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Cigarette Smoke Reduces the Efficacy of Cisplatin in Head and Neck Cancer Cells – Role of ABCG2

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Abstract. *Background/Aim: We evaluated the influence of smoking on head and neck squamous cell carcinomas (HNSCC), which are in their majority tobacco-driven. Tobacco smoke is expected to influence the expression of ABCG2-transporters involved in multidrug resistance. The aim of the study was to evaluate the effect of cigarette smoke condensate (CSC) on ABCG2 expression on HNSCC cells, to demonstrate the adverse effects of cigarette smoke during anticancer treatment in vitro and to assess the prevalence of ABCG2 expression in HNSCC. Materials and methods: HNSCC cell lines were treated with CSC and basal and induced ABCG2 expression was examined. The impact of CSC on cellular viability/proliferation during cytotoxic drug treatment was also evaluated. ABCG2 expression levels in HNSCC were correlated with the smoking history of patients. Results: HNSCC cells showed low basal ABCG2 expression. CSC treatment resulted in a threefold increase in the expression of ABCG2 and in resistance to cisplatin. Tumor samples of never smokers showed significantly higher ABCG2 expression compared to ever smokers. ABCG2 expression correlated with pack years of cigarette consumption. Conclusion: Tobacco consumption is linked to an inducible and increased ABCG2 protein expression and has an impact on drug resistance.*

Head and neck squamous cell carcinomas (HNSCC) comprise two major, distinct cancer entities referred to as "classic" tobacco-driven tumors and cancers that result from persistent infections with human papillomaviruses (HPV) predominantly arising in the oropharynx (1, 2). "Classical" HNSCC are induced by a multitude of chemical carcinogens contained in tobacco smoke, which lead to genetic alterations, accumulation of mutations, and ultimately malignant transformation of epithelial cells (3). In HNSCC caused by persistent HPV infections, viral oncoproteins induce proteolytic degradation and inactivation of the prominent tumor suppressors p53 and pRb in host cells, thus preserving the replicating potential of infected cells and ensuring viral reproduction (4). Accordingly, genetic alterations and mutations are found to a lesser extent in HPV-positive HNSCC (5).

In 1993, Browman and colleagues reported that patients with head and neck cancer who continue to smoke during radiation therapy have lower rates of response and survival than patients who stopped smoking (6). In a study of more than 1800 HNSCC patients who received either postoperative or definitive (chemo-) radiotherapy, smoking during treatment led to significantly lower local control and survival rates (7). Gillison and co-workers showed that the risk of progression or death increases by 1% per pack-year or 2% per year of smoking in patients with oropharyngeal cancer treated with radiotherapy and chemoradiation (8).

According to the evidence reviewed in the 2014 Surgeon General's Report, continued smoking by cancer patients represents a major cause of increased overall mortality, cancer-specific mortality and risk of second primary carcinomas. This is the result of increased tumor aggressiveness and/or altered response to treatment (9).

As reviewed by Warren *et al*., numerous molecular changes were found in cancer cells exposed to cigarette smoke. It was shown to increase ligand-dependent activation and autophosphorylation of epidermal growth factor receptor leading to increased proliferation. It modifies the regulation of cell cycle and senescence, and activates the expression of vascular endothelial growth factor and matrix metallo-proteinases. Long-term exposure of breast cancer cells to cigarette smoke enhanced their invasiveness and metastatic potential (10).

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Approximately 30% of primary and 70% of recurrent HNSCC show resistance to cytotoxic drugs (11). ATP-binding cassette (ABC) transporters play a critical role in the development of this resistance. ABC transporters are transmembrane proteins that extrude many different, structurally divergent substrates. ABCG2, also known as breast cancer resistance protein (BRCP), was initially cloned from drug resistant breast cancer cells and was later detected on cancer cells from several solid tumors (12, 13). ABCG2 expressing head and neck cancer cells were shown to be resistant to cisplatin (14).

The term multidrug resistance (MDR) originates from the discovery of ABC proteins on cancer cells resistant to various cytotoxic drugs. Furthermore, MDR is inducible. Malignant cells that developed resistance to a single anticancer drug often show cross-resistance to other drugs. ABC transporters are part of a general defense against xenobiotics in living organisms and highly conserved in evolution. In the human body, ABC transporters are particularly expressed in tissues that operate as interfaces between the organism and the environment (15).

Being chronically exposed to cigarette smoke, a complex mixture of numerous chemicals including more than 60 proven carcinogens, it seems reasonable, that epithelial cells in the upper aerodigestive tract learn to defend themselves from these harmful compounds. ABCG2 extrudes the major tobacco carcinogen benzo(a)pyren and its conjugates and might play a critical role in defense against mutagenesis and carcinogenesis in the upper aerodigestive tract (16, 17). Tobacco smoke-induced ABCG2 expression in epithelial cells could be a cause of resistance to anticancer drugs after malignant transformation. Cigarette smoke has been shown to increase ABCG2 expression in esophageal, lung and head and neck cancer cells (18, 19).

The aim of the study was to evaluate the effect of cigarette smoke condensate (CSC) on ABCG2 expression on cancer cells, to demonstrate adverse effects of cigarette smoke during anticancer treatment *in vitro* and to assess the prevalence of ABCG2 expression in HNSCC. We treated HNSCC cell lines (FaDu and PiCa) with CSC and examined basal and induced ABCG2 expression. We then evaluated the impact of CSC on cellular viability and proliferation during cytotoxic drug treatment. We also detected ABCG2 by immunohistochemical staining in tissue samples of HNSCC and correlated expression levels with smoking history of patients. Furthermore, we analyzed a selection of cases of the cancer genome atlas (TCGA) for ABCG2-mRNA in correlation to smoking history.

Materials and Methods

Fluorescence-activated cell sorting. All FACS measurements were performed on an LSRII cytometer, and the data were analyzed with FACSDiva Software. ABCG2 expression was determined using the BV421 Mouse Anti-Human CD338 antibody and the isotype control BV421 Mouse IgG2b, κ.

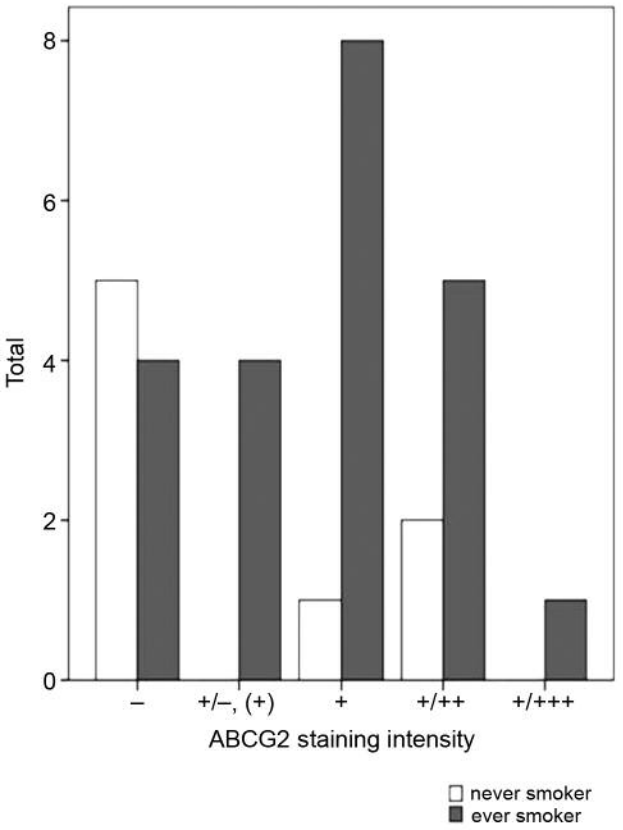

Figure 1. *Semiquantitative evaluation of ABCG2 staining intensity in oropharyngeal carcinomas (n=30) in relation to smoking.*

Cell lines were cultured in DMEM, supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. CSC was added at a final concentration of 10 μg/ml for 24 h to 48 h. At the indicated time points, cells were harvested and stained with either ABCG2-specific or isotype control antibodies.

The cell line FaDu was obtained from Leibniz Institute German Collection of Microorganisms and Cell Culture (DSMZ). The identity of all cell lines was validated by STR-typing. The cell line PiCa was generated in-house from surgical specimens (20). ABCG2 expression levels were evaluated as ratio to untreated controls.

Cell culture. PiCa and FaDu cells were kept in a humidified incubator at 37.5˚C and 5% CO_2 (20, 21). To generate spheroids, 1×10^3 cells were seeded on a 96 well ultra-low attachment plates and cultured for 1 week.

Chemicals. Cigarette smoke condensate (CSC) was obtained from Chemisches und Veterinäruntersuchungsamt, (Sigmaringen, Germany). Total smoke of 40 filter cigarettes was passed through a filter, which was then cut into small pieces, placed in a 50 ml tube and covered with 30 ml DMSO. After 24 h, 20 ml DMSO were added, the solution was filtered and diluted until a concentration of 10 mg/ml CSC was reached. Dose-response incubations were performed using six different concentrations of CSC (10, 20, 30, 50, 70 & 100 μg/ml), cisplatin (1, 5, 10, 25, 50 & 100 μM) and 5-fluoruracil (5-FU; 1, 10, 30, 50, 100 & 1000 μM) in monolayer cultures.

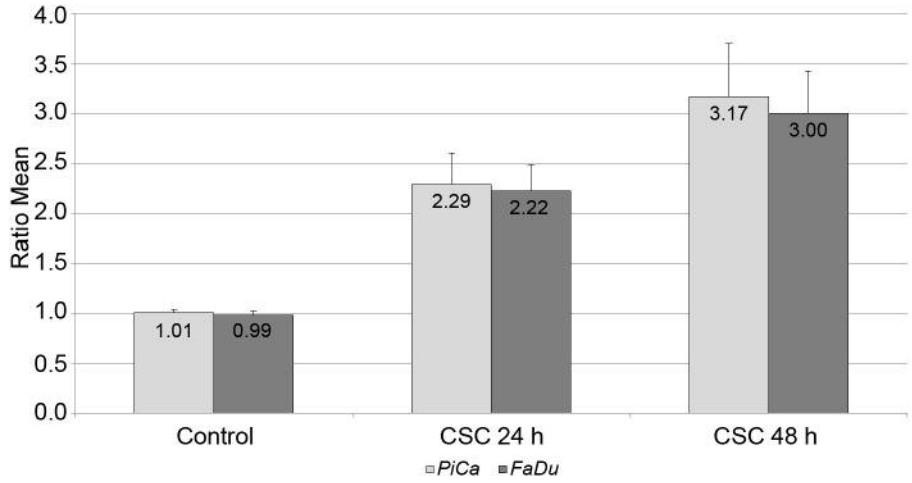

Figure 2. *Expression of ABCG2 in PiCa and FaDu cells before (control) and 24 and 48 h after incubation with cigarette smoke condensate (CSC) as a ratio to control.*

Determination of resistance to chemotherapy. Being kept in DMEM, cells were co-incubated with CSC and cisplatin or 5-FU, respectively, for 24 h. Cultures were subsequently washed using phosphate-buffered saline and re-suspended in DMEM/BEGM. We further incubated cells with cisplatin or 5-FU for 24 h to secure that cytotoxic drugs were present during a complete doubling time. Cell cultures were washed again in PBS and dissociated using StemPro Accutase Cell Dissociation reagent. Single cells were suspended in DMEM and transferred to a 96 well standard plate for adhesion during 24 h. Cellular viability was determined using the colorimetric WST-8 cell viability kit according to the manufacturer's instructions. Cellular proliferation was evaluated by the colorimetric cell proliferation BrdU-kit.

Immunostaining. Snap-frozen tissue samples were processed to 4μm consecutive sections. The ABCG2-specific antibody was used for immunohistochemical detection of antigens by using the avidin-biotin-peroxidase method (22). Staining intensity was assessed in a semiquantitative manner (Figure 1).

ABCG2 expression in TCGA HNSCC patients. To characterize the mRNA expression of the ABCG2 gene in the TCGA HNSCC cohort, RNAseq gene quantification data of primary tumors were downloaded from the Genomic Data Commons GDC data portal. Curated clinical data of appropriate patients were downloaded from CBioportal. The following subgroups were extracted from the TCGA HNSCC cohort: 19 patients with smoking history status "current", 23 "former smoking" patients and 19 "never smoking" patients. Differences in ABCG2 expression in the three groups was examined in all patients with known HPV status, in HPV-positive only and HPV-negative only.

Statistical analysis. All results were tested regarding normal distribution using the Kolmogorov-Smirnov test. For normally distributed variables, we applied the student's *t*-test, whereas the Wilcoxon- and Kruskal-Wallis test we used for non-normally distributed parameters. Associations between ordinal and nominal scaled factors were evaluated using the Chi-square test.

p-Values <0.05 were considered statistically significant. To analyze the results of the incubation experiments, multiple testing between cell lines and treatment groups was necessary. Because of multiple testing within the groups, the p-level was set to 0.005 according to the Bonferoni correction.

Results

ABCG2 expression in HNSCC cell lines. Two HPV-negative HNSCC cell lines (PiCa, FaDu) were assessed for ABCG2 expression levels. All cell lines were either kept untreated or treated with CSC (10 μg/ml) for 24 h and 48 h. Expression levels are given as the difference between the ABCG2 and isotype control antibodies. Basal expression levels were low for Pica and FaDu. PiCa were produced from a laryngeal carcinoma of a current smoker and, thus, represent tobacco-driven HNSCC (23). Basal ABCG2 expression was low in this cell line with a significant 2-fold induction after incubation with CSC for 24 h ($p<0.001$, *t*-test) and a 3-fold induction after incubation with CSC for 48 h (both $p<0.001$, *t*-test). This behavior of HPV-negative HNSCC was confirmed by analysis of FaDu cells, which also showed a significant 3-fold induction of ABCG2 after incubation with CSC for 48 h (Figure 2, $p<0.001$ both 24 h and 48 h, t-test). In both cell lines, the increase of the ABCG2 signal from 2-fold to 3-fold between 24h and 48h of incubation with CSC was significant (PiCa $p=0.003$, FaDu $p=0.002$, *t*-test).

Impact of cigarette smoke condensate (CSC), cisplatin and 5-FU on cellular viability and proliferation. To exclude major cytotoxicity caused by CSC we chose a concentration of 20 μg/ml, as the viability of PiCa monolayer cultures continuously decreased from 94.5% (10 μg/ml) to 38.3% (100 μg/ml) after 24 h of incubation. In order to assess if CSC influences tumor cell treatment, PiCa and FaDu HNSCC cell lines were treated with CSC and cisplatin or 5-FU, respectively, in the indicated

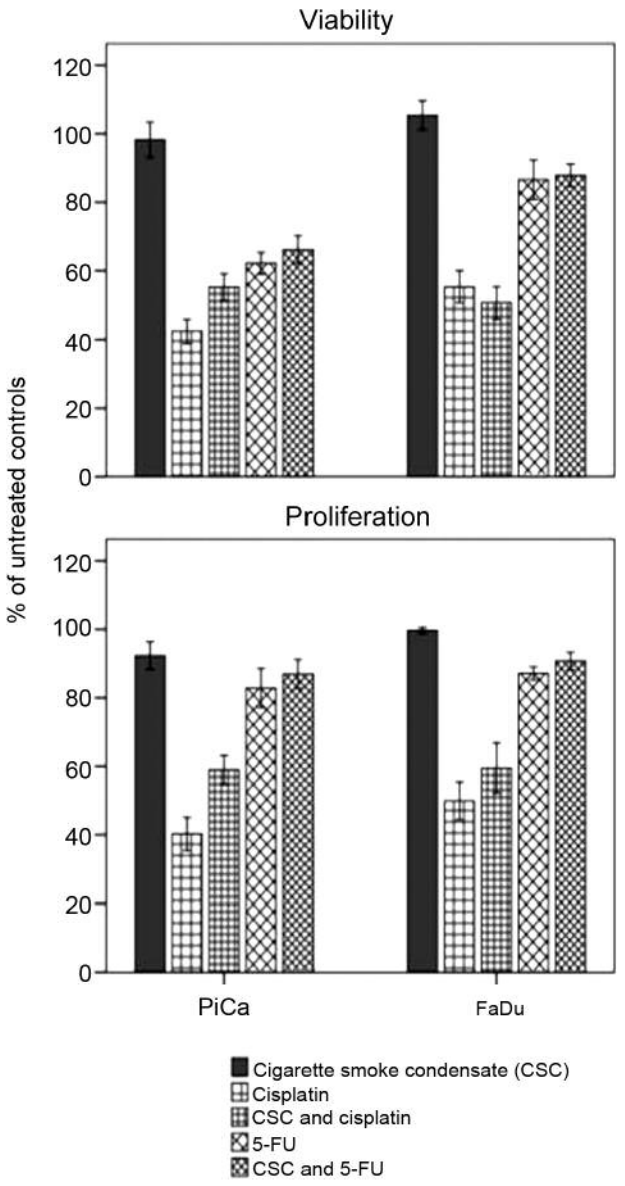

Figure 3. *Cytotoxic (WST8 assay) and anti-proliferative (Brdu assay) effect of cisplatin and 5-FU with and without co-incubation with cigarette smoke condensate (CSC) in spheroid cultures of PiCa and FaDu cells (bars indicate 95% confidence interval).*

combinations. CSC had no significant influence on the viability and proliferation of PiCa [Figure 3, p=0.654, Wilcoxon signed-rank test (WSRT)] and FaDu cultures (Figure 3, p=0.021, WSRT). In PiCa cells, 20 μg/ml CSC significantly decreased proliferation (p=0.002, WSRT).

As seen in Figure 4, incubation with cisplatin led to a decrease of spheroid size in both cell lines. It also significantly reduced viability and proliferation compared to untreated controls in both cell lines (Figure 3; all p<0.001, WSRT). It was more cytotoxic in PiCa compared to FaDu cells (p<0.001, Mann-Whitney-*U*-test). No such difference was seen regarding proliferation (p=0.015, Mann-Whitney-*U*-test).

5-FU also significantly decreased viability and proliferation of both cell lines compared to untreated controls (Figure 3; all p<0.001, WSRT). PiCa cells were more sensitive to 5-FU as compared to FaDu cells (Figure 3; p<0.001 Mann-Whitney-*U*-test). No difference between the cell lines were observed regarding the effect of 5-FU on proliferation (p=0.550, Mann-Whitney-*U*-test).

Incubation of PiCa cells with CSC significantly enhanced resistance to cisplatin. Cisplatin reduced viable cell counts to 46.9%. Incubation with CSC and cisplatin caused a decrease to only 52.9% (p<0.001, WSRT). This is also reflected by the BrdU test-results. Cisplatin decreased the proportion of proliferating cells to 40.3%. Incubation with CSC and cisplatin resulted in 59.0% proliferation rate cells (p<0.001, WSRT).

In FaDu cells CSC also significantly enhanced resistance to cisplatin as the proportion of proliferation cells decreased to 49.5% after incubation with cisplatin compared to a decrease to only 58.6% after incubation with cisplatin and CSC (p<0.001, WSRT). However, no significant effect on the viable cell count of FaDu cells could be shown as cisplatin reduced the viable cell count to 55.3% and incubation with CSC and cisplatin caused a decrease to 50.7% (p=0.040, WSRT).

CSC had no influence on the cytotoxic effects of 5-FU in both cell lines (p=0.012 and p=0.478, WSRT). It had also no effect on 5-FU-induced reduction of proliferation (PiCa cells: p=0.351, WSRT; FaDu cells: p=0.014, WSRT).

ABCG2 expression in oropharyngeal squamous cell carcinomas

Study population. Table I summarizes the patient characteristics of the cohort. Consistent with the etiology, we found fitting smoking histories of patients with p16-negative carcinomas HNSCC. At the time of diagnosis, 100% of p16-negative patients were formerly or currently exposed to cigarette smoke. In this group, 64.3% were current smokers. Former and current p16-negative smokers consumed 50.6 pack-years on average, whereas smokers of the p16-positive control group consumed only 8.9 pack-years (p<0.001, Mann-Whitney-*U*-test).

ABCG2 immunohistochemistry. ABCG2 expression was assessed by immunohistochemistry staining of kryosections of tumor samples (exemplified in Figure 5). Semi-quantitative evaluation of ABCG2 expression in 30 tissue samples of oropharyngeal squamous cell carcinomas revealed an association between ABCG2 expression and smoking history of patients. Never smokers (HPV-positive control group) showed significantly lower expression levels than ever smokers (p=0.019, Chi-Square test; Figure 1). Moreover, ABCG2 expression correlated with tobacco consumption as measured in pack-years (Table I; p=0.025, Chi-Square test).

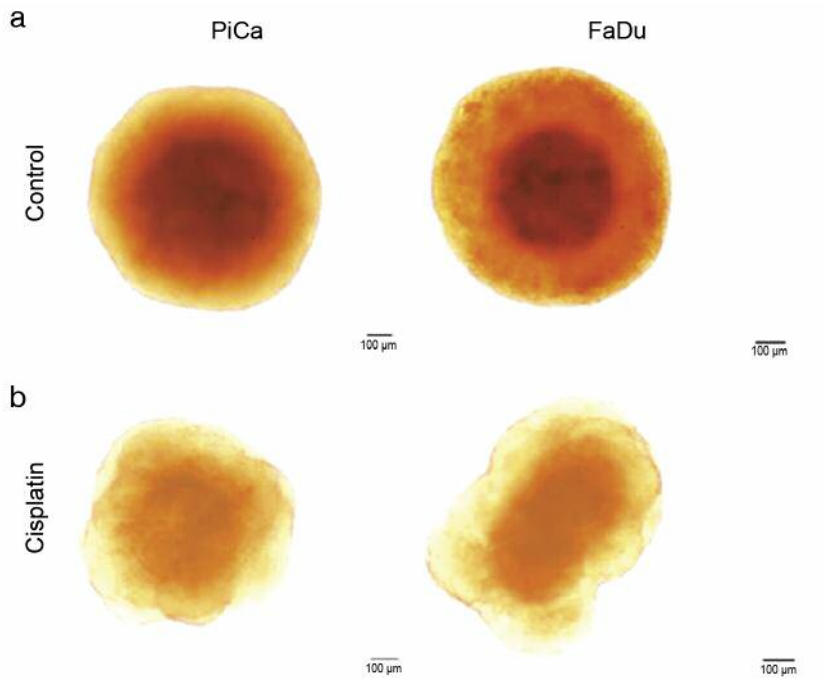

Figure 4. *Spheroid cultures of a) PiCa and FaDu cells, untreated control and b) PiCa and FaDu cells, after incubation with cisplatin for 48 h.*

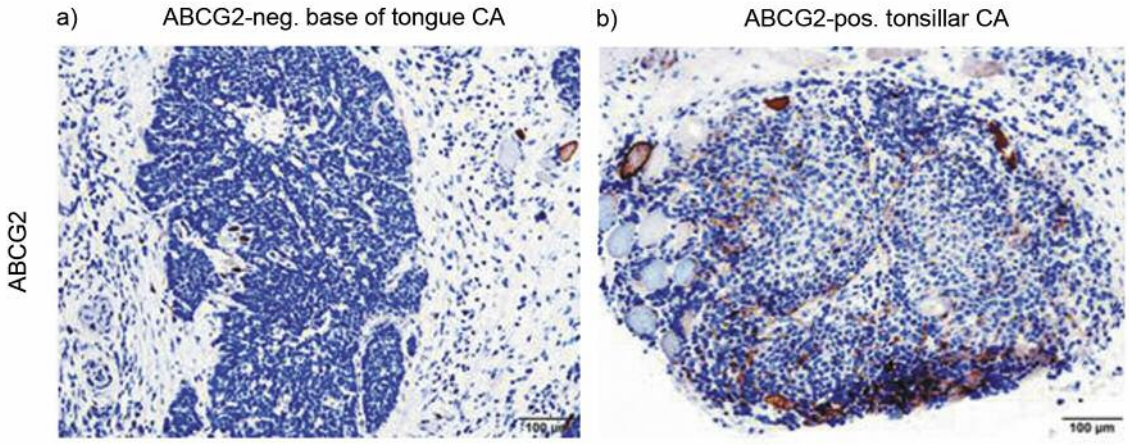

Figure 5. *Immunohistochemical staining of ABCG2 in an a) ABCG2-negative carcinoma of base of tongue. b) ABCG2-positive tonsillar carcinoma (+/++).*

ABCG2 gene expression in TCGA HNSCC patients. No differential expression of the *ABCG2* gene could be shown between current, former and never smokers or HPV-negative or -positive HNSCC.

Discussion

Three-dimensional spheroid cultures of cells were used for the evaluation of chemoresistance. as spheroids were shown to maintain morphology, polarity and cell-to-cell interactions. Cancer cell spheroids contain heterogeneous cell populations with regard to metabolic activity, a major determinant of response to cytotoxic drugs (20, 21).

Continued smoking after being diagnosed with cancer has been recently put into focus because of its detrimental effects on several clinical parameters including cancer-specific mortality, more severe treatment-related toxicities and poorer response to therapy (10). According to available literature, continued smoking leads to more recurrences and second primary tumors in patients suffering from tobacco-driven

Table I. *Patient characteristics in regards to ABCG2 immunostaining.*

	ABCG2-positive	ABCG2-negative	No. of patients	*p*-Value
Age (years)	61.4	57.9	30	0.283
Gender				
Female	7 (100%)	0 (0%)	7	0.048
Male	14 (60.9%)	9 (39.1%)	23	
No. of patients	21	9	30	
Staging				
pT1	1 (50%)	1 (50%)	2	0.051
pT2	15 (88.2%)	2 (11.8%)	17	
pT3	4 (60%)	6 (60%)	10	
pT4a	1 (100%)	0 (0%)	1	
No. of patients	21	9	30	
pN0	8 (88.9%)	1 (11.1%)	9	0.378
pN1	4 (57.1%)	3 (42.9%)	7	
pN2a	1 (100%)	0 (0%)	1	
pN2b	4 (57.1%)	3 (42.9%)	7	
pN2c	3 (75%)	1 (25%)	4	
pN3	0 (0%)	1 (100%)	1	
No. of patients	20	9	29	
Grading				
G1	-	-	-	0.300
G2	6 (85.7%)	1 (14.3%)	7	
G3	15 (65.2%)	8 (34.7%)	23	
No. of patients	21	9	30	
p16				
Positive	10 (62.5%)	6 (37.5%)	16	0.338
Negative	11 (78.6%)	3 (11.4%)	14	
No. of patients	21	9	30	
Smoking				
Never	3 (37.5%)	5 (62.5%)	8	0.019
Ever	18 (81.8%)	4 (18.2%)	22	
No. of patients	21	9	30	
Tobacco consumption (pack years)				
0	3 (37.5%)	5 (62.5%)	8	0.025
1-10	5 (100%)	0 (0%)	5	
>10	13 (81.3%)	3 (18.7%)	16	
No. of patients	21	8	29	

cancers (24). Bjarnason and colleagues have shown that head and neck cancer patients who received radiotherapy and did not stop smoking during treatment, had significantly higher grades of mucositis when they were treated in the afternoon, after they smoked cigarettes, compared to patients who were treated in the morning (25). With respect to response to therapy, resistance to anticancer drugs plays a significant role in long-term survival. Cytotoxic pressure imposed by therapeutic agents on cancer cells is a persistent stimulus for the cells to adapt and to develop mechanisms of resistance. Part of this resistance is provided by members of the ABC transporter family. As efflux pumps, they are involved in the development of multidrug resistance, in which an individual tumor acquires cross-resistance to many different compounds that can be chemically and functionally unrelated (26).

Tobacco-associated head and neck carcinogenesis is commonly seen as the result of heavy smoking. It is likely that epithelial cells in the upper aerodigestive tract establish defense mechanisms against the multitude of tobacco-contained chemicals over time, potentially by increasing expression and/or function of efflux carriers like ABCG2, given that tobacco carcinogens are substrates of ABCG2 (16, 27). Cigarette smoke has been shown to rapidly induce ABCG2 expression in various cell lines, *e.g.* lung and esophageal cancer cells followed by a rapid decline after the stimulus was removed (18). In head and neck cancer cells, CSC has been shown to cause expression of ABCG2 and membrane localization of the transporter within 24 h after exposure (19).

Drug resistance could partly be the result of metabolic imprinting during carcinogenesis as cells maintain their preparedness to defend themselves against xenobiotics. Accordingly, increased ABCG2 expression has been shown to be a common feature in HNSCC cells resistant to cisplatin (28-30).

Both, PiCa und FaDu cells had low basal ABCG2 expression. Incubation of PiCa and FaDu cells with cigarette smoke resulted in an increased resistance to cisplatin. CSC led to a 3-fold increase of ABCG2 expression in PiCa and FaDu cells. FaDu cells showed significantly greater resistance to 5-FU. CSC did not modify 5-FU resistance in both cell types. This observation could be explained by the greater affinity of ABCG2 for cisplatin, particularly sulfated conjugates of cisplatin (31, 32). Cisplatin, but not 5-FU, is highly reactive towards glutathione, building sulfated complexes (28, 30).

These results are in agreement with our hypothesis that resistance to cytotoxic drugs is a consequence of a permanent xenobiotic impact of tobacco-contained carcinogens on the upper aerodigestive tract mucosa. It has also previously been shown that HPV-positive HNSCC and cervical cancer cell lines possess an intrinsic resistance to chemotherapy, independent of tobacco-related carcinogenesis (33, 34).

Considering the distinct etiologies of tobacco- and HPV-related HNSCC, tobacco-exposed PiCa and FaDu cells may have been primed to respond to cigarette smoke with increased ABCG2 expression and/or membrane localization to fend off carcinogens (27). The reported low inducibility of ABCG2 expression in HPV-postive HNSCC cells could be the result of HPV-specific carcinogenesis and may rather reflect stem-cell-like properties and increased proliferative capacity (35, 36).

Immunohistochemical analysis showed that ABCG2 is expressed more frequently in carcinomas of ever smokers compared to never smokers. Expression levels correlated with lifetime tobacco consumption as measured in pack years. These results indicated an association between tobacco consumption and ABCG2 expression on the protein level in PiCa cells. Although p16-positive carcinomas occurred in patients who smoked significantly less, these tumors did not

differ in ABCG2 expression from p16-negative cancers in all subgroups. We found no such differences in ABCG2 expression on the mRNA level.

There are several limitations of the present study that need to be discussed. Firstly, the study lacks a HPV-positive cell line. We were not able to produce spheroids of HPV-positive cell lines, in part due to a comparably slow growth. Nevertheless, we believe that three-dimensional cell cultures are an indispensable prerequisite for experiments with cytotoxic drugs. Secondly, regarding the tumors used for immunostaining, p16 was used as a surrogate marker for HPV-association. The presence of HPV was not confirmed by in-situ hybridization or PCR because our study focusses on the impact of tobacco consumption and p16 is a sufficient surrogate marker according to the 8th edition of the TNM staging system.

Conclusion

There is growing evidence that ABCG2 plays a critical role in drug resistance of head and neck cancer cells and that smoking influences its expression. Our results confirm these observations. Although PiCa and FaDu cells showed low basal ABCG2 expression, after stimulation with CSC both cell lines readily increased ABCG2 expression threefold and became resistant to cisplatin. ABCG2 immunostaining revealed significantly higher ABCG2 expression in HNSCC of ever compared to never smokers. Carcinomas of patients who smoked >10 pack years were significantly more often ABCG2-positive. No such correlations were found on the mRNA-level by TCGA analysis.

Conflicts of Interest

The Authors have no conflicts of interest to declare regarding this study.

Authors' Contributions

Florian Simon: data analysis, preparation and revision of manuscript; Sabina Schwenk-Zieger: data acquisition; Sven Becker: data analysis; Kristian Unger: data acquisition, data analysis; Olivier Gires: data acquisition, data analysis; Philipp Baumeister: preparation and revision of manuscript.

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