia **14,** 1018–1024 https://doi.org/10.1038/sj.leu.2401796

Coculova M, Imrichova D, Seres M, Messingerova L, Bohacova V, Sulova Z, Breier A (2016): The expression of P-glycoprotein in leukemia cells is associated with the upregulated expression of nestin, a class 6 filament protein. Leuk. Res. **48,** 32–39 https://doi.org/10.1016/j.leukres.2016.05.021

Gao F, Dong W, Yang W, Liu J, Zheng Z, Sun K (2015): Expression of P-gp in acute myeloid leukemia and the reversal function of As2O3 on drug resistance. Oncol. Lett. **9,** 177–182 https://doi.org/10.3892/ol.2014.2692

Goncalves Silva I, Yasinska IM, Sakhnevych SS, Fiedler W, Wellbrock J, Bardelli M, Varani L, Hussain R, Siligardi G, Ceccone G, et al. (2017): The Tim-3-galectin-9 secretory pathway is involved in the immune escape of human acute myeloid leukemia cells. EBioMedicine **22,** 44–57 https://doi.org/10.1016/j.ebiom.2017.07.018

Imrichova D, Coculova M, Messingerova L, Sulova Z, Breier A (2014): Vincristine-induced expression of P-glycoprotein in MOLM-13 and SKM-1 acute myeloid leukemia cell lines is associated with coexpression of nestin transcript. Gen. Physiol. Biophys. **33,** 425–431 https://doi.org/10.4149/gpb_2014015

Imrichova D, Messingerova L, Seres M, Kavcova H, Pavlikova L, Coculova M, Breier A, Sulova Z (2015): Selection of resistant acute myeloid leukemia SKM-1 and MOLM-13 cells by vincristine-, mitoxantrone- and lenalidomide-induced upregulation of P-glycoprotein activity and downregulation of CD33 cell surface exposure. Eur. J. Pharm. Sci. **77,** 29–39 https://doi.org/10.1016/j.ejps.2015.05.022

Laemmli UK (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227,** 680–685 https://doi.org/10.1038/227680a0

Lelianova VG, Davletov BA, Sterling A, Rahman MA, Grishin EV, Totty NF, Ushkaryov YA (1997): Alpha-latrotoxin receptor, latrophilin, is a novel member of the secretin family of G protein-coupled receptors. J. Biol. Chem. **272,** 21504–21508 https://doi.org/10.1074/jbc.272.34.21504

O'Donnell MR, Abboud CN, Altman J, Appelbaum FR, Arber DA, Attar E, Borate U, Coutre SE, Damon LE, Goorha S, et al. (2012): NCCN clinical practice guidelines acute myeloid leukemia. J. Natl. Compr. Canc. Netw. **10,** 984–1021 https://doi.org/10.6004/jnccn.2012.0103

O'Donnell MR, Tallman MS, Abboud CN, Altman JK, Appelbaum FR, Arber DA, Bhatt V, Bixby D, Blum W, Coutre SE, et al. (2017): Acute myeloid leukemia. Version 3.2017, NCCN Clinical Practice Guidelines in Oncology. J. Natl. Compr. Canc. Netw. **15,** 926–957 https://doi.org/10.6004/jnccn.2017.0116

Pavlikova L, Seres M, Imrichova D, Hano M, Rusnak A, Zamorova M, Katrlik J, Breier A, Sulova Z (2016): The expression of P-gp in leukemia cells is associated with cross-resistance to protein N-glycosylation inhibitor tunicamycin. Gen. Physiol. Biophys. **35,** 497–510 https://doi.org/10.4149/gpb_2016039

Sugita S, Ichtchenko K, Khvotchev M, Sudhof TC (1998): alpha-Latrotoxin receptor CIRL/latrophilin 1 (CL1) defines an unusual family of ubiquitous G-protein-linked receptors. G-protein coupling not required for triggering exocytosis. J. Biol. Chem. **273,** 32715–32724 https://doi.org/10.1074/jbc.273.49.32715

Sumbayev VV, Goncalves Silva I, Blackburn J, Gibbs BF, Yasinska IM, Garrett MD, Tonevitsky AG, Ushkaryov YA (2016): Expression of functional neuronal receptor latrophilin 1 in human acute myeloid leukaemia cells. Oncotarget. **7,** 45575–45583 https://doi.org/10.18632/oncotarget.10039

Towbin H, Staehelin T, Gordon J (1992): Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Biotechnology **24,** 145–149

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