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ORIGINAL ARTICLE

Minor role of pregnane-x-receptor for acquired multidrug resistance in head and neck squamous cell carcinoma in vitro

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Abstract
Purpose Acquired multidrug resistance (MDR) has been linked to overexpression of drug-metabolising and transporting proteins mediated by pregnane-x-receptor (PXR). The aim of this work was to establish the relevance of PXR for MDR in head and neck squamous cell carcinoma (HNSCC).
Methods Using eight HNSCC cell lines, we determined the efficacy of paclitaxel, cisplatin and 5-fluorouracil (5-FU) via proliferation assays and determined the expression and activity of PXR through quantitative real-time polymerase chain reaction, western blotting and luciferase-based reporter gene assay. PXR knockdown approaches using shRNA-encoding vectors were applied to estimate the role of PXR for native MDR.
Results Drug resistance ranged between 5.2 and 620 nM for paclitaxel, varied between 4.5 and 58 μM for cisplatin, and varied between 1.1 and 5,467 μM for 5-FU. Lack of PXR mRNA expression was mostly accompanied by the absence of mRNA expression of cytochrome P450 3A4 (*CYP3A4*) and P-glycoprotein (P-gp, *ABCB1*) expression. Neither mRNA nor protein expression of PXR correlated with drug resistance. However, PXR activity tended to correlate with IC_{50} values of paclitaxel ($p = 0.08$). Knockdown of PXR in one of the cell lines had a slight but not significant impact on paclitaxel efficacy compared to scrambled sequence control. Surprisingly, only in two cell lines, PXR activity was increased by the well-known inductor rifampicin.
Conclusion This study suggests a malfunctioning of PXR and thus a minor relevance for iatrogenic chemotherapy resistance in HNSCC.

Keywords Cytostatics · Head and neck squamous cell carcinoma · Multidrug resistance · Pregnane-x-receptor

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Introduction

When head and neck squamous cell carcinoma (HNSCC) is treated with cytostatic drugs, multidrug resistance (MDR) can hamper the effectiveness of chemotherapy. Iatrogenic MDR acquired during previous cycles of chemotherapy seems to be of particular relevance, because studies enroling such patients for second-line treatment demonstrated considerably lower response rates than studies excluding pre-treated patients [1]. ATP-binding cassette (ABC) transporters are known to play an important role for MDR in various tumours. These energy-driven efflux pumps are considered to confer MDR by extruding anticancer agents

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or their metabolites from cells [2]. In HNSCC, many of these drug transporters are expressed and their expression levels are increased upon exposure to cytostatics used in HNSCC therapy, such as cisplatin, paclitaxel or 5-fluorouracil (5-FU) [3]. The expression of ABC-transporters is regulated by xenobiotic-sensing nuclear factors affecting the transcription of such target genes. Pregnane-x-receptor (PXR, NR1I2), also known as steroid and xenobiotic receptor, belongs to the orphan nuclear receptor family and is involved in the regulation of drug eliminating systems being the cause for drug–drug interactions and chemotherapy resistance [4–6]. The importance of PXR for drug-based cancer therapy is underlined by several studies demonstrating PXR to be activated by many different chemotherapeutic agents [5, 7, 8]. In addition, it has been shown that endometrial cancer tissues and cell lines express PXR variably and that the expression and inducibility of target genes are correlated with PXR levels [9]. From a clinical point of view, PXR has also been reported to be associated with the efficacy of chemotherapy and survival in particular cancer entities [10–12].

So far, for HNSCC, nothing is known about the relevance of PXR for cytostatics' efficacy. To assess the role of PXR for native and acquired MDR in HNSCC, this in vitro study investigated the association of mRNA, protein and activity levels of PXR with in vitro MDR in order to estimate whether the development of MDR and overexpression of drug transporters during chemotherapy is mechanistically linked to PXR activation. Thus, we first evaluated the differences in drug resistance towards paclitaxel, cisplatin and 5-FU within a set of human HNSCC cell lines differing in PXR mRNA and protein expression. Basal and drug-induced PXR activity was determined using a reporter gene approach, and in one cell line, also expressional and functional consequences of PXR knockdown were evaluated. Taken together, PXR might contribute to native paclitaxel resistance in HNSCC in vitro, but seems to be of minor importance for increased MDR acquired during chemotherapy due to obvious malfunctioning in PXR signalling.

Materials and methods

Materials

Culture media, medium supplements, antibiotics, aprotinin, dimethyl sulfoxide (DMSO), CITCO (6-(4-chlorophenyl) imidazo[2,1-b][1,3]thiazole-5-carbaldehyde-O-(3,4-dichlorobenzyl)oxime, phosphate-buffered saline (PBS), 5-FU, paclitaxel, the anti-β-actin antibody A-5316, the pLKO.1-puro-PXR2 vector and the pLKO.1-puro-scrambled vector were purchased from Sigma-Aldrich (Taufkirchen, Germany). The anti-PXR antibody H-11 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and the anti-mouse IgG, peroxidase linked secondary antibody NA931V from GE Life Sciences (Freiburg, Germany). Foetal calf serum (FCS) was purchased from PAA (Cölbe, Austria). Crystal violet, Tris (2-amino-2-(hydroxymethyl)-propan-1,3-diol), sodium dodecyl sulphate (SDS), glycerol, Tween 20, dithiothreitol (DTT) and rifampicin were purchased from AppliChem (Darmstadt, Germany). RIPA buffer was obtained from Thermo Scientific (Rockford, IL, USA). Methanol was obtained from Roth (Karlsruhe, Germany) and the BCA Protein Assay Kit and the ECL western blotting substrate from Pierce (Rockford, IL, USA). 96-well microtiter plates were from Nunc (Wiesbaden, Germany), and cell culturing bottles and white 96-well plates with clear bottom were from Greiner (Frickenhausen, Germany). Nitrocellulose membranes were from Schleicher & Schuell BioScience (Dassel, Germany). Cisplatin was obtained from the University Hospital's pharmacy. RNeasy Mini-Kit was obtained from Qiagen (Hilden, Germany), the RevertAid^{TM} H Minus First Strand cDNA Synthesis Kit from Fermentas (St. Leon-Rot, Germany) and the qPCR SYBR Green Mix from Abgene (Hamburg, Germany). Pefabloc was obtained from Serva (Heidelberg, Germany) and leupeptin, pepstatin and bromphenol blue from Biomol (Hamburg, Germany). pGL4.21, pGL4.74 [hRluc/TK] renilla vector and the Dual-Glo^{TM} Luciferase Assay System were obtained from Promega (Madison, WI, USA). Amaxa Cell Line Nucleofector Kit V was purchased from Lonza (Cologne, Germany). Coomassie blue was purchased from BioRad (Munich, Germany).

Cell lines

The HNSCC cell lines have been derived from intraoperatively obtained samples, established and characterised as reported previously [13]. Cell lines were classified according to their mRNA levels of PXR into PXR^{-} [expression under the detection limit in quantitative real-time polymerase chain reaction (qRT-PCR) using LightCycler technology] and PXR^{+} (high expression of PXR mRNA; Table 1). Suitability of cell lines for the representation of HNSCC was recently confirmed by comprehensive tumour cell biological characterisation [14]. Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10 % heat-inactivated FCS, 100 U/ml penicillin and 100 μg/ml streptomycin sulphates.

Proliferation assay

Cell proliferation was quantified as described previously [15]. Briefly, 30,000 cells per well were seeded into 96-well plates. Twenty-four hours after cell seeding, cell

Table 1 Patient data of HNSCC cell lines with (PXR+) and without (PXR−) detectable PXR mRNA expression

mRNA expression	Patient	Localisation	Age	TNM	G	Therapy	PFS	OS	M
PXR−	HNO124	Oral cavity	48	T2N2bM0	3	Op + Rad + adj Chemo	5	9	+
	HNO150	Larynx	47	T3N2bM0	2	Op + Rad	15	23	+
	HNO210	Larynx	69	T3NxMx	3	Op + Rad	2	17	−
	HNO97	Oral cavity	72	T3N2bM0	4	Op + Rad	1	6	−
	HNO432	Hypopharynx	58	T2N2bM0	2	Op + Rad + adj Chemo	68	68	−
PXR+	HNO206	Oropharynx	62	TxN3M0	3	Op + Rad	6	18	+
	HNO388	Nasal cavity	65	T2N0M0	3	Op + Rad	5	62	−
	HNO413	Oropharynx	83	T2N2bM0	2	Op	44	44	−

Localisation localisation of tumour; *Age* age in years at the time of diagnosis; *TNM* initial tumour staging according to UICC, *G* histological grading, *Op.* operation, *Rad.* radiation, *adj Chem.* adjuvant chemotherapy, *PFS* progression free survival in months, *OS* overall survival in months after surgery, *M* occurrence of distant metastases

culture medium was replaced by a fresh medium containing cytostatics drugs. For each cytostatic (paclitaxel, cisplatin, 5-FU), eight different concentrations (paclitaxel: 0.01–500 nM; cisplatin: 0.01–200 μM; 5-FU: 0.001–50 mM) were applied ensuring concentration ranges between no and profound proliferation inhibition. After 48 h of drug exposure, extent of proliferation inhibition was determined using crystal violet staining of surviving cells. Absorption of crystal violet was measured using a Multiskan RC photometer (ThermoFisherScientific, Bonn, Germany) with 555-nm absorption wavelength. Proliferation was expressed as proliferation index by calculating crystal violet absorption intensity as percentage relative to baseline (absorption intensity of wells with no cells, but medium only [set to 0 %]) and native proliferation (absorption intensity of untreated cells [set to 100 %]). Each concentration was tested in octuplet and each assay was performed four times.

Quantification of mRNA expressions by quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

RNA was isolated using the RNeasy Mini-Kit, and cDNA was synthesised with the RevertAid^{TM} H Minus First Strand cDNA Synthesis Kit according to the manufacturers' instructions. Expression of mRNA was quantified by qRT-PCR with a LightCycler® 480 (Roche Applied Science, Mannheim, Germany) using the SYBR Green format with the Absolute QPCR SYBR Green Mix. Primer sequences were published previously [16–18]. The following genes were quantified: *PXR*, *CAR* (encoding for constitutive androstane receptor), *CYP3A4* and *ABCB1* (encoding for P-glycoprotein [Pgp]). The most suitable housekeeping gene for normalisation was identified using geNorm (version 3.4, Center for Medical Genetics, Ghent, Belgium). Among the housekeeping genes tested (*β2-microglobulin, glucose-6-phosphate dehydrogenase, glucuronidase β, ribosomal protein L13* (*RPL13*), *hypoxanthine-phosphoribosyl-transferase 1, 60S* (*human*) *acidic ribosomal protein P1*), *RPL13* proved to be the most stable in the used HNSCC cell lines under the assay conditions. Data were evaluated by calibrator-normalised relative quantification with efficiency correction using LightCycler® 480 software as published previously [19]. Results are expressed as the ratio target gene/housekeeping gene divided by the corresponding ratio of the calibrator (HNO206 cell line). All samples were amplified in triplicate.

Quantification of protein expression by western blotting

After harvesting, cells were washed with PBS and lysed in RIPA buffer supplemented with leupeptin (5 μg/ml), pepstatin A (1 μg/ml), aprotinin (1 μg/ml) and pefabloc (1 mg/ml). Lysates were passed through a 25G needle and subjected to protein determination using BCA Protein Assay Kit. SDS-PAGE was performed with 8–40 μg protein per lane as described previously [18]. Proteins were blotted to a nitrocellulose membrane and incubated in blocking buffer (3 % skimmed milk in Tris-buffered saline with 0.3 % Tween 20) for 1 h. Membranes were incubated with primary antibodies for PXR (1:100, overnight) or β-actin (1:40,000, 1 h), washed 6 times for 7 min with TBS-Tween (0.3 %) buffer, incubated with the secondary antibody (1:2,000 in blocking buffer, 1 h) and further washed 4 times for 7 min with TBS-Tween (0.3 %) and 2 times for 5 min with TBS. Protein bands were developed with ECL western blotting substrate. Optical density of the bands was quantified using Image J Software (NIH, Bethesda, MD, USA). In knockdown experiments, PXR expression was normalised to β-actin expression. For the evaluation of basal PXR expression of the eight HNSCC cell lines, PXR protein expression was normalised to total lane protein content determined after staining with coomassie blue, because HNSCC cell lines exhibited different β-actin expression levels.

Reporter gene plasmid construction

A PXR reporter gene assay was applied to investigate the basal and drug-mediated increase in PXR activity in HNSCC cell lines.

Most of the PXR-responsive genes are characterised by the presence of conserved sequences such as direct repeats and everted repeats known as PXR-responsive elements in their 5′ regulatory sequences. PXR binding to these repeats favours the recruitment of the transcription machinery stimulating the target gene expression. The insertion of these regulatory sequences from a typical PXR target gene like human CYP3A4 upstream of a reporter gene and its further activation by a PXR ligand is considered a valid approach to assess PXR activation [20, 21].

The used reporter plasmid pGL4.21-CYP3A4-Luc was constructed according to Gu et al. [22], but using the vector pGL4.21 instead of pGL3-basic. Briefly, the CYP3A4 promoter (proximal response element module, PREM) comprising −362/+53 region and xenobiotic response element module (XREM; −7,836/−7,208) were amplified from human genomic DNA using specific primers, digested with respective restriction enzymes and consecutively cloned upstream from the firefly luciferase open reading frame of pGL4.21. Correct insertion was verified by sequencing (Eurofins MWG Operon, Ebersberg, Germany). Validity of the cloned reporter gene construct and feasibility of luciferase induction was proved using LS180 cells [23].

Transfection of HNSCC cells

2×10^6 cells were transfected with 2 µg of pGL4.21-CYP3A4-Luc and 0.2 µg of the pGL4.74 [hRluc/TK] renilla vector using Amaxa Cell Line Nucleofector Kit V and the electroporation device Nucleofector (Lonza, Cologne, Germany).

Measurement of PXR and CAR activity

After 24 h of transfection, medium was replaced by respective drug containing cell culture medium. Cells were incubated for 24 h with 20 µM rifampicin (PXR activator) or 1 µM CITCO (selective activator of CAR), respectively.

The reporter gene assay was performed with the Dual-Glo™ Luciferase Assay System according to manufacturer's instructions with minor modifications. Briefly, drug-containing medium was replaced by 40 µl of fresh cell culture medium and 40 µl of luciferin containing buffer. After 10 min of cell lysis on a plate shaker at room temperature, firefly luminescence was measured using a Glomax 96 microplate luminometer (Promega Corporation, Madison, WI, USA) after transfer into white-walled 96-well plates. After addition of 40 µl of Stop&Glo reagent and subsequent incubation under conditions mentioned above, renilla luminescence was measured.

Drug-induced increases of PXR activity were calculated by division of firefly luminescence by renilla luminescence (transfection efficiency control).

Knockdown of PXR

Sub-confluent HNO97 cells were trypsinised, diluted and transfected with 2 µg of pLKO.1-puro-PXR vector encoding a short hairpin RNA (shRNA; sense strand 5′-CGAGCTGTGTCAACTGAGATT-3′) directed against protein-coding region (1,840–3,144 bp) of the human PXR mRNA (transcription variant 1). Alternatively, cells were transfected with 2 µg of control vector (pLKO.1-puro-scrambled) encoding a non-silencing shRNA. Transfected cells were seeded either in T-25 culture flasks (for western blotting) or in 96-well plates for the evaluation of paclitaxel efficacy using proliferation assays. For PXR activity measurements, cells were additionally transfected with 2 µg of pGL4.21-CYP3A4-Luc and 0.2 µg of the pGL4.74 [hRluc/TK] plasmids. PXR activity was measured 72 h after transfection.

Statistical analysis

Statistical differences in drug resistance between cell lines with or without *PXR* mRNA expression were evaluated using unpaired, two-sided Student's *t*-test. Differences in basal PXR were evaluated using one-way ANOVA with Bonferroni's multiple comparison tests. Difference in reporter gene activation within the respective cell line with or without exposure to rifampicin or CITCO was evaluated using unpaired, two-sided Student's *t*-test. Correlation between PXR activity and paclitaxel, cisplatin, or 5-FU IC_{50} values was evaluated using Spearman correlation test with 95 % confidence intervals. Differences in PXR protein expression, PXR activity and paclitaxel IC_{50} values upon PXR knockdown were evaluated using unpaired, two-sided Student's *t*-test. A p value of ≤ 0.05 was considered significant.

Results

Cytostatic drug resistance of HNSCC cell lines

The HNSCC cell lines used are derived from distinct localisations within the upper aerodigestive tract and exhibit highly different clinical and pathological properties. For characterisation concerning drug resistance, proliferation assays were conducted. There were no statistical differences in the mean IC_{50} values between cell lines with or without *PXR* mRNA expression, respectively (Table 2).

mRNA expression levels of HNSCC cell lines

In general, PXR mRNA expression levels in our HNSCC cell lines were rather low. When quantification was reliably possible (PXR+ cell lines), crossing point values varied from 32 to 35. For comparison, in LS180 cells (established model for PXR expression and activity), crossing points are usually 22–24 and in the hepatoma cell line, HepG2 cells, the crossing points lay between 23 and 25. Besides mRNA levels of *PXR*, *CAR* and *ABCB1* encoding the most prominent ABC-transporter, we also evaluated expression levels of *CYP3A4*, an additional target gene of PXR. HNO206, HNO388 and HNO413 express mRNA of both *PXR* and *ABCB1*, whereas *CYP3A4* was only expressed in HNO206 and HNO388, but not in HNO413 (Fig. 1; Table 2). HNO97, HNO124, HNO150 and HNO210 did not show detectable levels of *PXR, ABCB1* or *CYP3A4*. In contrast, HNO432 demonstrated no mRNA expression of *PXR*, but expression of *CYP3A4* and very high levels of *ABCB1*. None of the cell lines expressed detectable CAR amounts at the mRNA level (Table 2).

PXR protein expression of HNSCC cell lines

Western blotting was used for the evaluation of PXR protein expression. Expression levels were quantified by normalising densitometry values to total protein content of the blotting membrane, because β-actin highly differed among HNSCC cell lines evaluated. PXR expression levels varied among HNSCC cell lines. HNO210 exhibited the highest PXR expression, whereas HNO124 showed the lowest protein expression (Fig. 2b). Protein expression levels of PXR did not correlate with mRNA levels. Moreover, there was no significant correlation between IC_{50} values of cytostatics and PXR protein expression.

PXR activity in HNSCC cell lines

Besides the evaluation of mRNA and protein expression level of PXR, we applied a reporter gene assay in order to scrutinise the basal and drug-induced activity of PXR. Rifampicin was used to selectively activate PXR.

Basal PXR activity varied among HNSCC cell lines used. HNO97 exhibited significantly higher basal activity than all other cell lines (Fig. 2a, white bars). When cell lines were exposed to 20 μM rifampicin for 24 h, only PXR activity in HNO124 and HNO150 was increased (Fig. 2a, black bars). PXR protein expression did not correlate with PXR activity. However, there was at least a borderline correlation between PXR activity and IC_{50} of paclitaxel ($p = 0.0831$; Spearman $r = 0.666$; Fig. 3).

As a control experiment, to estimate possible interferences with CAR, cell lines were also exposed to CITCO, a CAR activator. Firefly luminescence was never increased by CITCO (data not shown) excluding an influence of CAR on the reporter gene assay.

Knockdown of PXR

Because HNO97 exhibited the highest intrinsic PXR activity and the highest drug resistance, we performed a PXR knockdown and evaluated functional consequences. Compared to HNO97 cells being transfected with a control vector encoding a scrambled shRNA (set to 100 %), anti-PXR vector led to a significant 35 % reduction in PXR protein expression (Fig. 4a). This protein reduction was accompanied by a significant reduction to 46 % of PXR activity after 72 h (Fig. 4b). Moreover, IC_{50} of paclitaxel decreased from 5.7 to 4.0 nM (Fig. 4c). However, this sensitisation did not reach statistical significance.

Table 2 Summary of the investigated characteristics of the HNSCC cell lines

Cell line	PXR expression		PXR activity (arbitrary unit)		Drug resistance (IC_{50} values)			mRNA expression		
	mRNA	Protein	Basal	Rifampicin exposed	Paclitaxel (nM)	Cisplatin (μM)	5-FU (μM)	ABCB1	CYP3A4	CAR
HNO124	–	~	8.6 ± 0.7	17.4 ± 0.9[b]	9.2 ± 0.5	11.1 ± 0.6	3.1 ± 1.6	–	–	–
HNO150	–	++	2.6 ± 0.5	16.6 ± 8.3[b]	8.4 ± 0.4	15.6 ± 0.6	5.5 ± 1.2	–	–	–
HNO210	–	++	16.7 ± 4.0	12.2 ± 5.3	37.0 ± 9.7	10.9 ± 1.2	2.2 ± 1.7	–	–	–
HNO97	–	~	35.9 ± 6.8[a]	31.8 ± 11.4	620 ± 185	58.7 ± 4.2	5,467 ± 814	–	–	–
HNO432	–	+	1.1 ± 0.2	1.0 ± 0.2	27.5 ± 4.2	20.6 ± 4.1	1.1 ± 0.7	++	++	–
HNO206	+	+	0.8 ± 0.4	1.0 ± 0.5	5.2 ± 0.4	4.5 ± 0.5	1.2 ± 0.3	+	++	–
HNO388	+	++	2.8 ± 0.3	3.4 ± 0.2	147 ± 56	39.1 ± 1.1	31.8 ± 12.8	+	+	–
HNO413	++	++	2.2 ± 0.5	2.3 ± 0.5	19.7 ± 2.0	34.0 ± 1.1	36.6 ± 8.2	+	–	–

[a] Statistically different basal PXR activity (one-way ANOVA with Bonferroni's multiple comparison tests)

[b] Statistically different PXR activity after 24 h exposure to 20 μM rifampicin (unpaired, two-sided Student's *t*-test)

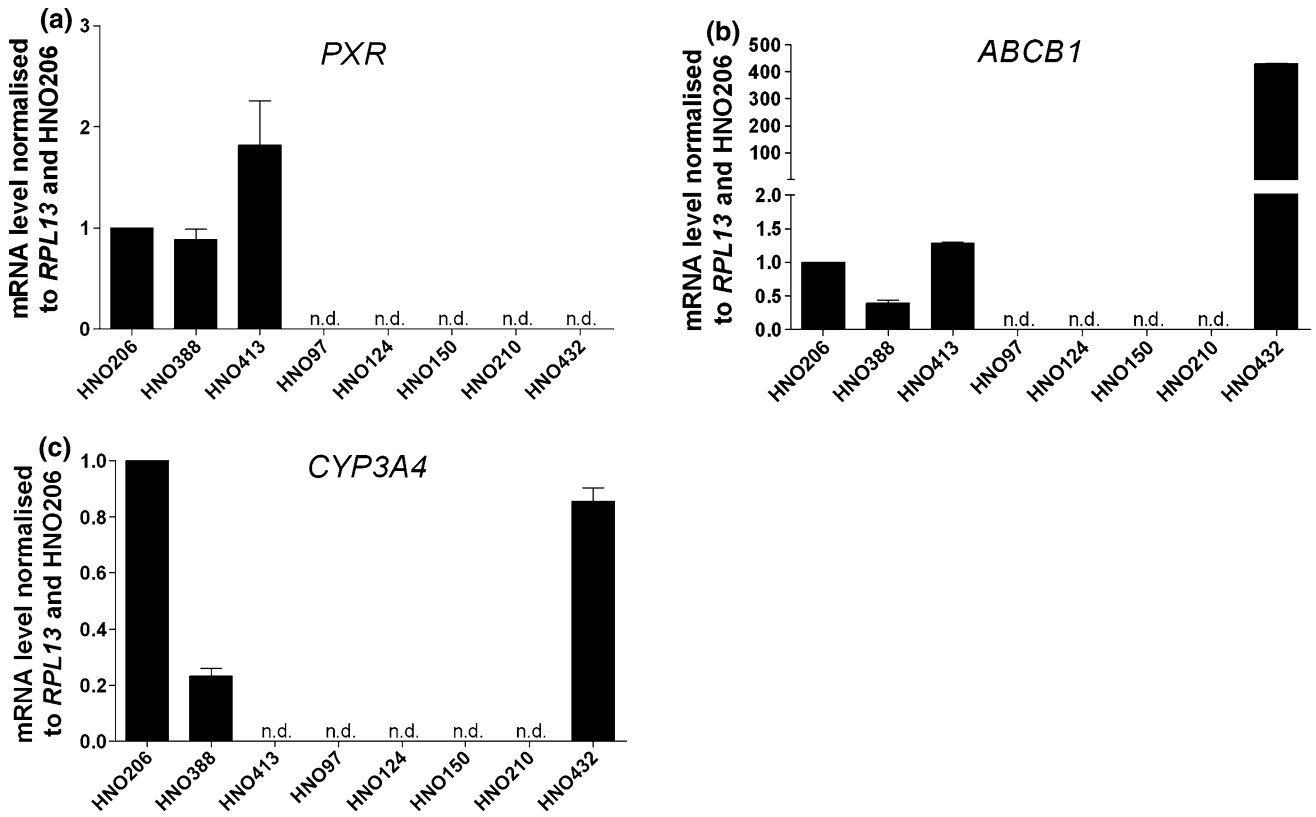

Fig. 1 mRNA expression of *PXR* (**a**), *ABCB1* (**b**) and *CYP3A4* (**c**), in HNSCC cell lines. mRNA expression was normalised to *RPL13* gene. The expression in HNO206 cells was used as calibrator. Data are expressed as mean ± SD of $n = 3$. *n.d* not detectable

Discussion

Disease recurrence is an unsolved problem in the therapy of HNSCC. The relapsing tumours are generally considered aggressive and platinum-resistant. Patients with previous chemotherapy demonstrated lower response rates to second-line treatment than patients who were not pretreated [1]. This indicates a crucial role of acquired MDR in HNSCC. However, the molecular characteristics and mechanistical tropism of this condition have not been thoroughly investigated so far. Such missing information precludes the development of targeted strategies or optimised dosing schedules to overcome acquired MDR or to predict the course of disease.

Xenobiotic-sensing factors have been linked to the induction of drug-metabolising and transporting proteins and subsequent alterations of drug kinetics and efficacy [4–6]. Amongst other transcription factors, PXR is considered a "master regulator" for drug elimination and its role in chemotherapy resistance has been experimentally underlined [24]. However, in case of HNSCC, the impact of PXR on drug resistance is unknown. Moreover, it also remains debatable whether increased MDR and drug transporter overexpression of tumours during chemotherapy is truly caused by increased transcription of target genes mediated by nuclear receptors, such as PXR [25]. Alternatively, it might also result from selection processes promoting intrinsically resistant cells [26]. For instance, genetic instability as a prerequisite for such phenomena [27, 28] has already been described in HNSCC [29, 30]. To the best of our knowledge, this is the first study comprehensively examining the role of PXR activity in HNSCC.

In the cell lines used, lack of *PXR* mRNA expression was mostly associated with undetectable levels of *ABCB1* and *CYP3A4* (Table 2). Such interdependences have already been proposed in other cancer cells [31]. Except HNO432, HNSCC cell lines lacking *PXR* mRNA expression did also not express detectable mRNA levels of *ABCB1* confirming a tight relationship of *ABCB1* with *PXR* [5]. Strikingly, mRNA levels of PXR did hardly match with protein expression. For instance, HNO150 exhibited considerable protein expression, but undetectable levels of mRNA. In contrast, HNO206 showed obvious mRNA expression of PXR, but the respective protein was only weakly expressed. These findings indicate that in HNSCC, the transition from PXR mRNA to protein might be complexly regulated by post-transcriptional mechanisms, such as miRNA-148a [32]. This regulatory RNA binds sequence specifically at the mRNA of PXR and can subsequently diminish its translation into protein. Since neither the

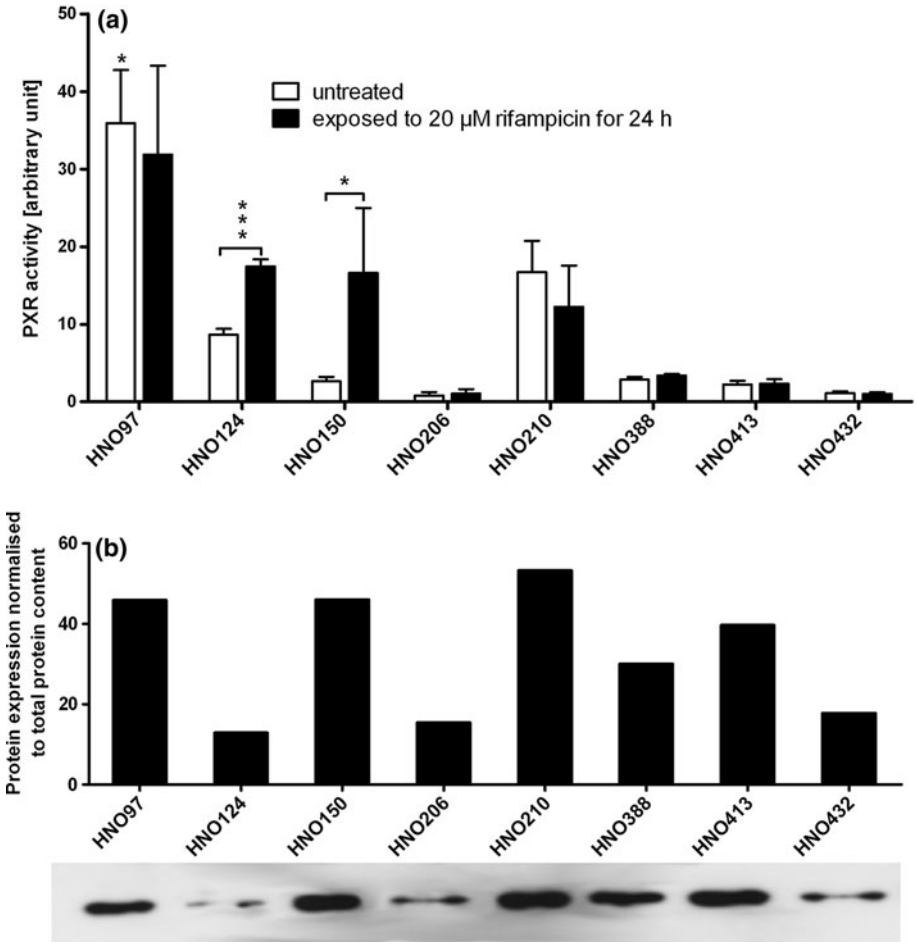

Fig. 2 **a** PXR activity in HNO cells. Basal PXR activity (*white bars*) and PXR activity after 24 h exposure to 20 µM rifampicin (*black bars*) were evaluated by the measurement of luciferase luminescence normalised to renilla luminescence. Data are expressed as mean ± SD of $n = 3$. *$p < 0.05$; ***$p < 0.001$. **b** Protein expression of PXR in HNO cells. Western blot analysis of whole cell lysates of HNSCC cell lines. PXR protein expression (evaluated by densitometry) was normalised to total protein content (evaluated by coomassie blue staining of blotting membrane). Western blots were performed in triplicate and a representative experiment is shown

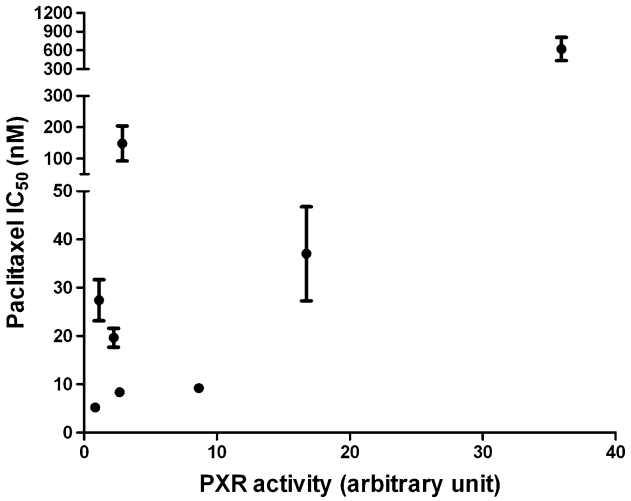

Fig. 3 Correlation of basal PXR activity with paclitaxel cytotoxicity. IC_{50} values obtained after 48 h treatment with paclitaxel (*y*-axis) were correlated with the basal activity results from reporter gene assays (*x*-axis). Spearman correlation coefficient ($r = 0.666$) was evaluated with 8 XY pairs corresponding to the 8 assessed cell lines. $p = 0.0831$

growth rate (unpublished results) nor mRNA or protein expression levels of PXR correlated with IC_{50} values of the cytostatics evaluated, we also determined PXR activity. In general, basal PXR activity tended to correlate with drug resistance to paclitaxel (Fig. 3). In particular, HNO97 exhibited both considerable drug resistance (Table 2) and high basal activity of PXR (Fig. 2b, white bars). Assuming a causal relationship, we performed a PXR knockdown approach in HNO97 to reduce PXR activity. Down-regulation of PXR protein expression and activity (Fig. 4a, b) only slightly diminished paclitaxel resistance (Fig. 4c). To assess whether increased expression of drug transporters after chemotherapy is due to the activation of tumour cell endogenous PXR, we intended to evaluate whether HNSCC cell lines' PXR is activatable. We exposed the HNSCC cell lines to 20 µM rifampicin, a well-known and potent activator of PXR. Very surprisingly, rifampicin increased PXR activity in only two out of eight cell lines (Fig. 2b) proposing a functional perturbation in PXR signalling. PXR activation only worked properly in those cell lines (HNO124, HNO150) that have been derived from

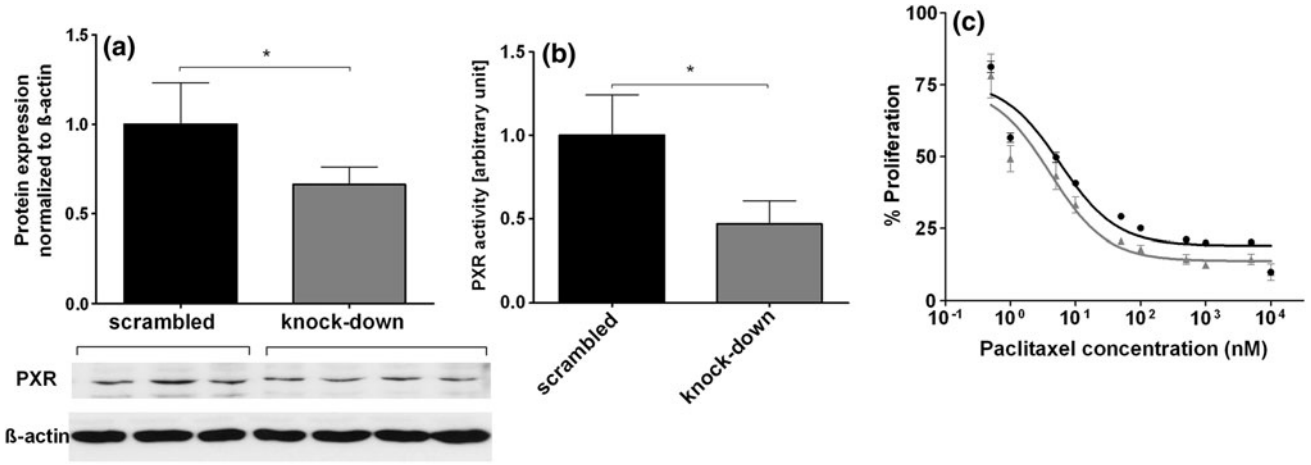

Fig. 4 Expressional and functional consequences of PXR knockdown in HNO97. **a** PXR protein (scrambled control *black bar*, knockdown *grey bar*) expression was normalised to β-actin expression; **b** PXR activity (scrambled control *black bar*, knockdown *grey bar*) was determined using reporter gene assay; **c** proliferation inhibition by paclitaxel in scrambled control cells (IC_{50}: 5.7 nM, *black curve*) and knockdown cells (IC_{50}: 4.0 nM, *grey curve*). Data are expressed as mean ± SD of $n = 3$–4. *$p < 0.05$

patients suffering from distant metastasis (Table 1) suggesting additional role of PXR for malignant phenotype [33]. One possible reason for the observed PXR dysfunction might be polymorphisms leading to altered protein structure and function. For instance, there are at least two documented mutations leading to altered potency of PXR to enhance transcription of its target genes [34]. The generally missing vigour of rifampicin treatment could additionally be explained by low expression of essential cofactors or post-translational regulation of PXR through cyclic AMP-dependent kinases [35] also explaining the striking association of high PXR protein expression with low activity in some of our cell lines. However, these causalities were not our primary object, although these molecular reasons for PXR malfunctioning in HNSCC are well worthy to be evaluated. In contrast, we intended to properly document this PXR dysfunction in HNSCC for the first time. Thus, in consequence, these results suggest acquired MDR and transporter overexpression upon chemotherapy in HNSCC to be seldom caused by transcriptional mechanisms mediated by PXR. Although the cell lines tested did not express CAR mRNA, we also evaluated CITCO, a well-known and selective activator of CAR, to exclude interferences of this xenobiotic sensor with the cloned response elements known to bind both PXR and CAR. However, CITCO did not show any effect either suggesting these nuclear receptors to be of minor relevance for acquired increases of drug resistance in HNSCC.

Our results demonstrate PXR expression in HNSCC and propose that basal PXR activity in HNSCC might at least in part determine sensitivity to cytostatics, such as paclitaxel. However, in contrast to findings in other tumour entities, activity of PXR is hardly increasable in HNSCC, thus questioning the hypothesis of enhanced MDR gene transcription as a consequence of PXR activation.

In conclusion, acquired chemotherapy resistance and overexpression of drug transporters in HNSCC upon exposure to cytostatics is unlikely related to increased transcription of MDR genes mediated by PXR but possibly caused, for example, by selection of intrinsically resistant cells.

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Conflict of interest None.

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