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Human papilloma virus (HPV) 18 proteins E6 and E7 up-regulate ABC transporters in oropharyngeal carcinoma. Involvement of the nonsense-mediated decay (NMD) pathway

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ABSTRACT

Oropharyngeal cancer incidence increased dramatically in the last decades, being infection with human papillomaviruses (HPV) a determinant of this trend. Concerning etiology, treatment response and prognosis, HPV^+ and HPV^- oropharyngeal cancers constitute different disease entities. The underlying molecular background is not completely understood. ATP-binding cassette (ABC) transporters mediate the efflux of anticancer drugs and are regulated by changes in the intracellular milieu. Furthermore, a role in cancer pathogenesis besides drug transport was reported. We evaluated the effect of transfection with E6 and E7 oncogenes from HPV16 and HPV18 on ABC transporters in oropharyngeal cancer cells. HPV18E6/E7 up-regulated P-glycoprotein (P-gp), multidrug resistance-associated protein 1 (MRP1) and MRP2 expression in HNO206 cells and breast cancer resistance protein (BCRP) in HNO206 and HNO413 cells. While P-gp was regulated translationally, MRP1, MRP2 and BCRP up-regulation resulted from mRNA stabilization. For MRP1 and MRP2, the nonsense-mediated decay pathway was involved. In general, resistance to substrates of up-regulated transporters was increased. Transfection with oncogenes individually indicated a major role of HPV18E7. Our findings suggest ABC transporters as molecular players leading to differences in the pathogenesis of HPV^+ and HPV^- oropharyngeal cancer.

1. Introduction

Oropharyngeal squamous cell carcinoma accounts for about 3% of all cancer cases worldwide. Only in the United States, more than 16000 new cases are diagnosed every year [1]. While the incidence of other types of head and neck squamous cell carcinomas (HNSCC) has decreased in the last years, probably due to a reduction in tobacco and alcohol consumption, the incidence of oropharyngeal carcinoma exhibited an alarming increase. In this regard, infection with high risk types of the human papillomavirus (HPV) (e.g. HPV16, HPV18) increased from about 16% of the oropharyngeal cancer cases between 1984 and 1989 to more than 70% between 2000 and 2004, probably contributing to this increasing trend [2]. HPV exerts its oncogenic effect by interaction of the viral oncoproteins E6 and E7 with several host proteins. Among them, inhibition of the function of tumor suppressor proteins like p53 and retinoblastoma (Rb) results in loss of control of the cell cycle and cell proliferation, contributing to malignant transformation [3]. In addition, several other cellular targets of E6 and E7 were identified, whereby the interaction profile clearly differs between oncoproteins from different virus types [4]. This strong modification of the intracellular milieu by HPV contributes to HPV^+ oropharyngeal cancer being a different disease entity from the HPV^- disease.

ABC (ATP-binding cassette) transporters are transmembrane

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proteins localized in the plasma membrane of normal and tumor cells, where they mediate the efflux of a wide variety of endo- and xenobiotics. Since most of the chemotherapeutic agents administered in the clinical practice are substrates of one or more ABC transporters, the latter are considered key determinants of the response to chemotherapy and disease prognosis [5]. Among the ABC superfamily, P-glycoprotein (P-gp/*ABCB1*), multidrug resistance-associated proteins 1 and 2 (MRP1/*ABCC1* and MRP2/*ABCC2*) and the breast cancer resistance protein (BCRP/*ABCG2*) have been related to multidrug resistance and therapy failure in several tumor diseases [6]. Due to their major role in the maintenance of intracellular homeostasis, ABC transporters are usually regulated in response to fluctuations in the intracellular environment, as could be a viral infection [5,7]. Transcriptional- (e.g. nuclear receptors), post-transcriptional- (e.g. mRNA stabilization), translational- (e.g. microRNAs) and post-translational- (e.g. dynamic localization) mechanisms have been reported to be involved in ABC transporter modulation [5,7,8]. Although transporter expression in HNSCC is well-acknowledged [9,10], little is known about the effect of HPV infection on ABC transporter expression. Therefore, the aim of the current work was to evaluate the modulation of P-gp, MRP1, MRP2 and BCRP by E6 and E7, viral proteins involved in most interactions with the host cell, from the high-risk types HPV16 and HPV18 in oropharyngeal cancer cell lines as well as the underlying molecular mechanisms.

Our data indicate transporter up-regulation mainly by HPV18 derived oncoproteins. Two different molecular mechanisms consisting of translational regulation for P-gp and mRNA stabilization for MRP1, MRP2 and BCRP were identified, the latter being, at least in part, due to a negative modulation of the nonsense-mediated decay (NMD) pathway by HPV18 oncoproteins. These results suggest a potential role of ABC transporters contributing to the differential pathogenesis of HPV^{+} oropharyngeal carcinoma.

2. Materials and methods

2.1. Materials

Cell culture media and supplements, cisplatin, LY335979 (zosuquidar), MK571 and the GenElute Mammalian Total RNA Miniprep Kit were from Sigma-Aldrich (Taufkirchen, Germany). RevertAid H Minus First Strand cDNA Synthesis Kit and Absolute QPCR Mix SYBR Green, no ROX were from Thermo Fisher Scientific (Waltham, USA). Primers for qRT-PCR were from Eurofins Genomics (Ebersberg, Germany). Actinomycin D and cycloheximide were from Santa Cruz Biotechnology (Heidelberg, Germany). Fumitremorgin C (FTC) and NMDI14 were from Merck (Darmstadt, Germany). Crystal violet, 5-fluorouracil (5-FU) and paclitaxel were from Applichem (Darmstadt, Germany). All reagents were of analytical grade purity or higher.

2.2. Cell lines and transfection

HNO cell lines were derived from intraoperatively acquired samples. The generation and characterization of the cell lines have already been described [11]. For the current study HNO206 and HNO413 HPV^{-} oropharyngeal cancer cells were used (see supplementary material). Culture media and conditions were already described [12]. Cells transiently expressing E6 and E7 from HVP16 or HPV18 were generated by electroporation using the Cell Line Nucleofector Kit V (Lonza, Basel, Switzerland). Briefly, cells were pelleted and resuspended in V-solution (2×10^6 cells in 100 μL). Following, the plasmids pcDNA3-HPV16E6 and pCMV-HPV16E7-HA-Flag (HPV16 E6/E7 cells), pcDNA3-HPV18E6 and pCMV-HPV18E7-HA-Flag (HPV18 E6/E7 cells) or the empty vectors (0.5 μg each) were added and cells were transfected using a Nucleofector 2b device (Lonza, Basel, Switzerland). All plasmids were kindly provided by Drs. Felix and Karin Hoppe-Seyler (German Cancer Research Center, Heidelberg, Germany) [13]. To assess the separated effects of E6 or E7 from HPV18, cells were transfected either with both empty vectors, with pCMV (empty) and pcDNA3-HPV18E6 plasmids (E6 expressing cells) or with pcDNA3 (empty) and pCMV-HPV18E7-HA-Flag plasmids (E7 expressing cells). Transfected cells were seeded in T-25 flasks (2×10^6 cells/flask) for protein and mRNA expression studies or 96-well plates (14000 cells/well and 19000 cells/well for HNO206 and HNO413 cells, respectively) for cell viability assays, cultured for 72 h and processed as described below. Expression of the viral oncogenes was evaluated at the mRNA level through RT-PCR followed by agarose gel electrophoresis (see supplementary material).

2.3. mRNA and protein expression analysis

mRNA expression was assessed in HPV16 E6/E7-, HPV18 E6/E7- and empty vector-transfected cells through qRT-PCR using a LightCycler®480 (Roche Applied Science, Mannheim, Germany) as described [14]. Primer sequences were described previously [15]. β2-microglobuline (β2-mg) was used as housekeeping gene, after being the most stable within a panel of 7 potential housekeeping genes, as determined using geNorm (version 3.4., Center for Medical Genetics, Ghent, Belgium) [12].

Protein expression was evaluated through Western blot as previously described [12]. Primary antibodies used were anti-P-gp (C219, Merck Millipore, Darmstadt, Germany), anti-MRP1 (MRPr1, Kamiya Biomedical Company, Tukwila, USA), anti-MRP2 (M_2-III-6, Enzo Life Sciences, Farmingdale, USA) and anti-UPF1 (C6), anti-BCRP (BXP21) and anti-GAPDH (G9) (Santa Cruz Biotechnology, Heidelberg, Germany). Optical density was analyzed using ImageJ (National Institutes of Health, Bethesda, USA).

2.4. mRNA and protein stability determination

For transporters showing modulation by E6/E7 at the mRNA level, we evaluated whether this effect results from transcriptional up-regulation or mRNA stabilization. For this purpose, cells were transfected and incubated with the transcription inhibitor actinomycin D (5 μg/ml) for different times (0–21 h) before sample preparation. Following, RNA was isolated and qPCR was performed as described in 2.3.

To elucidate the mechanism underlying P-gp up-regulation by HPV18 E6/E7 at the protein level, cells were transfected and incubated with cycloheximide (100 μM) for 0–16 h, as a translation inhibitor. Subsequently, protein samples were obtained and P-gp expression was analyzed as described in 2.3.

2.5. Participation of the nonsense-mediated decay (NMD) pathway

NMD involvement in E6/E7 effect on ABC transporter expression was first evaluated using NMDI14 as inhibitor of the pathway (50 μM, added 2 h after transfection). NMDI14 mechanism of action bases on the disruption of the interaction between UPF1 (up-frameshift protein 1) and SMG7 [16]. After NMDI14 addition, cells were further cultured for 70 h and mRNA samples were prepared and analyzed as described in 2.3.

In addition, we evaluated NMD participation using a loss-of-function model. For this purpose, cells were simultaneously transfected with the corresponding E6 and E7 plasmids as described in 2.2 and with the plasmid pLKO.1-UPF1 (clone TRCN0000412485, Sigma-Aldrich) codifying a shRNA directed against human UPF1 (UPF1 kd cells) or with pLKO.1-Scr vector,

codifying a scrambled non-silencing shRNA. 72 h after transfection, cells were collected and UPF1 protein expression was analyzed as described in 2.3.

2.6. Drug resistance assays

To evaluate whether E6/E7 effects on ABC transporter expression lead to changes in the resistance towards substrate drugs, transfected cells were exposed to paclitaxel (0.01–500 nM), cisplatin (0.01–200 μM) and 5-FU (0.0005–25 mM), chemotherapeutic agents used in HNSCC treatment and substrates of P-gp [5], MRP2 [5] and BCRP [17], respectively. Participation of ABC transporters was verified coincubating the cells with LY335979 (P-gp inhibitor, 1 μM), MK571 (MRP inhibitor, 20 μM) or FTC (BCRP inhibitor, 1 μM) [18,19]. Cell viability was evaluated through crystal violet staining as described [12].

2.7. Statistical analysis

All data are expressed as mean ± standard deviation. All statistical analyses were performed using GraphPad Prism 7.0 (GraphPad Software, La Jolla, USA). Statistical comparisons were performed by using the Student's t-test or one-way ANOVA followed by Tukey's post-hoc test for two or more than two experimental groups respectively. For mRNA and protein stability assays, expression data were plotted as a function of the exposure time to actinomycin D or cycloheximide and fitted to a linear model. $t_{1/2}$ was obtained by interpolation in the curve equation. Cell viability data was fitted to a sigmoidal curve by non-linear regression and IC_{50} values were calculated as the concentration of chemotherapeutic agent leading to a decrease of 50% in cell viability.

3. Results

3.1. Effect of HPV E6/E7 on ABC transporter expression

The effects of E6/E7 from HPV16 and HPV18 on ABC transporters were evaluated at protein and mRNA levels. HPV18 E6/E7 resulted in a clear increase in P-gp (Fig. 1a), MRP1 (Fig. 1b), MRP2 (Fig. 1c) and BCRP (Fig. 1d) protein levels in HNO206 cells. No changes in the protein expression of either transporter by HPV16 E6/E7 in HNO206 cells were observed (Fig. 1a–d). Similarly, an increase in BCRP protein levels only by HPV18 E6/E7 in HNO413 cells was observed (Fig. 1e). No alterations in P-gp, MRP1 or MRP2 protein expression either by HPV16- or by HPV18 E6/E7 in HNO413 cells were observed (data not shown).

Contrarily to the observations at the protein level, *ABCB1* mRNA expression (codifying P-gp) exhibited a clear decrease in HPV18 E6/E7-expressing HNO206 cells (Fig. 1f), evidencing a dissociation between protein and mRNA levels and suggesting a translational or

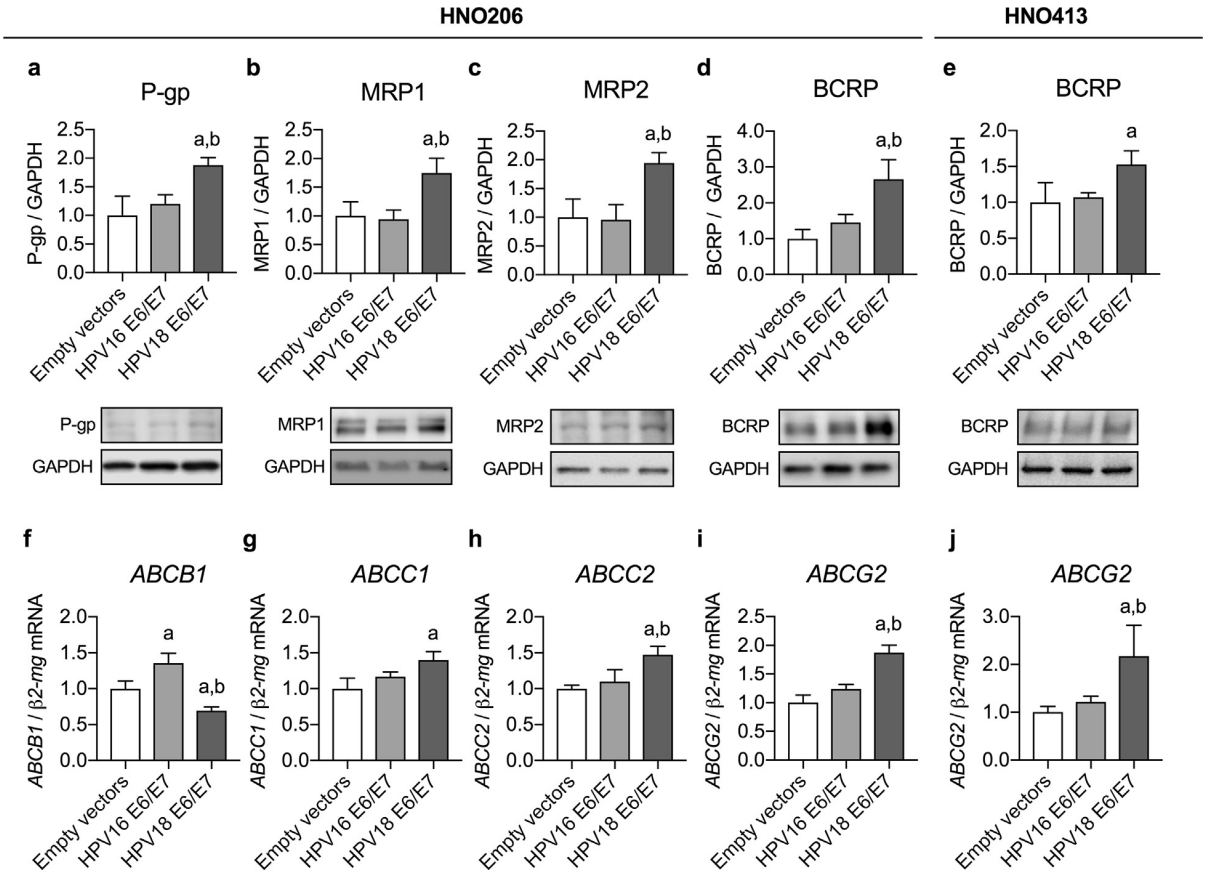

Fig. 1. Effect of HPV E6/E7 on ABC transporter expression. Oropharyngeal cancer cells were transfected with E6 and E7 from HPV16 or HPV18. Control cells were transfected with the corresponding empty vectors. Protein expression was determined through Western blot for P-gp (**a**), MRP1 (**b**), MRP2 (**c**) and BCRP (**d**) in HNO206 cells and for BCRP in HNO413 cells (**e**). The optical density of the target proteins was normalized to the optical density of GAPDH (loading control). Representative blots are shown. mRNA expression was evaluated through qRT-PCR for *ABCB1* (**f**), *ABCC1* (**g**), *ABCC2* (**h**) and *ABCG2* (**i**) in HNO206 cells and for *ABCG2* (**j**) in HNO413 cells. *β2-mg* was used as housekeeping gene. All results are presented as % of control and expressed as mean ± standard deviation. a: statistically different from empty vectors, b: statistically different from HPV16 E6/E7. $p < 0.05$, $n = 3–4$.

post-translational mechanism. Conversely, *ABCC1* and *ABCC2* mRNA levels (codifying MRP1 and MRP2, respectively) exhibited a significant increase by HPV18 E6/E7 in HNO206 cells (Fig. 1g and h, respectively), agreeing well with the increase at the protein levels described above and indicating a transcriptional or post-transcriptional modulation. Similarly, higher *ABCG2* mRNA levels (codifying BCRP) in response to HPV18 E6/E7 were observed in both HNO206 (Fig. 1i) and HNO413 cells (Fig. 1j), also correlating with the increased protein levels in these cells. These data support the presence of, at least, two different regulatory mechanisms (i.e. modulation at the protein level for P-gp and modulation at the mRNA level for MRP1, MRP2 and BCRP) both resulting in the increased protein levels.

3.2. Effect of HPV18 E6/E7 on protein and mRNA stability

As a first approach to elucidate the mechanism leading to increased mRNA or protein expression, we evaluated the mRNA- or protein decay in the presence of actinomycin D or cycloheximide, respectively. In HNO206 cells, P-gp $t_{1/2}$ was not significantly modified between empty vector- and HPV18 E6/E7 transfected cells (Fig. 2a), thus ruling out a protein stabilization by E6/E7 and indicating a translational mechanism. *ABCC1* (Fig. 2b) and *ABCC2* mRNAs (Fig. 2c) in HNO206 cells as well as *ABCG2* mRNA both in HNO206 (Fig. 2d) and HNO413 cells (Fig. 2e) exhibited a clear stabilization by HPV18 E6/E7, as evidenced by the respective increases in their $t_{1/2}$.

3.3. Involvement of the nonsense-mediated decay (NMD) pathway in ABCC1, ABCC2 and ABCG2 stabilization by HPV18 E6/E7

The NMD pathway represents a RNA surveillance mechanism leading to degradation of mRNAs with premature termination codons. Recently, an important NMD role also in the degradation and thus in the modulation of the expression of physiological mRNAs was demonstrated [20]. We hypothesized a negative modulation of the NMD by HPV18 E6/E7 as a possible mechanism leading to the observed increase in the transporter mRNA stability. To evaluate this possibility, we used the synthetic inhibitor NMDI14, disrupting the key step of the NMD. Indeed, addition of NMDI14 fully prevented the increases in *ABCC1* and *ABCC2* mRNA expression by HPV18 E6/E7 in HNO206 cells compared to empty vector transfected cells (Fig. 3a and b, respectively), clearly suggesting a participation of this pathway. On the contrary, an increase in *ABCG2* mRNA levels by HPV18 E6/E7 was still observed in presence of NMDI14 in both HNO206 and HNO413 cells (Fig. 3c and d, respectively), thus indicating a minor role of a UPF1-SMG7-dependent NMD mechanism in *ABCG2* stabilization by E6/E7.

Furthermore, we evaluated NMD participation in a loss-of-function model (UPF1 knockdown). Under these conditions, a significant reduction in UPF1 protein expression in HNO206 (Fig. 4a) and HNO413 cells (Fig. 4b) was observed. Moreover, UPF1 knockdown fully prevented *ABCC1* (Fig. 4c) and *ABCC2* mRNA increase (Fig. 4d) by HPV18 E6/E7 in HNO206 cells, in line with the results using NMDI14. Similarly, UPF1 knockdown prevented the increase in MRP1 (Fig. 4e) and MRP2 protein expression (Fig. 4f) by HPV18 E6/E7, further supporting a role of the NMD pathway. On the contrary, UPF1 knockdown did not prevent *ABCG2* up-regulation by HPV18 E6/E7 in HNO206 (Fig. 4g) or HNO413 cells (Fig. 4h), also agreeing with the above-presented data obtained with NMDI14 and arguing against a role of this pathway in BCRP up-regulation.

3.4. Effect of HPV18 E6/E7 on chemoresistance

The functional relevance of ABC transporter up-regulation by HPV18 oncoproteins was evaluated assessing the resistance to cytostatic drugs usually administered in oropharyngeal cancer chemotherapy. Results showed an increase in resistance to paclitaxel by HPV18 E6/E7 in HNO206 cells (Fig. 5a). The partial prevention of the effect by LY335979 indicates an involvement of P-gp. Similarly, we observed an increased resistance to cisplatin (Fig. 5b) and 5-FU (Fig. 5c) by HPV18 E6/E7 in the same cell line. In this case, the effects were totally prevented by MK571 and FTC, inhibitors of the MRPs and BCRP, respectively, thus indicating transporter up-regulation by E6/E7 as the major mechanism leading to the enhanced chemoresistance. In HNO413, increased resistance to 5-FU by HPV18 E6/E7 was also observed (Fig. 5d), in principle agreeing with BCRP up-regulation by the viral oncoproteins. Although FTC led to a clear sensitization of the cells towards 5-FU, the residual increase in the chemoresistance by E6/E7 in the presence of the inhibitor suggests the presence of additional resistance mechanisms triggered by E6/E7.

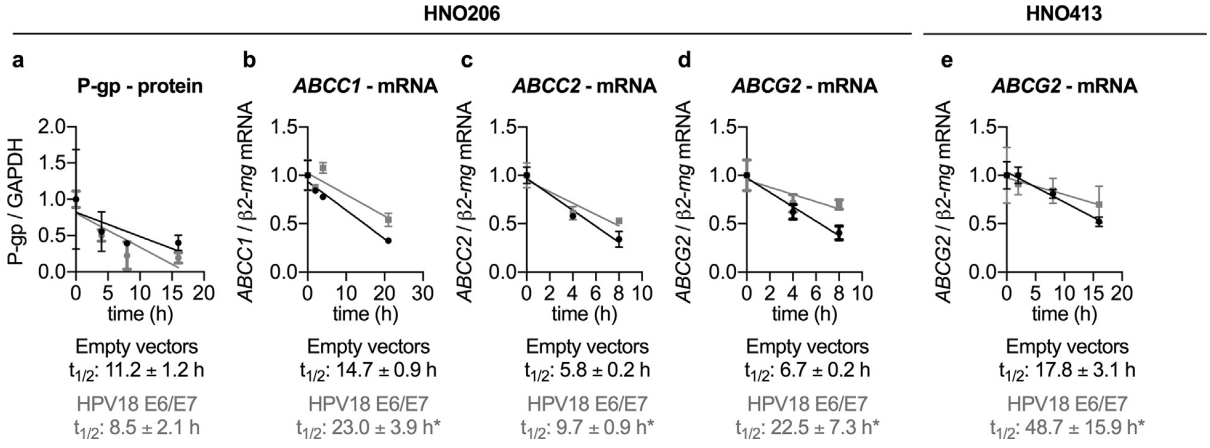

Fig. 2. Effect of HPV18 E6/E7 on transporter protein and mRNA stability. Oropharyngeal cancer cells were transfected with HPV18 E6 and E7 or with the corresponding empty vectors and incubated either with cycloheximide (100 μM) or actinomycin D (5 μg/ml), to assess protein or mRNA stability, respectively. Protein stability was determined for P-gp in HNO206 cells (**a**). P-gp protein expression was determined through Western blot with GAPDH as a loading control. Normalized protein expression was plotted as a function of the time after cycloheximide addition and $t_{1/2}$ values were obtained by linear regression. mRNA stability was determined for *ABCC1* (**b**), *ABCC2* (**c**) and *ABCG2* (**d**) in HNO206 cells and for *ABCG2* in HNO413 cells (**e**). Normalized mRNA expression was plotted as a function of the time after actinomycin D addition and $t_{1/2}$ values were obtained by linear regression. All results are expressed as mean ± standard deviation. * Statistically different from empty vector transfected cells, $p < 0.05$, $n = 3$.

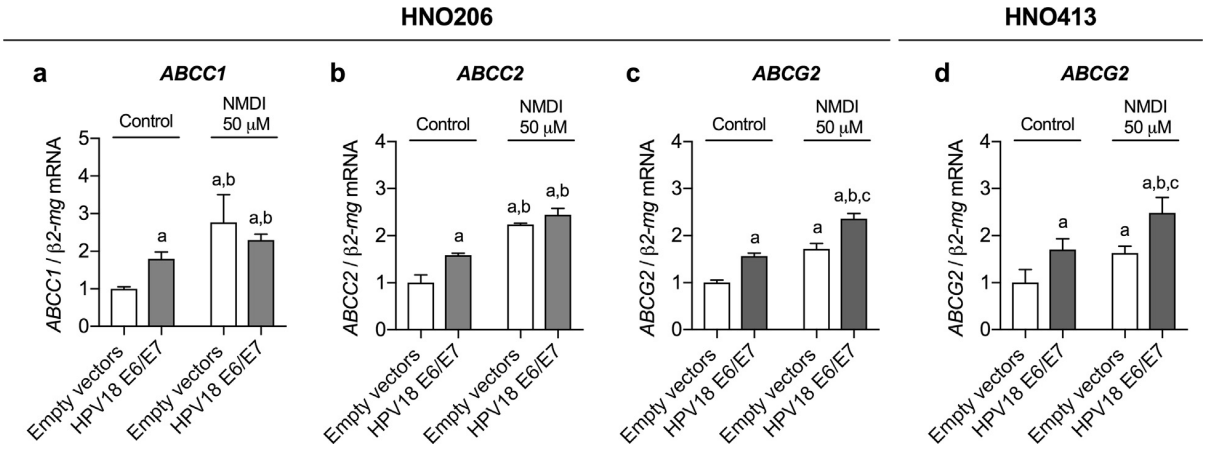

Fig. 3. Effect of NMDI14 on transporter mRNA up-regulation by HPV18 E6/E7. Oropharyngeal cancer cells were transfected with HPV18 E6 and E7 or with the corresponding empty vectors and treated with NMDI14 (NMDI, 50 μM). Control cells were transfected as described and exposed to DMSO. mRNA expression of *ABCC1* **(a)**, *ABCC2* **(b)** and *ABCG2* **(c)** in HNO206 cells and *ABCG2* in HNO413 cells **(d)** was determined through qRT-PCR with *β2-mg* as housekeeping gene. All results are presented as % of expression in empty vector-transfected control cells and expressed as mean ± standard deviation. a: statistically different from control empty vector transfected cells, b: statistically different from control HPV18 E6/E7 transfected cells, c: different from NMDI 50 μM empty vector transfected cells, $p < 0.05$, $n = 3$.

3.5. Individual role of E6 and E7 in ABCC1, ABCC2 and ABCG2 modulation

To assess the individual contribution of each HPV18 oncoprotein on the transporter mRNA stabilization, HNO206 and HNO413 cells were transfected only with HPV18 E6 or HPV18 E7 and mRNA expression was analyzed. A similar pattern characterized by no changes by HPV18 E6 and mRNA up-regulation by HPV18 E7 was observed for *ABCC1* (Fig. 6a), *ABCC2* (Fig. 6b) and *ABCG2* (Fig. 6c) in HNO206 cells, as well as for *ABCG2* in HNO413 cells (Fig. 6d). Moreover, mRNA increase achieved by HPV18 E7 alone was similar to the induction achieved after expression of both E6 and E7 (Fig. 6 a–d), thus attributing HPV18 E7 the main role in the transporter induction presented here.

4. Discussion

HPV$^+$ oropharyngeal carcinoma incidence increased by more than 200% in the last decades [2]. Concerning etiological factors, therapeutic response and prognosis, HPV$^+$ tumors constitute a distinct disease entity [21]. Although interaction of viral proteins, mainly E6 and E7, with host proteins can be hypothesized as a major factor underlying these differences, the whole interactome and the transcriptome changes resulting from these interactions are far from being completely elucidated. ABC transporters play a key role in the efflux of chemotherapeutic agents, thus contributing to chemoresistance and therapy failure [5,8]. In addition, a further role of these proteins in cancer pathogenesis, beyond drug efflux, has been described [22]. In the current work, we demonstrated an up-regulation of the major drug transporters P-gp, MRP1, MRP2 and BCRP by HPV18 E6/E7 (Fig. 1). In contrast, a previous study on the expression of multidrug-resistance genes in 20 HNSCC specimens failed to find an association between transporter levels and HPV infection [23]. Noteworthy, the analysis did not take into consideration the different localization of the tumors. Only 8 cases had oropharyngeal origin and, among them, only 2 were HPV$^+$. Therefore, an association could have remained underestimated. Conversely, a study with cervix carcinoma samples demonstrated a significant association between moderate and high P-gp expression and infection with HPV16 or HPV18, thus highlighting the HPV potential to modulate ABC transporters [24]. Although numerous reports about ABC modulation by changes in the intracellular conditions (e.g. exposure to endo- and xenobiotics) are available [5,7], there is less evidence associating viral infections, also triggering important intracellular changes, with transporter regulation. For instance, a study in hepatocellular carcinoma cells described a decrease in P-gp and an increase in BCRP expression after transfection with the nonstructural 5A protein from the hepatitis C virus (HCV) and after transfection of a full-length HCV replicon [25]. Our data constitutes the first report describing transporter modulation by HPV E6 and E7 in oropharyngeal cancer cells, the most frequent HNSCC subject to HPV infection.

Our data evidences P-gp up-regulation by E6/E7 at the translational level (Fig. 2a). Several microRNAs (miRNAs) have been reported to negatively modulate P-gp translation [26]. A transcriptome analysis pointed out a set of 10 miRNAs majorly down-regulated in HPV$^+$ oropharyngeal cancer [27]. Among them, an *in-silico* analysis using the TargetScan platform [28], indicated potential of miR-223-3p and miR-381-3p to bind *ABCB1* mRNA. However, in our experimental setting, no changes in miR-223-3p or miR-381-3p expression by HPV18 E6/E7 were observed (Rigalli et al., unpublished results), thus arguing against a biological relevance of these miRNAs in the observed effects. Noteworthy, the above-mentioned transcriptome analysis did not distinguish between HPV types [27]. Considering the lower incidence of HPV18, the set of down-regulated miRNAs identified could be more representative of HPV16$^+$ tumors. A similar analysis for HPV18$^+$ specimens may help identifying miRNAs mediating the increase in P-gp translation described in the present work.

In contrast to P-gp regulation, MRP1, MRP2 and BCRP up-regulation takes place through mRNA stabilization by HPV18 E6/E7 (Fig. 2b–e), probably involving the NMD pathway. Indeed, the NMD constitutes an RNA surveillance mechanism originally described to target mRNAs harboring a premature termination codon. More recently, this pathway was also described to modulate up to 10% of non-mutated physiological mRNAs [20]. UPF1 constitutes the most important protein mediating the NMD. One of the main processes leading to mRNA decay consists of recruitment of SMG5-SMG7 heterodimers by UPF1 to the mRNA, followed by recruitment of deadenylating and decapping enzymes. Finally, mRNAs lacking 5′ cap and 3′ poly(A) tail are intrinsically instable and exhibit exonucleolytic decay [20]. NMDI14 prevents the

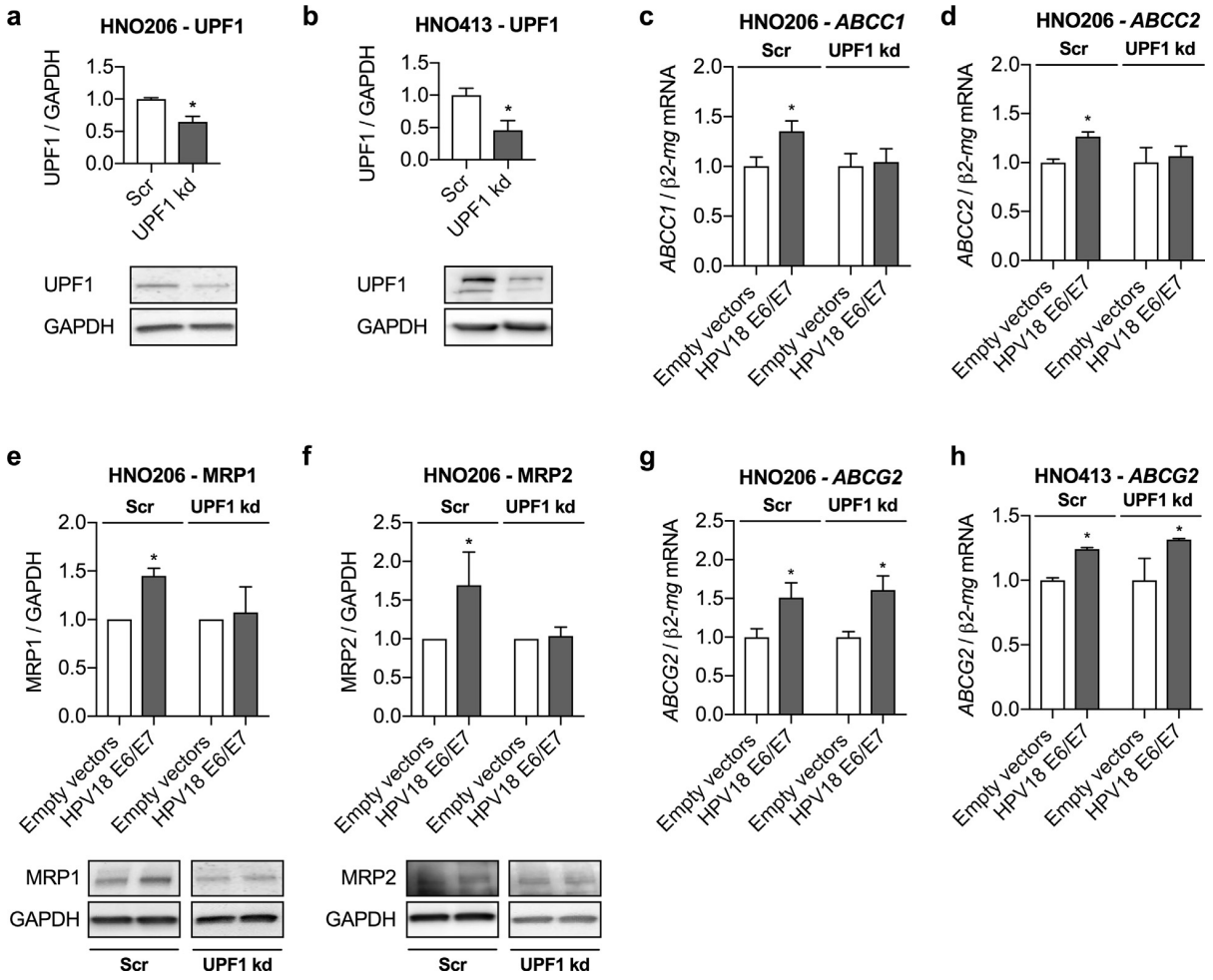

Fig. 4. Effect of UPF1 knockdown on transporter mRNA up-regulation by HPV18 E6/E7. UPF1 was silenced by transfection with a plasmid encoding a shRNA against human UPF1 (UPF1 kd cells). Control cells were transfected with a plasmid encoding a scrambled non-silencing shRNA (Scr). Efficacy of the knockdown was verified at the protein level in HNO206 (**a**) and HNO413 cells (**b**). * Statistically different from Scr, $p < 0.05$, $n = 3$. Scrambled and UPF1 kd cells were simultaneously transfected with plasmids encoding HPV18 E6/E7 or the corresponding empty vectors. Transporter expression was assessed at the mRNA level by qRT-PCR for *ABCC1* (**c**), *ABCC2* (**d**) and *ABCG2* (**g**) in HNO206 cells and for *ABCG2* (**h**) in HNO413 cells. *β2-mg* was used as housekeeping gene. * Statistically different from the respective empty vector transfected cells, $p < 0.05$, $n = 4$ (c -UPF1 kd cells-; d and g -except Scr empty vector cells-) or $n = 3$ (c -Scr cells-, g -Scr empty vector cells- and h). Protein expression was determined through Western blot for MRP1 (**e**) and MRP2 (**f**) in HNO206 cells. The optical density of the target proteins was normalized to the optical density of GAPDH (loading control). Representative blots are shown. * Statistically different from the respective empty vector transfected cells, $p < 0.05$, $n = 3$. All results are expressed as mean ± standard deviation.

formation of the UPF1-SMG7 (or UPF1-SMG5) heterodimers, thus impairing NMD [16]. Our data showing a prevention of HPV18 E6/E7-mediated *ABCC1* and *ABCC2* mRNA up-regulation by NMDI14 indicate a participation of this pathway. Furthermore, experiments with UPF1 knockdown (Fig. 4) also show prevention of transporter induction by E6/E7, supporting NMD involvement. On the contrary, no prevention of BCRP up-regulation either by NMDI14 (Fig. 3c and d) or by UPF1 knockdown (Fig. 4g and h) was observed. Interestingly, our results agree with a transcriptome-wide analysis where *ABCC1* and *ABCC2* rank around the places 1900th and 330th of the most significant NMD targets, while *ABCG2* ranks around the 11600th place, clearly depicting a lack of *ABCG2* modulation by NMD [29]. Furthermore, MRP2 modulation by UPF1 was also demonstrated in a model of hepatocellular carcinoma [30]. To our knowledge, our work constitutes the first evidence of NMD modulation by HPV oncoproteins and may contribute to the understanding of the different molecular background exhibited by HPV^{+} and HPV^{-} tumors.

Our study highlights a differential effect of HPV18 oncoproteins, with HPV16 E6/E7 exhibiting negligible effects on ABC transporters. Moreover, experiments with expression of E6 or E7 alone indicate E7 as the major mediator of the effects observed (Fig. 6). Type-specific protein-protein interactions have been already reported for E7. For instance, HPV16 E7 and HPV48 E7 target p130 host protein and thus overcome cell cycle block either by direct binding to p130 or by an indirect mechanism, respectively [31]. A study on the interactomics of E7 from different HPV types pointed the products of the genes *SARS2* (mitochondrial seryl-tRNA synthethase), *ENC1* (kelch-related family of actin-binding proteins) and *SERPINB7* (protease inhibitor) as proteins differentially interacting with HPV18 E7 [32]. Whether HPV18 E7 interaction with any of these gene products plays any role in the regulation of NMD pathway and this way in the resulting ABC modulation still has to be determined.

Besides the increase in transporter expression by HPV18 E6/E7, our data demonstrates a higher resistance towards chemotherapeutic agents being substrates of the up-regulated transporters (Fig. 5). Prevention by specific transporter inhibitors highlight a

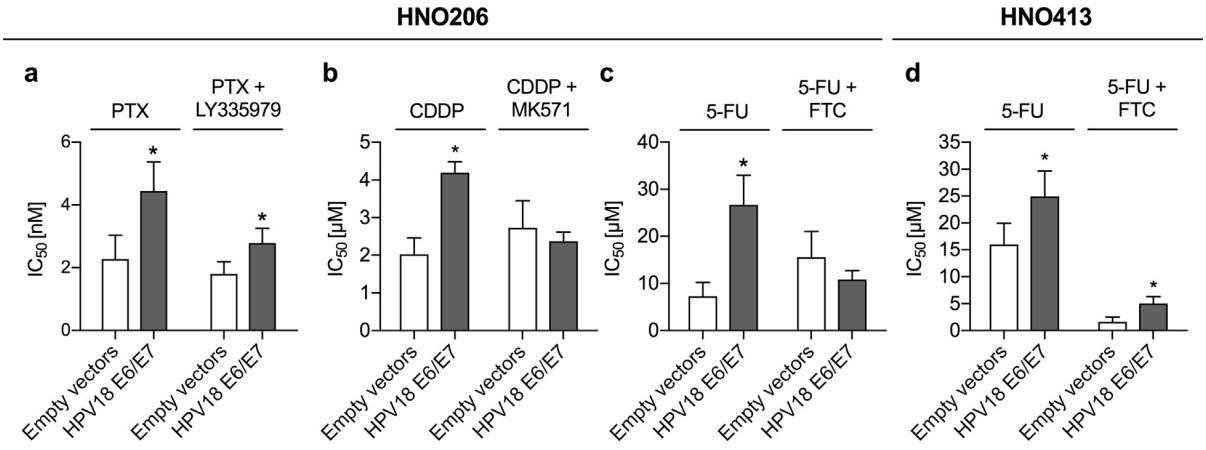

Fig. 5. Effect of HPV18 E6/E7 on chemoresistance. Oropharyngeal cancer cells were transfected with HPV18 E6 and E7 or with the corresponding empty vectors and treated with different concentrations of paclitaxel (PTX) **(a)**, cisplatin (CDDP) **(b)** or 5-fluorouracil (5-FU) (**c** and **d**) in presence or absence of LY335979, MK571 or fumitremorgin C (FTC) as P-gp, MRP and BCRP inhibitors, respectively. Cell viability was determined through crystal violet staining and adjusted to a sigmoidal curve. Data are presented as mean ± standard deviation of the IC_{50} values (concentration leading to a decrease of 50% in cell viability). * Statistically different from the corresponding control, $p < 0.05$, $n = 4$ (except for Fig. 5a and d -empty vector with inhibitor groups-, $n = 3$).

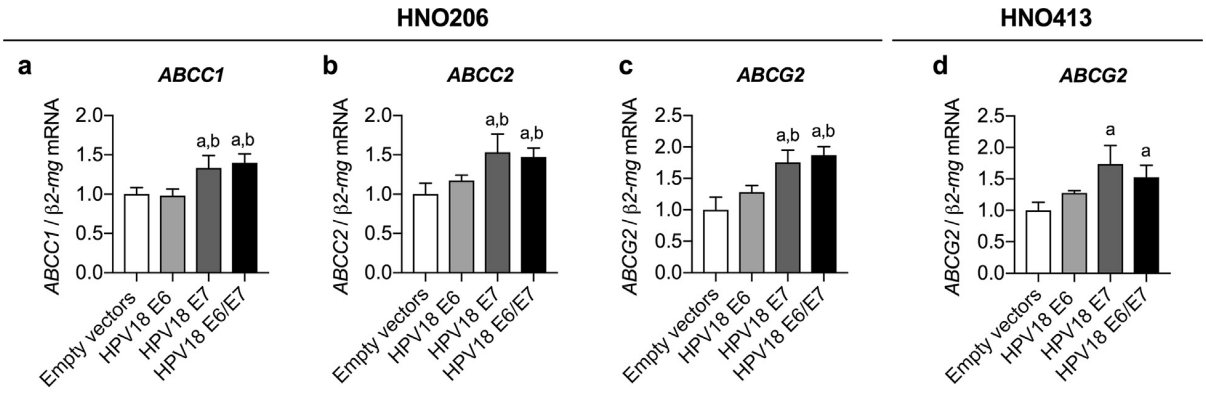

Fig. 6. Effect of HPV18 E6 or E7 on transporter mRNA expression. Oropharyngeal cancer cells were transfected either with HPV18 E6 or HPV18 E7 (or the corresponding empty vectors). mRNA expression was evaluated through qRT-PCR for *ABCC1* **(a)**, *ABCC2* **(b)** and *ABCG2* **(c)** in HNO206 cells and for *ABCG2* **(d)** in HNO413 cells. *β2-mg* was used as housekeeping gene. mRNA expression after transfection with both HPV18 E6 and E7 is shown for comparison purposes. All results are expressed as mean ± standard deviation. a: statistically different from empty vectors, b: statistically different from HPV18 E6. $p < 0.05$, $n = 3$ (for empty vector- and HPV16 transfected cells), $n = 4$ (for HPV18 transfected cells).

role of P-gp, MRPs and BCRP. In general, HPV^+ HNSCC patients exhibit a better prognosis than HPV^- patients, whereby the differential role of the immune system seems to play a key role [21]. In addition, differential prognosis within HPV^+ patients have been described for patients harboring different virus types. In this regard, HPV18 constitutes an independent factor of poor prognosis in cervical cancer [33,34]. In oropharyngeal squamous cell carcinoma, poorer overall survival of non-HPV16 patients, including HPV18, was demonstrated [35]. Our results showing a general ABC transporter up-regulation by HPV18 E7 may contribute to explain this differential prognosis.

In conclusion, we demonstrated a significant up-regulation of P-gp, MRP1, MRP2 and BCRP by HPV18 E6/E7 in oropharyngeal squamous carcinoma cells. While P-gp modulation takes place through increased protein translation, MRP1, MRP2 and BCRP up-regulation can be attributed to mRNA stabilization, probably through negative modulation of the NMD by HPV18 E7. Furthermore, an increase in resistance to cytostatic drugs was demonstrated. These findings provide further insight into the differential molecular background exhibited by HPV^+ oropharyngeal carcinoma patients.

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Conflicts of interest

The authors declare that they have no conflict of interest.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.canlet.2018.04.036.

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