EGFR and P-GP Expression in Oropharyngeal Mucosa in Relation to Smoking

PHILIPP BAUMEISTER[1], MELANIE MÄRTE[2], MAXIMILIAN REITER[1], CHRISTIAN WELZ[1], SABINA SCHWENK-ZIEGER[2] and ULRICH HARRÉUS[1]

[1]*Department of Otorhinolaryngology,Head and Neck Surgery, University of Munich, Germany;*
[2]*Clinical Experimental Oncology, Department of Otorhinolaryngology, Head and Neck Surgery, University of Munich, Germany*

Abstract. *Background: About two thirds of head and neck squamous cell carcinoma (HNSCC) cases are attributable to heavy tobacco and alcohol consumption. Tobacco carcinogens cause cellular damage in large areas of the upper aerodigestive tract mucosa and contribute to distinct molecular changes, such as increasing levels of epidermal growth factor receptor (EGFR), during carcinogenesis. P-Glycoprotein (P-GP) is a multidrug-resistance transporter protein capable of extruding not only cytotoxic drugs, but also certain tobacco-related carcinogens. EGFR plays a major role in the transcriptional and functional regulation of P-GP and previous studies in our laboratory showed that stimulation of EGFR protection protected oropharyngeal cells from a carcinogen that is substrate of P-GP. Therefore, we evaluated expression levels of EGFR and P-GP and looked for a possible association with the smoking status of patients. Materials and Methods: Tissue cultures of healthy oropharyngeal mucosa were produced from 30 patients undergoing surgery at our Department. Expression levels of EGFR on P-GP were determined by immunohistochemical staining. To evaluate possible influences of EGFR on P-GP expression, we stimulated the receptor using transforming growth factor alpha (TGF-α) for 24, 48 and 72 h. Results: Current and former smokers had significantly higher EGFR/P-GP levels than never smokers. While EGFR expression was detected in almost all samples, P-GP expression was largely restricted to former and current smokers. TGF-α had no detectable effect on EGFR/P-GP levels. Conclusion: These results show an association between tobacco use and levels of both proteins. Since both these proteins are involved in drug resistance of head and neck cancer, this study might help to further understand the differences in response to therapy and prognosis of tobacco-related and -unrelated cancer.*

Correspondence to: Philipp Baumeister, University of Munich Hospital, Marchioninistr. 15, 81377 Munich, Germany. Tel: +49 89070950, Fax: +49 8970956869, e-mail: Philipp.Baumeister@med.uni-muenchen.de

Key Words: EGFR, P-Glycoprotein, oropharyngeal mucosa, TGF-a, HNSCC, smoking.

Head and neck squamous cell carcinoma (HNSCC), the sixth most common cancer type worldwide, was long thought to be a relatively homogenous disease compared to other tumor types (1, 2). In fact, recent insight has revealed that HNSCC is a far more heterogenous disease than expected. At least two types of HNSCC must be distinguished, human papilloma virus (HPV)-positive and HPV-negative. About 25% of HNSCC contain HPV genomic DNA, predominantly in oropharyngeal cancer (3). HPV-DNA-encoded oncoproteins E6 and E7 lead to proteolytic degradation of the tumor suppressors p53 and pRb, thus causing genetic instability (4). HPV-positive cancer arises more often in individuals who are non- or never-smokers and can develop independently of the presence of other carcinogens (3).

Approximately two-thirds of HNSCC can be attributed to tobacco smoking, which is classified as group 1 (carcinogenic to humans) by the International Agency for Research on Cancer (IARC). These types of cancer develop predominantly in men in their sixties (5, 6). Tobacco-containing carcinogens exert their harmful effects on upper aerodigestive tract mucosa in a chronic manner, leading to the accumulation of genetic alterations. In this regard, tobacco smoke literally reaches every corner and damages the entire epithelium lining the upper aerodigestive tract. According to the Slaughter's concept of field cancerization, HNSCC arises from a multifocal precancerous lesion within large areas of damaged mucosa (7).

Epidermal growth factor receptor. Epidermal growth factor receptor (EGFR) is a prototypal receptor tyrosine kinase with five genetically distinct ligands including epidermal growth factor (EGF) and transforming growth factor alpha (TGF-α).

It is expressed on all epithelial and stromal cells. In polarized epithelium, EGFR expression is largely restricted to basolateral aspects, allowing signal transduction from fibroblast-derived TGF-α in the stroma. Physiologically it plays a pivotal role during the maturation of epithelial organs in the neonatal period and organ-repair in adults (8).

EGFR biology in HNSCC. EGFR is an established oncogene in HNSCC. High expression levels of the receptor were detected in up to 90% of tumors. It activates several downstream pathways leading to a high proliferation rate, inhibition of apoptosis, enhanced invasion, metastasis and neoangiogenesis, and serves as a transcription factor (2, 9, 10). High EGFR expression is an independent prognostic marker associated with poor overall and disease-free survival (11). EGFR-antisense therapy using the monoclonal antibody cetuximab (Erbitux™) significantly increases 5-year survival of patients treated with radiotherapy (12). EGFR was also shown to influence sensitivity to cytotoxic drugs. Targeting EGFR in various cell lines enhanced sensitivity to cisplatin, a chemotherapeutic drug widely used in HNSCC (13, 14, 15). On the other hand, we showed that the stimulation of EGFR reduces cisplatin-induced DNA damage in oropharyngeal cells (16). In clinical trials, the combination of cetuximab with a platinum-based chemotherapy significantly prolonged overall survival by 2.7 month (17). Moreover, cetuximab was shown to overcome cisplatin-resistance (18).

There are still many open questions regarding EGFR biology in HNSCC. Studies reporting EGFR overexpression by immunohistochemical staining sometimes lack normal tissue controls. Moreover, we certainly do not know the exact mechanisms of all tyrosine phosphorylation sites within EGFR (at least 13) and their different downstream effects (2). At present, EGFR-specific proliferation is most probably not accessible to EGFR-targeted therapy (8).

The role of EGFR in head and neck carcinogenesis. Alterations in EGFR expression can be detected early in head and neck carcinogensis. In 1993, Grandis and Tweardy reported increased production of *EGFR* mRNA in histologically normal mucosa of HNSCC patients (19). Three years later, by quantitative immunohistochemical analysis, the same group confirmed an almost two-fold higher EGFR protein expression in histologically normal mucosa from HNSCC patients compared to non-tumor controls (20). Intriguingly, elevated levels of TGF-α, were also detected. Thus, an autocrine EGFR stimulation loop was supposed to be a major contributor to malignant transformation. In later stages of carcinogenesis, EGFR expression was shown to be gradually elevated in normal tissue adjacent to the tumor, hyperplasia and dysplasia. A dramatic increase of EGFR levels was detected in the step from dysplasia to squamous cell carcinoma (21). Thus, elevated EGFR levels are an established marker for head and neck field cancerization (2).

The underlying mechanisms of increasing EGFR expression during carcinogenesis remain elusive. Gene amplification might be one cause and was found to coincide with elevated EGFR expression in 30 % of oral squamous cell carcinoma (22). When we examined healthy mucosa from HNSCC patients and non-tumor controls, we found *EGFR* amplification in 7.7% of tumor patients, but none in the controls (23).

Since increased levels of EGFR and its specific ligand TGF-α are already present in histologically normal mucosa of HNSCC patients, we looked for the receptor's role in chemical mutagenesis. Tissue cultures produced from oropharyngeal mucosa of HNSCC patients and controls were stimulated with TGF-α for 24 h. During a 1 hour incubation, DNA damage was introduced using benzo(a)pyrene diol epoxide (BPDE), the activated metabolite of benzo(a)pyrene, a tobacco-related carcinogen classified as group 1 by IARC (24). In cultures derived from HNSCC patients, TGF-α stimulation resulted in a decrease of BPDE-induced DNA damage by 36%. In controls, TGF-α did not cause any significant change (25). In a subsequent study we showed that this effect was completely abrogated when EGFR was blocked before stimulation, indicating an EGFR-specific mechanism (26).

P-Glycoprotein. P-Glycoprotein (P-GP) is the archetypal member of the ATP-binding cassette (ABC) transporter family, which are also referred to as multidrug-resistance (MDR) proteins. P-GP, also called MDR1, is product of the *ABCB1* gene and is the first MDR discovered, the best studied and has the broadest substrate specifity. About 40 years ago, clinical oncologists became aware of the phenomenon that certain tumors exhibit inherent resistance to cytotoxic drugs while others developed resistance during therapy. Moreover, cancer that had become resistant to one single chemotherapeutic drug suddenly also showed resistance to many other drugs; 30 years later, researchers clearly established that this phenomenon is due to the expression of MDR transporters. Today we know that MDR transporters are highly conserved during evolution and are present in practically all living organisms. Although still referred to as multidrug-resistance pumps, these proteins did not evolve to protect cancer cells, but rather participate in a general chemoimmunity defense system (27).

P-GP in head and neck cancer. In 1993, Kelley and colleagues detected P-GP expression in 60% of HNSCC specimens. P-GP levels accurately predicted clinical response to chemotherapy (28). In oral squamous cell carcinoma and premalignant lesions, P-GP was shown to positively correlate with p53 protein expression, which is suspected to influence P-GP levels (29). In a study of 111 patients with advanced HNSCC treated with concurrent chemoradiation, co-expression of p53 and P-GP led to a significantly lower

disease-free and overall survival. In this study, 62% of carcinomas were P-GP positive (30). In another study evaluating 45 oral carcinomas P-GP was detected in only 18% and had no significant influence on survival (31). Knock down of P-GP in laryngeal cancer cells led to increased sensitivity to cytotoxic drugs (32). Together, these data suggest a major role of P-GP in drug resistance of HNSCC.

P-GP in xenobiotic defense. High expression of P-GP is observed in tissue that forms some kind of barrier (27). This is exemplified by *ABCB1* knockout mice lacking a functional blood brain barrier which were accidently killed when their cages were treated with ivermectin, a neurotoxin used to extinguish mites (33). Evaluation of P-GP expression in the upper aerodigestive tract led to controversial results: in a study of oral cancer patients in Europe, 4 out of 6 samples of healthy oral mucosa exhibited P-GP expression (34). Yet in a Japanese study, healthy oral epithelium from tumor patients did not show any P-GP expression (35). P-GP expression is inducible by various stress factors (36, 37). In 1992, Yeh *et al*. demonstrated, that P-GP is capable of extruding benzo(a)pyrene (BPDE) from multidrug resistant cancer cells (38). Myllynen *et al*. reported 1.5- to 2-fold increased formation of BPDE-DNA adducts after P-GP inhibition in the same cell line (39). Thus, it became evident that P-GP plays an important role in the protection of cells against mutagens and carcinogens (40, 41). In a case control study of more than 400 smokers and tobacco chewers, individuals with the TT genotype of *ABCB1*, which is associated with significantly lower P-GP expression, had a greatly increased risk of developing upper aerodigestive tract cancer (42).

Available data indicates a pivotal role of EGFR and P-GP in drug resistance of HNSCC. Both proteins are associated with poor prognosis. EGFR-targeted therapy was shown to be effective in drug-resistant cancer (18). Moreover, the receptor was shown not only to regulate the transcription of MDR proteins (43 - 45), but also to directly enhance P-GP activity (46). Since P-GP is likewise crucial in the protection against tobacco carcinogens (38-41) and its expression is inducible (36, 37), we investigated the expression of EGFR and P-GP in healthy oropharyngeal mucosa and looked for a possible association with smoking. We also examined the influence of EGFR stimulation on the expression of P-GP. To gain results comparable to previous studies of our laboratory, we again used the model of oropharyngeal mucosa cultures.

Materials and Methods

Tissue culture. The study was approved by the Ethics Committee of the University of Munich, Germany. All biopsy donors were informed by the investigators and signed an informed consent statement.

We prepared tissue cultures from freshly biopsied mucosa. Samples were harvested during resection of oropharyngeal squamous cell carcinomas and tonsillectomy. Biopsies of tumor patients were taken from macroscopically normal mucosa close to the tumor-free resection margins. For these patients, tumor resection with or without selective neck dissection was the primary treatment. In patients diagnosed with cancer of unknown primary, tissue samples were taken during tonsillectomy. Diagnosis and staging of all patients are shown in Table I.

Specimens were dissected into cubes of 1 mm^3, excluding deeper layers, and were washed three times in bronchial epithelial cell basal medium (BEGM, supplemented with bovine pituitary extract, insulin, hydrocortisone, epinephrine, triiodothyronine, transferrin and retinoic acid; Promocell, Heidelberg, Germany). Cubes were placed one to each well of 24-well plates, coated with 0.75% Noble Agar (Difco, Detroit, MI, USA) and dissolved in Dulbecco's modified eagle's medium (Gibco), 10% fetal calf serum (Gibco), nonessential amino acids (Gibco) and amphotericin B (all Gibco, Eggenstein, Germany). After about 20 days in 250 μl BEGM at 37.5˚C, 5% CO_2 and 100% relative humidity, tissue cultures were completely coated with epithelium. BEGM was replaced every second day during cultivation. Every seventh day, multiwell plates were changed to avoid adherence (47).

Stimulation of EGFR expression. To evaluate possible effects on the expression of EGFR and P-GP, we stimulated the receptor with TGF-α (50 ng/ml; Sigma-Aldrich, Steinheim, Germany) for 24, 48 and 72 hours. After all incubations, BEGM was replaced twice.

Immunohistochemical staining. All tissue cultures were embedded in Tissue-Tek® and frozen in liquid nitrogen. Cryosections were cut (5 μm-thick), airdried overnight and fixed in acetone. Epitopes were stabilized and fixed in parafomaldehyde for 5 minutes before slides were washed with hydroxyethylpiperazine-N'-2-ethanesulphonic-acid-(HEPES)-buffered Ringer solution buffer.

EGFR. Staining of the EGFR was carried out according to the EGFR pharmDx™ protocol (Dako Corp., Carpinteria, CA, USA). In brief, specimens were covered with proteinase K solution for 5 minutes, before intrinsic peroxidase was blocked using hydrogen peroxide (5 minutes). The primary antibody, or the negative control reagent, was added for 30 minutes, before specimens were covered with the labeled polymer (horseradish peroxidase) solution for half an hour. Finally, DAB+ substrate chromogen solution was added and specimens incubated for 10 minutes (see Figure 1). Counterstaining was carried out using hematoxylin (Dianova, Hamburg, Germany).

P-GP. For the evaluation of P-GP expression, the primary p170 antibody (BioPrime, Gaithersburg, MD, USA) was used. Slides were stained by a streptavidin-biotin immunoperoxidase technique using the NOVA Detect HRPO/DAB standard streptAB system kit (Dianova). For counterstaining, hematoxylin (Dianova) was used. The specimens were incubated with the primary antibody overnight at 4˚C, followed by the secondary, biotin-labeled antibody. The results were visualized with diaminobenzidine. For positive control, specimens of a human hepatocellular carcinoma were used; for negative controls, normal mouse IgG at the same concentration as the primary antibody was applied.

After each step, slides were rinsed using phosphate buffered saline, excess buffer was tapped off the slides. All steps were

Table I. *Patient characteristics; expression levels ==> 0=0% of cells stained. 1=10-20% of cells stained; 2=20-30% of cells stained; 3=30-60% of cells stained; 4=>60% of cells stained.*

	Gender	Age (years)	Diagnosis	EGFR expression				P-GP expression				Smoking status			Alcohol Consumption
				0h	24h	48h	72h	0h	24h	48h	72h	Never	Former	Current	
1	Female	25	Chronic tonsillitis	3	2	2	2	0	0	0	0	X			0
2	Female	49	Chronic tonsillitis	0	0	1	1	0	0	0	0	N/A	N/A	N/A	N/A
3	Male	22	Chronic tonsillitis	1	1	1	2	0	2	2	2	X			0
4	Female	15	Chronic tonsillitis	1	2	1	2	0	0	0	0	X			0
5	Male	30	Chronic tonsillitis	1	1	1	2	0	0	0	0	X			0
6	Female	18	Chronic tonsillitis	1	1	1	2	0	0	0	0	X			0
7	Female	50	Chronic tonsillitis	2	2	1	1	0	0	0	0	X			0
8	Female	21	Chronic tonsillitis	2	1	1	1	0	0	0	0	X			N/A
9	Female	22	Chronic tonsillitis	2	2	2	2	2	1	1	2	X			0
10	Female	19	Chronic tonsillitis	2	2	3	1	2	3	3	3		1.5 PY		0
11	Female	30	Chronic tonsillitis	2	3	4	2	3	4	3	4		12 PY		0
12	Male	34	Chronic tonsillitis	0	0	1	2	1	1	2	3	X			0
13	Female	23	Chronic tonsillitis	N/A	0	0	1	N/A	0	0	0	X			0
14	Male	38	Chronic tonsillitis	2	3	2	2	0	0	0	0	X			0
15	Male	21	Chronic tonsillitis	1	3	2	2	0	0	0	0	X			0
16	Male	41	pT2 pN2c cM0 base of tongue	3	3	3	3	1	1	1	2	N/A	N/A	N/A	N/A
17	Male	71	Cancer of unknown primary	2	2	2	3	1	1	0	0		80 PY		Former abuse
18	Male	53	pT1 pN2b cM0 right tonsil	3	4	4	3	3	3	2	2			>20/d	Not regularly
19	Male	47	Cancer of unknown primary	3	4	4	3	2	2	2	1			20/d	Former abuse
20	Male	67	pT3 pN2c cM0 right tonsil	2	3	2	2	0	2	1	1		60 PY		Not regularly
21	Female	69	pT2 pN0 cM0 right tonsil	1	2	2	2	N/A	2	2	2		40 PY		Not regularly
22	Male	75	pT2 pN2c cM0 left tonsil	0	1	1	0	3	3	3	3			>20/d	Not regularly
23	Male	78	pT2 pN0 pM1 right tonsil	3	2	N/A	2	1	1	N/A	1	N/A	N/A	N/A	N/A
24	Male	56	pT3 pN2b cM0 base of tongue	3	2	2	3	3	3	3	3			20/d	7.5 Units/d
25	Male	48	pT3 pN2b cM0 right tonsil	3	3	3	3	3	3	3	2			>20/d	25 Units/d
26	Male	66	pT2 pN0 cM0 left tonsil	1	1	1	2	2	2	1	2		25 PY		2.5 Units/d
27	Male	54	pT2 pN0 cM0 right tonsil	3	2	2	2	3	2	3	2			>20/d	5 Units/d
28	Male	61	pT2 pN1 cM0 right tonsil	2	2	2	2	1	2	2	2			20/d	10 Units/d
29	Male	67	pT2 pN2a cM0 right tonsil	2	2	3	3	1	0	0	1		40 PY		Not regularly
30	Male	74	pT2 pN0 cM0 base of tongue	3	3	3	3	2	1	1	2			20/d	10 Units/d

Smoking status ==> PY=Pack years; 20/d=20 cigarettes per day; Alcohol ==> 1 beer (500 ml)=2.5 units.

performed in a humid chamber. Protein expression was scored semi-quantitatively. For each slide, the area with the most intensive staining was selected for analyses. The number of positively stained cells was divided by the total number of evaluated cells and multiplied by 100. Thus, slides were categorized as follows: no detectable expression ==> 0; 10 to 20% positive cells ==> 1; 20 to 30% ==> 2: 30 to 60% ==> 3; > 60% ==> 4. Sections were reviewed and scored independently by two investigators (MM and SSZ).

Statistical analysis was performed using SPSS software (Munich, Germany).

Results

Patient characteristics and individual expression levels are displayed in Table I. To determine, if our results agree with other studies (15, 16), we initially grouped the patients according to their diagnosis into tumor cases and non-tumor controls. There were significantly higher levels of EGFR and P-GP in the tumor group compared to controls for the basal expression (0 hours) and for all expression levels after the different stimulation periods (24 h, 48 h and 72 h), except for P-GP expression after 72 hours of stimulation with TGF-α (Mann Whitney *U*-test, see Figures 2 and 3) confirming the results of previous studies. No influence of duration of incubation with TGF-α on EGFR and P-GP levels was detected after 24, 48 and 72 hours of incubation (Wilcoxon signed rank test).

Table II shows the overall EGFR and P-GP expression according to smoking habit. Since all biopsies were taken from normal mucosa, for all further analyses we no longer distinguished between cases and controls. As there was no significant difference between EGFR/P-GP expression immediately after cultivation (0 hours) and following different periods of stimulation with TGF-α, the average levels of all available (Σ) slides are also shown in Table II. Smoking status for 26 patients was available for analyses. Patients were grouped into three categories: never, former

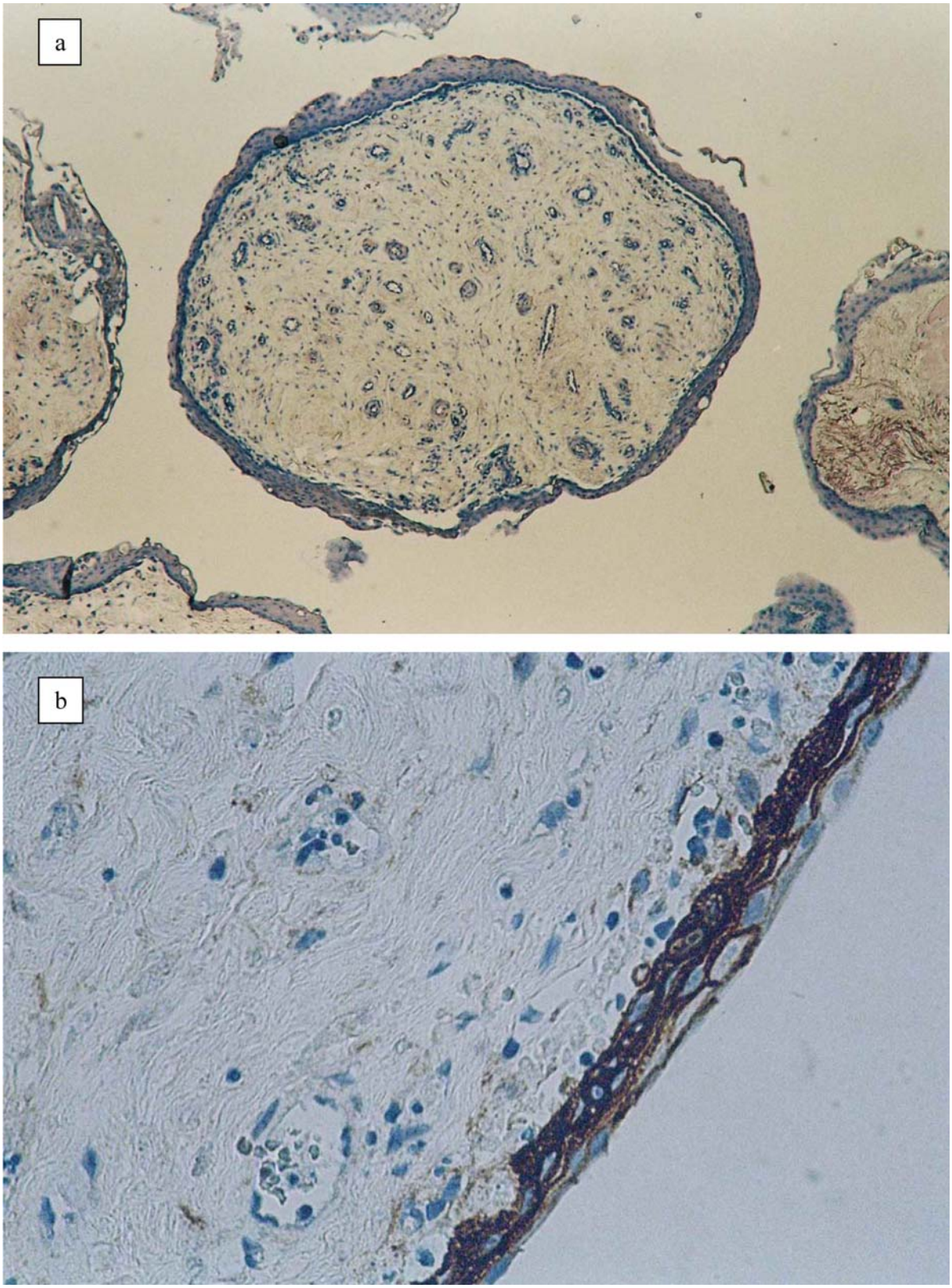

Figure 1. *Histological slide of a mini-organ culture (hematoxylin/eosin staining, ×500; a) and immunohistochemical staining of epidermal growth factor receptor (b).*

Table II. *Epidermal growth factor receptor and P-GP expression levels grouped by smoking status.*

Never smokers		Current smokers	
EGFR (0 h); n=11	1.33	EGFR (0 h); n=8	2.5
EGFR (Σ); n=47	1.47	EGFR (Σ); n=32	2.53
P-GP (0 h); n=11	1.5	P-GP (0 h); n=8	2.5
P-GP (Σ); n=47	0.4	P-GP (Σ); n=32	2.34
Former smokers			
EGFR (0 h); n=7	1.71		
EGFR (Σ); n=28	2.11		
P-GP (0 h); n=6	1.5		
P-GP (Σ); n=27	1.7		

Table III. *P-values (Mann Whitney U-test) for comparison of data shown in Table II. Epidermal Growth Factor Receptor/P-GP (0 h), initial expression after cultivation; EGFR/P-GP (Σ), average expression after 0, 24, 48 and 72 h of stimulation with Transforming Growth Factor α.*

EGFR (0h)		EGFR (Σ)	
Former *vs*. never	0.395	Former *vs*. never	0.032
Current *vs*. former	0.022	Current *vs*. former	0.162
Current *vs*. never	0.018	Current *vs*. never	0.010
P-GP (0h)		P-GP (Σ)	
Former *vs*. never	0.011	Former *vs*. never	0.004
Current *vs*. former	0.065	Current *vs*. former	0.268
Current *vs*. never	<0.001	Current *vs*. never	<0.001

and current smokers. Never smokers did not consume tobacco so far, former smokers stopped smoking at least six month before their operation and current smokers currently smoked at the time of the procedure. Evaluating slides without EGFR stimulation, there was a significantly higher expression of the receptor in current smokers compared to former smokers and to never smokers. No difference was seen between former and never smokers. When all available slides were considered, current smokers expressed significantly more EGFR than never smokers as did former smokers, whereas there was no difference between current and former smokers (see Table III).

Regarding initial P-GP expression levels, 17/28 cultures (60.7%) in total, 14/15 cultures (93.3%) from patients with smoking history and 2/11 cultures (16.7%) from never smokers, were P-GP positive. Again there was no statistically significant difference between expression in those of current and former smokers, but there was between current and never smokers, as well as former and never smokers. Analyses of all slides confirmed these findings. Expression levels are illustrated in Figures 4 and 5, *p*-levels are summarized in Table III.

Discussion

Our results indicate a clear association between EGFR and P-GP levels on one hand, and smoking on the other hand. Stimulation of EGFR with TGF-α had no influence on expression of either protein. Thus, at least within 72 hours, EGFR did not increase P-GP transcription. The antimutagenic effect of TGF-α that we showed in previous studies using the same tissue cultures must therefore be interpreted as a consequence of the direct enhancement of P-GP function by EGFR signaling. Smoking seems to have a long lasting impact on oropharyngeal cells, since 7/8 cultures from current smokers and 7/7 cultures from former smokers showed EGFR expression. P-GP was detectable in 8/8 cultures of current and 5/6 cultures of former smokers. P-GP seems to be a more sensitive marker for smoking since 10/11 cultures of never smokers showed EGFR, but only 2/11 cultures of never smokers P-GP expression (initial levels).

The results of this study clearly indicate that exposure of oropharyngeal mucosa to cigarette smoke leads to increasing P-GP expression and that EGFR plays a major role in the functional regulation of this transporter. Moreover, the results offer a good explanation for previous findings of our laboratory that EGFR stimulation protects oropharyngeal cells from BPDE-induced DNA damage (25, 26).

Seen from the oncologist's perspective, the proposed mechanism harbors a significant problem. Tumors arising from tissues with high P-GP expression usually have the same high levels themselves (48). If smoking induces P-GP expression, about two-thirds of head and neck carcinomas arising in patients with a history of considerable tobacco consumption will highly express P-GP and, hence, increased drug resistance and poor prognosis.

The identification of HPV as a causative factor of oropharyngeal squamous cell carcinoma offers a good opportunity to study molecular features of tumors of a completely different etiology. Whereas overall incidences of head and neck cancer are slightly decreasing, most likely due to less tobacco consumption at least in the Western world, an increase in cancer in the base of tongue and tonsils has been noted during the last two decades (49). For these and other oropharyngeal squamous cell cancer a strong association with HPV, mainly type 16, was found (50). HPV-positive carcinoma of the tonsils and the base of tongue predominantly affect younger patients with less tobacco consumption (3, 51, 52). Consequently, these types of cancer frequently do not show the typical alterations of field cancerization; EGFR expression is distinctly lower than in

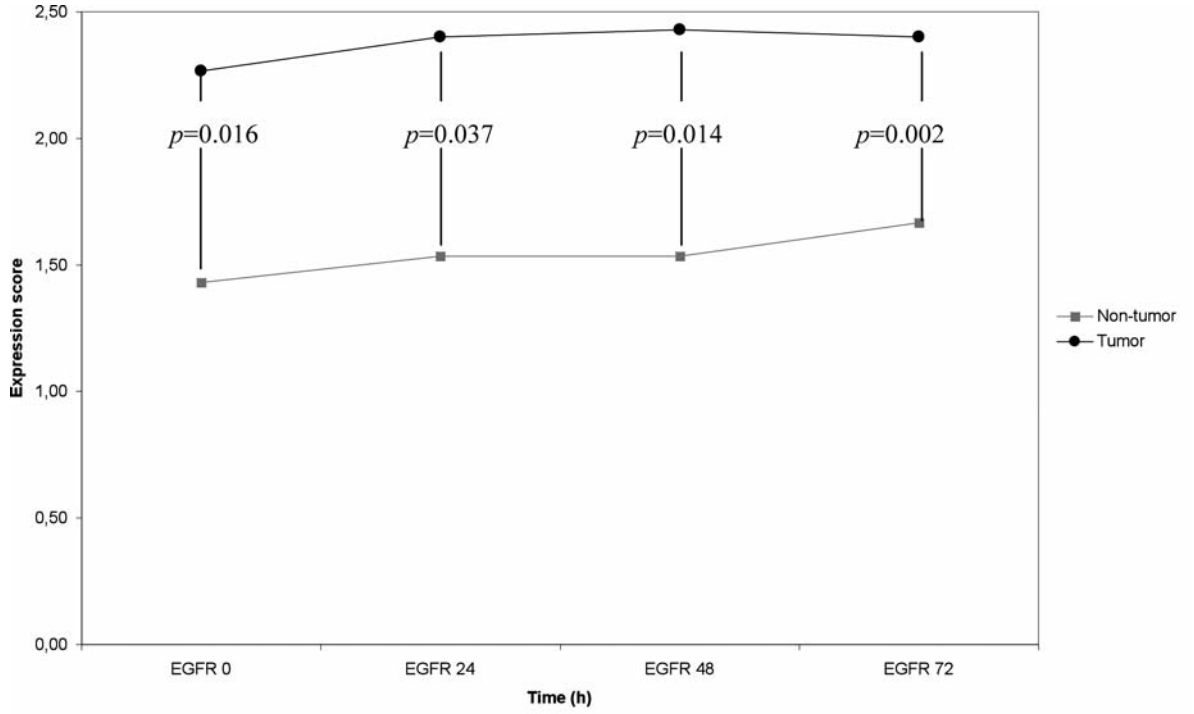

Figure 2. *Epidermal growth factor receptor expression after cultivation (0 h) and different stimulation periods using transforming growth factor α (50 ng/ml).*

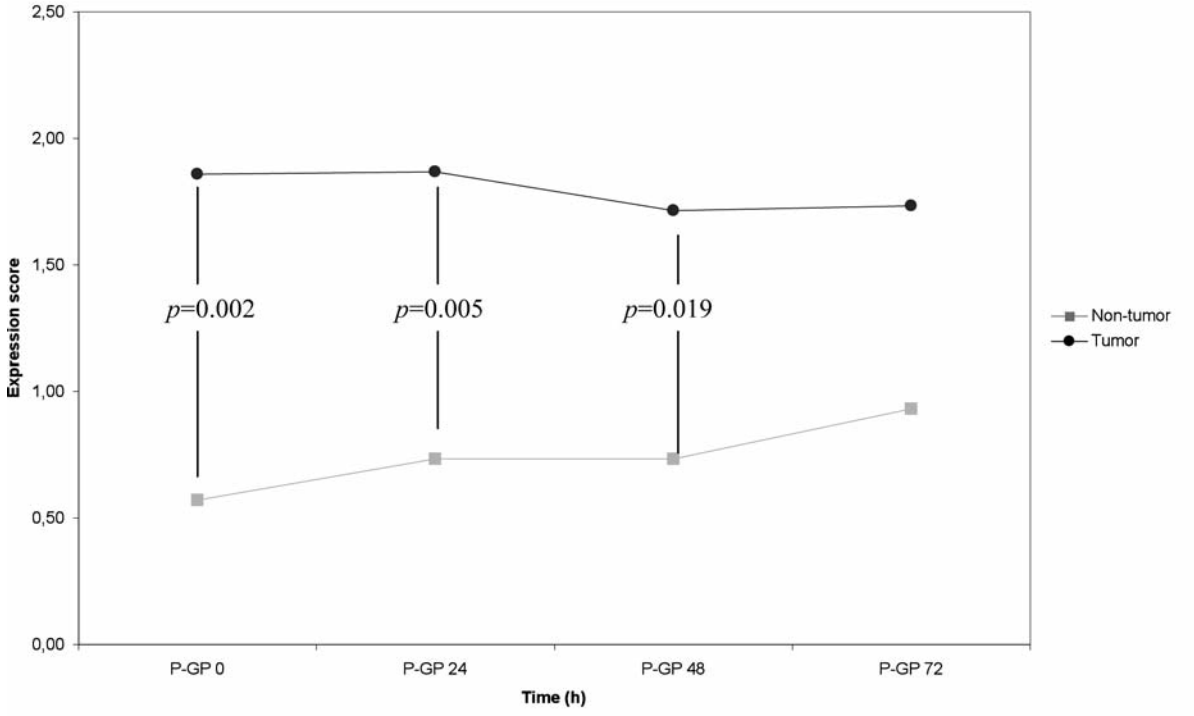

Figure 3. *P-GP expression after cultivation (0 h) and different stimulation periods using transforming growth factor α (50 ng/ml).*

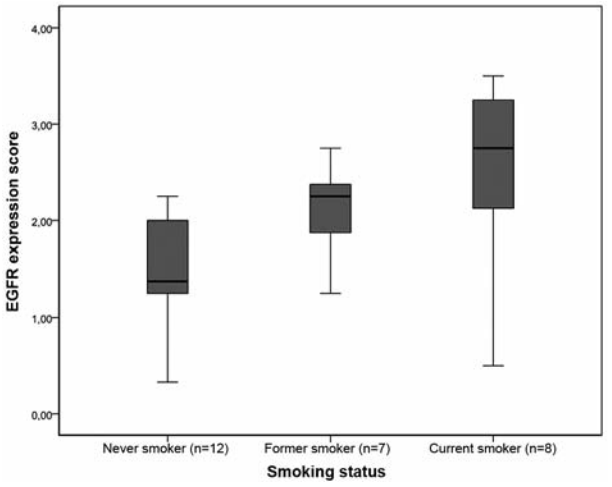

Figure 4. *Average Epidermal Growth Factor Receptor (Σ) expression according to smoking status.*

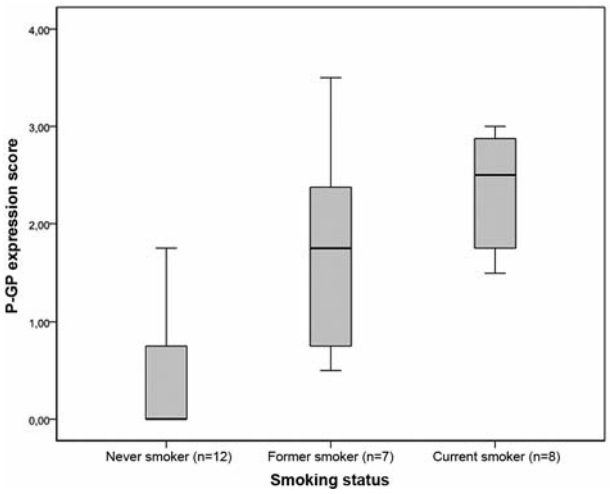

Figure 5. *Average P-GP (Σ) expression according to smoking status.*

tumors associated with tobacco use. Young *et al.* also reported increased *EGFR* gene copy number largely restricted to tumors negative for p16 expression, a robust surrogate marker for HPV infection. At the protein level, 8 out of 10 *EGFR*-negative carcinomas were p16 positive (53, 54). Similar results were found in a study 106 of newly diagnosed cases of oropharyngeal cancer. HPV associated tumors and EGFR levels were inversely correlated (55). Our institution routinely determines HPV association of HNSCC by p16 immunohistochemical staining. In this study, 13 tissue donors had histologically proven oropharyngeal cancer, 4/13 were p16-positive. In our study, there was no difference in EGFR and P-GP levels in tissue cultures derived from patients with p16-positive or negative tumors, most probably because all cancer patients were former or current smokers. This view is underlined by a study of Won and colleagues presented during the 2010 Annual Meeting of the American Society of Clinical Oncology reporting an inverse correlation of EGFR and HPV positivity in 66 tonsil cancer, but elevated EGFR levels in HPV-positive cancer of smokers (56).

The lack of molecular features of tobacco-induced field cancerization in many cases of HPV-positive oropharyngeal cancer has an immense impact on tumor biology. In a retrospective analysis of 111 cases of oropharyngeal cancers, enrolled in the TAX 324 trial and whose pretreatment biopsies were assessable, 5-year survival after induction chemotherapy followed by chemoradiation of patients with HPV-DNA positive cancers was 82% compared to 35% of HPV-negative tumors (57). In a prospective study of 66 patients with oropharyngeal cancer treated with induction chemotherapy (cisplatin/carboplatin plus 5-fluoruracil) followed by concurrent chemoradiation for responders and surgery for non-responders, p16 expression in pretreatment biopsies was inversely correlated with EGFR levels and low EGFR combined with high p16 expression were markers of good response to non-surgical therapy (58). The favourable cancer biology of HPV/p16-positive tumors is in some sense overruled by the negative effects of enhanced EGFR levels and heavy smoking, respectively. In a retrospective analysis of 71 cases of HNSCC, Szabó *et al.* could not observe any improved survival of HPV positive patients, most probably due to heavy tobacco and alcohol consumption by the majority of patients. Again, intensive EGFR expression was correlated with poor prognosis (59).

In summary, the results presented here, findings of previous studies in our laboratory, available data from other laboratories and clinical trials point to the following: i. EGFR and P-GP levels in healthy oropharyngeal mucosa are related to smoking history. ii. EGFR and P-GP mediate chemoimmunity of mucosal cell to mutagens and carcinogens. iii. In the case of malignant transformation, EGFR and P-GP contribute to drug resistance of cancer.

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Received March 5, 2012
Revised April 6, 2012
Accepted April 9, 2012