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Expression of Multi-drug Resistance Genes (*mdr1, mrp1, bcrp*) in Primary Oral Squamous Cell Carcinoma*

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Abstract. *The expression of resistance genes can cause the ineffectiveness of chemotherapeutics for the treatment of cancer. Therefore, known resistance genes were investigated in oral squamous cell carcinoma (OSCC) and the results were compared with clinico-pathological findings. Materials and Methods: Fresh frozen samples of 45 primary OSCC were investigated for the expression of mdr1 (p-glycoprotein-mediated multi-drug resistance), mrp1 (multi-drug resistance-related protein) and bcrp (breast cancer-related protein), using a reverse transcriptase PCR. The gene products were revealed immunohistochemically on representative slices of the same tumor sample. The results were compared with TNM stage grouping [SG, (UICC, 1987)], HPV infection and p53 mutations (exons 5-8). Results: The expression of the resistance genes was independent of age, sex, localisation of the tumor, HPV infection and p53 mutations. SG did not correlate to mdr1 and mrp1. On the other hand, bcrp expression increased 2.7-fold between SG III and IV OSCC. Loss of differentiation was associated with an increased expression of mdr1 ($p=0.06$), mrp1 ($p<0.01$) and bcrp ($p<0.01$). The bcrp expression correlated with shorter survival periods. Expression of mrp1 and mdr1 did not correlate positively in a linear pattern. Expression of mdr1 and bcrp moderately positively correlated ($p<0.01$). Discussion: Multi-drug resistance genes can be up-regulated in OSCC. The expression of at least one of these genes is up-regulated in SG-IV OSCC. Determining these genes could probably support current studies on therapeutic effects in OSCC, e.g. new cytostatic drugs.*

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Key Words: Multi-drug resistance, *mdr1, mrp1, bcrp*, oral squamous cell carcinoma, oral cancer, rt-pcr, p53, HPV infection.

The current estimation of new cases of patients with oral and oropharyngeal cancer is about 11,000 per year. Based on calculations of the Hamburg State Cancer Registry and comparing data from the last 30 years (1), this type of cancer is increasing in this region. Squamous cell carcinoma is the predominant histological type of oral cancer (2, 3). Ablative surgery and radiotherapy as an adjunct are the most effective treatment methods. Cytostatic drugs are only rarely used with curative intent but have proved to be effective in combination with radiotherapy and surgery and for palliative treatment (4). The effectiveness of cytostatic chemotherapy for OSCC is frequently restricted due to an inducible cellular mechanism called "chemoresistance" (5). The multifactorial resistance of carcinoma cells to cytostatics can vary during chemotherapy, shows similarities between pharmacological classes (6-16) and can also be induced by X-rays (17-19). This phenomenon is currently called "multi-drug resistance" (MDR) (20-35). MDR can also be induced *in vitro* and is used for the prediction of chemosensitivity of tumors to cytostatic drugs (33, 36-39). One cause for MDR is p-glycoprotein, which binds intracellulary to cytostatics and promotes exocytosis. The cDNA of *mdr1* and *mdr2* encode for p-glycoprotein resulting in MDR (10, 40-49). Following the discovery of p-glycoprotein further, drug-resistance-associated proteins were identified, *e.g.* multi-drug resistance-associated protein (*mrp1*) (8, 43, 50-53).The *mrp1* expression proved to be prognostic for neuroblastoma (54) and some leukemias (48, 55-57). A third pump mechanism not related to p-glycoprotein or *mrp1* was identified from breast cancer cells (58, 59). BCRP (*b*reast *c*ancer *r*elated-*p*rotein, *bcrp*), like *mdr1* and *mrp1*, belongs to the super family of ABC-transporters (*A*TP-*B*inding *C*assette) (60, 61).

Quantification of MDR in OSCC has yet not been undertaken. The aim of this study was to quantify the expression of MDR genes in OSCC and to correlate the findings to p53 mutations (62, 63), human papilloma virus infection status (64, 65) and clinical parameters (66-68).

Table I. *Permanent tumor cell lines.*

Alphanumeric Identification	Characteristics, Source	Reference
A2780	Breast carcinoma, *mrp1* in physiological range	70
K562	Erythroleukemia,	
K-562-RADR	*mdr1*-positive subline	38
EPG85-257	Gastric carcinoma,	71
EPG-85-257RNOV	*bcrp*-positive subline	

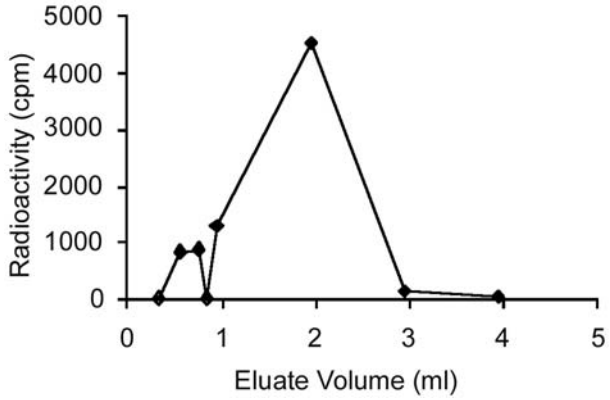

Figure 1. *Eluate profile of the RT product in a nick column. The first relative maximum is caused by the incorporated radioactively-marked nucleotides. The absolute maximum is caused by the radioactivity of the nucleotides that are not incorporated. The cDNA of the RT-PCR reaction is localized in the fraction 350 µl to 850 µl.*

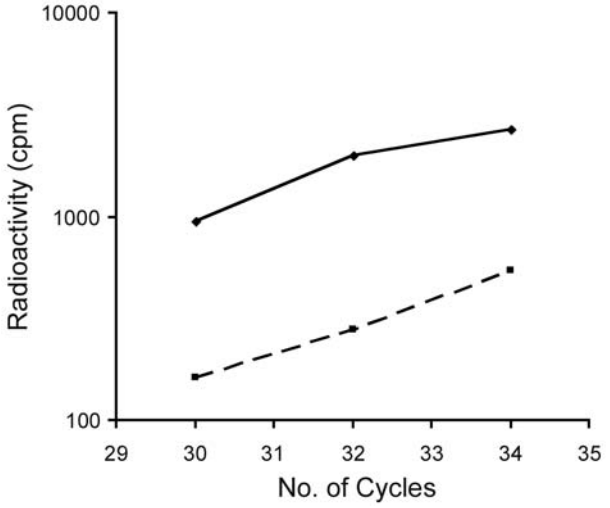

Figure 2. *Dependency of bcrp on the number of cycles, determined on the mitoxantrone-sensitive reference cells EPG85-257P (below) and the 185-fold mitoxantrone-resistant variant EPG85-257RNOV (above). Mean values from double determinations.*

Materials and Methods

Reference cell lines. An overview on the characteristics of the reference cell lines that were used to determine the expression level of MDR genes is provided in Table I.

The expression of the reference gene *gapdh* (glycerine aldehyde - 3 - phosphate-dehydrogenase) was determined in every sample as a control. The expression level of the MDR genes (*mdr1, mrp1, bcrp*) was defined as the quotient of the expression value of a single gene to the simultaneously determined value of the reference gene in each case. The following three cell lines with a known expression pattern were used for semi-quantitative calibration (69):

a) Leukemia cell line K562: The human erythroleukemia cell line K562 was stepwise selected with doxorubicin. The subline K562-RADR expresses p-glycoprotein.

b) Breast cancer cell line A2780: The resistance gene *mrp1* is expressed in the majority of normal tissues and in tumors. A normal range of expression has to be considered when analysing tumors. The cell line A2780 derived from a breast carcinoma was used as a reference for normal *mrp1* expression (70).

c) Gastric cancer cell line EPG85-257: The cell line EPG85-257 was generated from a gastric carcinoma (71). The subline EPG85-257RNOV shows a 185-fold mitoxantrone resistance. The cells do not express p-glycoprotein but the resistance factor *bcrp* 12-fold.

OSCC tissue samples. The OSCC tissue samples were from 45 patients treated at the Oral and Maxillofacial Surgery Department, Eppendorf University Hospital, Germany. The carcinomas were classified according to the UICC, 1987 (72). The mRNA extractions of these tumors (n=45) had been investigated in an earlier study for p53 mutations (65) and were provided for this study by Dr. S. Riethdorf, Department of Pathology, UKE. In 26 cases frozen tissue was available for immunohistochemistry. All tissues and preparations were immediately frozen in liquid nitrogen and stored at -80˚C. The ethics committee of the Hamburg Chamber of Physicians had approved these investigations (No. OB.96).

Immunohistochemical staining of MDR proteins. mRNA was identified immunohistochemically in specimens of 26 OSCC patients from whom paraffin sections were available. The p-glycoprotein was identified on 4-µm-thin slices of the OSCC using mouse monoclonal antibodies (C219, Alexis Biochemicals, Gruenberg, Germany, and JSB-1, Boehringer Mannheim Biochemica, Mannheim, Germany). The antibody MRPr1 was used to identify the *mrp* gene product (SanBio, Amsterdam, The Netherlands). The avidin-biotin method modified by Lage *et al.* (73) was used for the visualization of the antigen-antibody reactions (61, 74, 75).

In brief, the slices were dewaxed in xylol, dehydrated in graded ethanols, finally cleaned in H_2O and stored in TRIS-buffered saline (TBS, Merck, Darmstadt, Germany). The commercially available antibodies, supplied as liquids, were diluted in bovine serum albumin (BSA), goat serum albumin (both: Sigma, Deisenhofen, Germany) and TBS at a proportion of 1:20:79, *i.e.* C219 4 µg/ml,

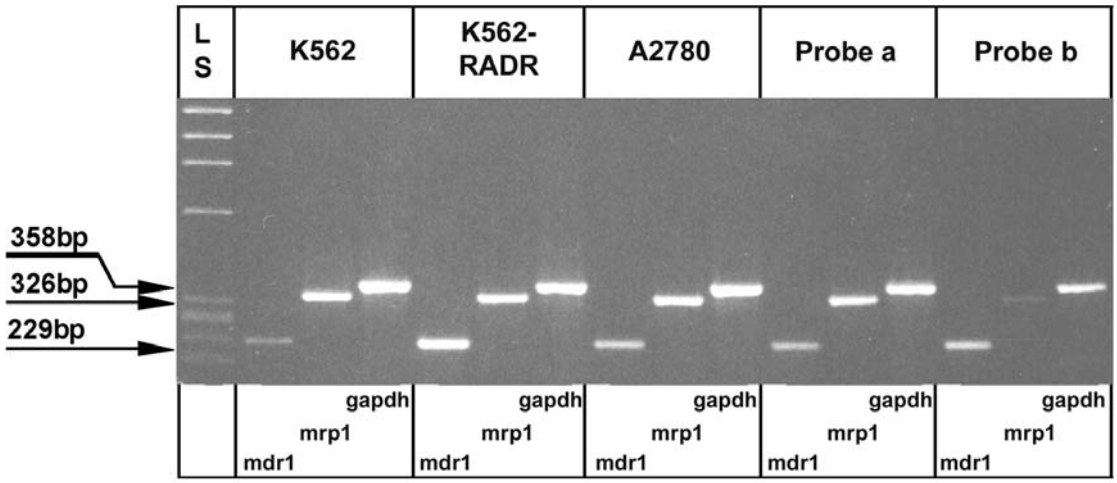

Figure 3. *Agarose gel with PCR findings. On the left side appears the length standard (LS). From the left to the right appear sequentially the PCR products of mdr1 (229 bp), mrp1 (326 bp) [for reference: (52)] and gapdh (358 bp), starting with the cell lines K562, K562-RADR and A2780, followed by an OSCC (probe a) and a colon carcinoma (probe b). For details see text.*

JSB-1 2μg/ml and MRPr 0.8 μg/ml. The slides were incubated with 100 - 150 μl of the diluted antibody and incubated for one hour in a liquid chamber. Then the slides were cleaned in TBS (250ml). Tween (250 μl, 0.1%; Serva, Heidelberg, Germany) and NaCl (1.5g; 100 mM) was added to this TBS diluent. Incubation with the second biotin-marked antibody was allowed for 30 min (Dianova, Hamburg, Germany, 2.6 μg/ml in TBS, including 1% BSA). Then the slides were cleaned in TBS and incubated with the AEC staining solution provided by Dako (Glostrup, Denmark; C219 and MRPr: 5 min; JSB-1: 7 min). Final rinsing of the slides in H_2O was followed by counterstaining with haemalaun and embedding in glycine.

Incubation with an anti-vimentin antibody (Dianova) with known reactivity in tumor cells and stroma served as a positive control for the staining system (76). Negative controls were created by omitting the primary antibody but maintaining the identical staining protocol. All tissues were judged on routinely processed haematoxylin-eosin-stained slices in order to re-evaluate the tissue and to compare the findings to the original histological diagnosis.

Determination of mRNA expression. Extracted RNA was prepared with RNAzol (RNAzol™, Cinna Biotecs, Houston, Texas, USA) according to Chevillard *et al.* (77, 78). Frozen tumor samples stored at -80˚C were homogenized with a cooled tungsten carbide pellet in the presence of frozen nazol (2ml/100mg tissue) and using a micro-dismembranator (Braun, Melsungen, Germany). Extraction of the RNA from the homogenate was facilitated by adding chloroform (1/10) to the preparation, shaking the substances for 15 sec and then setting the bottle on ice for 5 min. Then the samples were centrifuged at 15,000 g and 4˚C for 15 min. This results in two phases. The superior phase was decanted and replaced and incubated with the same volume of isopropanol for 20 min at -20˚C. After centrifugation, the supernatant was discarded and 1 ml ethanol (75%) was added. After the third centrifugation (4˚C, 7,500, 15 min) the ethanol was removed using pipettes and the pellet was dissolved in RNAse-free bidistilled water. The concentration of total RNA was determined photometrically at 260 nm. The purity of the sample was determined as the quotient of the extinction at 260nm/280nm (2.0-1.7). Samples with quotients lower than 1.6 were discarded. In addition, 5μg RNA in 1% agarose gels were regularly quality checked under denaturating conditions. The RNA was stored at -80˚C.

Table II. *Expression of the resistance genes mdr1 and mrp1 in reference cell lines, an OSCC (a) and colon carcinoma (b). The calculations are based on the measured radioactivity of the separated bands of the agarose gel, as shown in Figure 3.*

	K562	*K562-RADR*	*A2780*	*a*	*b*
mdr1/gapdh	0.03	0.59	0.12	0.16	0.49
mrp1/gapdh	0.61	0.58	0.45	0.69	0.03

Construction of cDNA (reverse transcriptase reaction). This method was described in detail by Reymann *et al.* (79) and was developed from a combination of the techniques described by Dietel *et al.* (37) and Gekeler *et al.* (80). A defined amount of radioactive-marked nucleotide is added to the RT-PCR. The determination of the radioactivity of the RT-PCR product allows the calculation of the cDNA amount.

Eight μg RNA were dissolved in 10 μl distilled water and incubated with 0.5 μg random hexanucleotide primer (Promega, Mannheim, Germany) for 10 min at 70˚C. The incubated RNA was put in a solution containing 10 μl First Strand Buffer 5x (250 mM Tris-HCl, 375 mM KCl, 15 mM $MgCl_2$, Gibco BRL), 5 μl dithiotreitol (0.1 M DTT, Gibco BRL), 5 μl dNTP mixture (each 10 mM dATP, dGTP, dCTP and dTTP), alpha-^{32}P dCTP pmol (Amersham, Freiburg, Germany) and 45 μl distilled water and heated at 42˚C for 3 min. Then Superscript II (200 U/μl, Gibco BRL) was mixed thoroughly with the solution (1 h, 42˚C).

Table III. Part 1. *Basic patient data, ordered according to the Stage Group (TNM, UICC 1987). Abbreviations: G=gender; f=female; m=male; Age=age at diagnosis in years; survival following diagnosis in months (d=deceased; a=alive); Loc.=localisation: 1=floor of the mouth; 2=cheek; 3=hypo-, oropharynx or tonsil; 4=larynx; 5=disseminated local recurrence; Grading: 1=well; 2=moderate; 3=poor; HPV status (Type 16 or 18): 1=proven infection; 0=no HPV infection; p53 status: 1=proven mutation; 0=without p53 mutation; -=not determined.*

No.	G.	Age	Survival	Loc.	Stage-Group	TNM-Classification	Grading	HPV Status	*p53* Status
1	f	81	17 (d)	1	1	T1N0M0	1	1	1
2	m	45	26 (d)	1	2	T2N0M0	2	1	1
3	m	40	108 (a)	1	2	T2N0M0	1	-	-
4	m	37	16 (d)	4	3	T3N0M0	2	0	0
5	m	52	113 (a)	1	3	T3N0M0	-	-	0
6	m	75	3 (d)	1	3	T2N1M0	2	-	0
7	f	69	11 (d)	1	3	T2N1MX	2	-	-
8	f	66	12 (d)	1	3	T2N1M0	2	-	-
9	m	65	7 (d)	1	3	T3N1M0	1	1	1
10	m	55	34 (d)	1	3	T3N1M0	2	0	0
11	m	69	12 (d)	4	3	T3N1M0	-	1	1
12	m	65	62 (a)	1	4	T2N2M0	2	1	0
13	f	75	12 (d)	1	4	T2N2M0	2	1	0
14	m	63	69 (d)	3	4	T2N2M0	2	-	1
15	m	64	85 (a)	1	4	T2N2M0	2	-	0
16	m	49	48 (d)	3	4	T3N2M0	2	0	1
17	m	64	5 (d)	1	4	T3N2M0	2	0	1
18	m	52	9 (d)	1	4	T3N2M0	2	1	1
19	m	46	18 (d)	3	4	T3N3M0	2	1	1
20	m	59	108 (a)	3	4	T3N3M0	3	1	0

Table III. Part 2. *Basic patient data ordered according to the Stage Group (TNM, UICC 1987). Abbreviations: G=gender; f=female; m=male; Age=age at diagnosis in years; survival following diagnosis in months (d=deceased; a=alive); Loc.=localisation: 1=floor of the mouth; 2=cheek; 3=hypo-, oropharynx or tonsil; 4=larynx; 5=disseminated local recurrence; Grading: 1=well; 2=moderate; 3=poor; HPV status (Type 16 or 18): 1=proven infection; 0=no HPV infection; p53 status: 1=proven mutation; 0=without p53 mutation; -=not determined.*

No.	G.	Age	Survival	Loc.	Stage-Group	TNM-Classification	Grading	HPV Status	p53 Status
21	f	76	37 (d)	1	4	T4N0M0	1	0	1
22	f	72	157 (a)	1	4	T4N0M0	2	1	0
23	m	74	104 (a)	4	4	T4N0M0	2	1	1
24	m	63	150 (a)	1	4	T4N0M0	1	0	1
25	m	68	6 (d)	1	4	T4N1M0	2	0	1
26	m	53	50 (d)	5	4	T4N1M1	3	0	0
27	f	85	12 (d)	2	4	T4N2M0	1	-	-
28	f	44	13 (d)	1	4	T4N2M0	2	-	1
29	m	65	5 (d)	1	4	T4N2M0	2	-	1
30	m	53	34 (d)	1	4	T4N2M0	2	-	1
31	m	54	26 (d)	1	4	T4N2M0	2	-	0
32	f	81	7 (d)	2	4	T4N2M0	2	1	1
33	m	49	6 (d)	1	4	T4N2M0	2	1	1
34	m	41	40 (a)	1	4	T4N2M0	2	0	1
35	f	41	9 (d)	1	4	T4N2M0	2	1	0
36	m	49	6 (d)	3	4	T4N2M0	2	-	1
37	f	70	13 (d)	3	4	T4N2M0	2	1	1
38	m	62	7 (d)	1	4	T4N2M0	-	0	1
39	m	52	7 (d)	1	4	T4N2M0	-	0	0
40	m	43	14 (d)	1	4	T4N2M0	-	0	1
41	m	55	12 (d)	1	4	TXNXM1	3	-	-

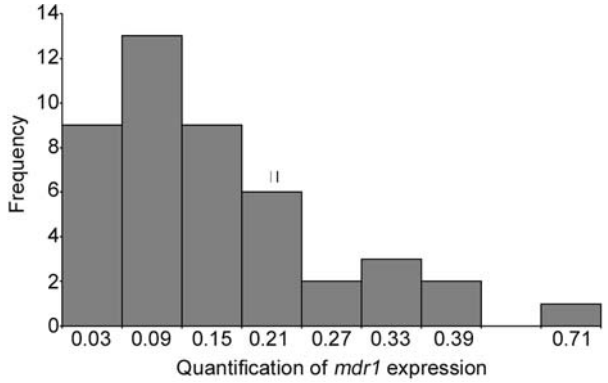

Figure 4. *Histogram of the quantified mdr1 expression. The values of the abscissa are means of the 0.06 units of each class (n=45).*

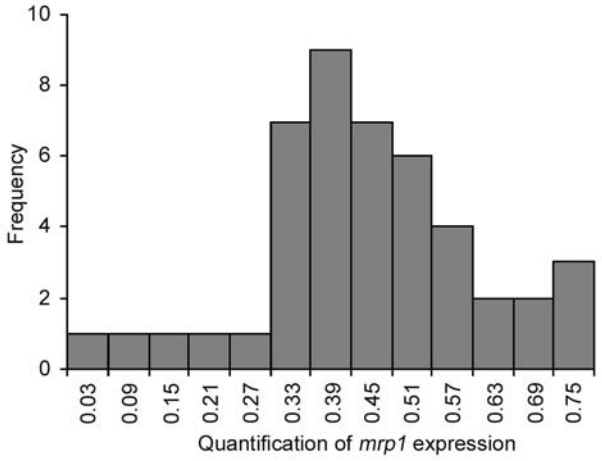

Figure 5. *Histogram of the quantified mrp1 expression. The values of the abscissa are means of the 0.06 units of each class (n=45).*

Cleaning of the cDNA. The RT-PCR product was applied to nick columns (Pharmacia Biotec, Uppsala, Sweden). The eluate was fractionated and the radioactivity of marked dCTP was calculated with a liquid scintillation spectrometer (Packard, Meriden, USA). Fractions of 350 µl up to 850 µl contained the cDNA. The eluate (500 µl) was precipitated with 1/10 10M ammonium acetate and 2.5-fold of ethanol, cooled at -80˚C for 30 min, then centrifuged for 30 min at 14,000 g and 4˚C. The sediment was rinsed in 500 µl of 70% ethanol. The incubation was repeated at -80˚C for 30 min. The sediment was dissolved in 30 µl distilled water.

The known proportion of labeled and non-labeled nucleotides allowed the calculation of the obtained cDNA (Figure 1).

PCR. The PCR was performed according to Reymann *et al.* (79). The cDNA preparation was diluted to 20 µg cDNA in 5 µl per attempt [exception: *gapdh*: cDNA 10 µg in 5 µl distilled water]. Then the solution was completed with 2.5 µl PCR-buffer, 10x (200 mmol/l Tris-HCl, pH=8.4, 500 mmol/l KCl, Gibco BRL), 2.5 µl dNTP Mix (2 mmol/l), alpha ^{32}P dCTP (0.15 pmol), 0.75 µl $MgCl_2$ (50 mmol/l, Gibco BRL) and 18 µl distilled water and 1 µl each of the specific primers (10 µmol/l, for details see Gekeler *et al.* (80)).

The amplification was performed in a thermocycler (Hybaid, Heidelberg, Germany) with 0.25 µl taq-polymerase (5 U/µl, Gibco BRL) (start: 94˚C, 10 min, then 32 cycles: denaturation 1 min and 91˚C, primer binding 1.5 min and 60˚C, replication 2 min and 70˚C, termination of the reaction at 70˚ for 10 min), (Figure 2).

Comparative analysis. The quantification of the PCR products followed after electrophoretic separation in an agarose gel, excision of the visible bands and determination of the radioactive extinction. An example is depicted in Figure 3 and Table II.

Statistics. The statistical calculations were performed according to Tallarida and Murray (81) and their recommendations for test requirements in statistics programs (SPSS™ version 9.0). The clinical parameter resulted in asymmetrical spot check values, *i.e.* small subgroups. Therefore, statistics were first of all performed with the rank sum test and Chi-square test and secondly with the ANOVA and *t*-test [further tests and descriptive values: Kaplan-Meier analysis, log-rank, Levene-test, Fisher's exact test, arithmetical mean, standard deviation, standard error of mean (SEM), multiple correlation coefficient, quantile]. The significance ($p<0.05$) and trends ($0.1 > p > 0.05$) of differences were calculated, the latter one restricted to differences >50%.

Table IV. *Stage grouping according to the TNM classification (72). One tumor was not determined for T- and N-stage. A further patient's N-stage was not clear. However, all patients were adequately categorized to stage groups.*

Stage/Group		No. of Patients	%
1. T-Stage	1	1	3
	2	9	23
	3	10	25
	4	20	50
2. N-Stage	0	9	23
	1	8	20
	2	21	53
	3	2	5
3. M-Stage	0	38	95
	1	2	5
4. Stage-Group	1	1	2
	2	2	5
	3	8	20
	4	30	73

Results

Patient data. The expression analysis was performed on 45 OSCC tumor samples. The clinical data of 41 patients were retrospectively evaluated (Tables III and IV). The classification and stage grouping was performed according to the recommendations of the Union International Contre le Cancer (UICC), (72). Data from an earlier study on the

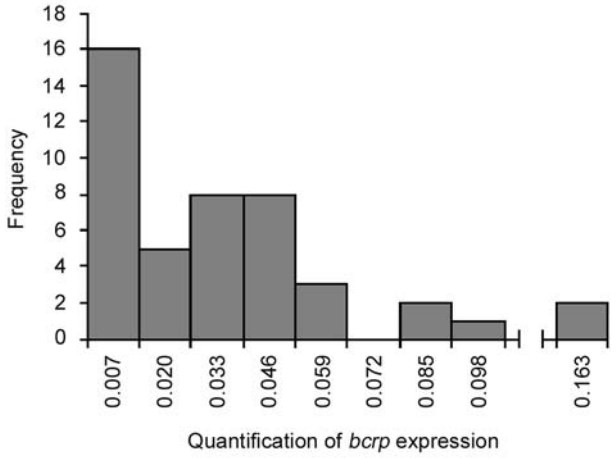

Figure 6. *Histogram of the quantified bcrp expression. The values of the abscissa are means of the 0.013 units of each class (n=45).*

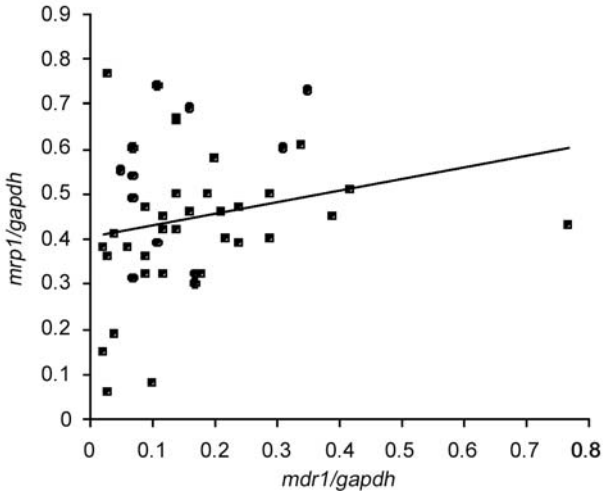

Figure 7. *Co-expression of mdr1 and mrp1. The mean mdr1 expression is 0.16. The mean mrp1 expression is 0.45. The expression patterns are different for both genes. The regression analysis reveals r=0.22, p=0.14 (n=45).*

Table V. *Relation of mdr1 expression (positive or negative) and mrp1 expression. The mrp1 expression is normal within the mean value ± 1 SD. The mdr1 expression is positive, if the value exceeds the 75% percentile of the reference cell line K562 (p=0.028, Pearson Chi-square-test, p=0.034, Fisher's exact test, n=45).*

	mdr1 expression negative n (%)	mdr1 expression positive n (%)
mrp1 expression normal n (%)	18 (40%)	17 (38%)
mrp1 expression subnormal/elevated n (%)	9 (20%)	1 (2%)

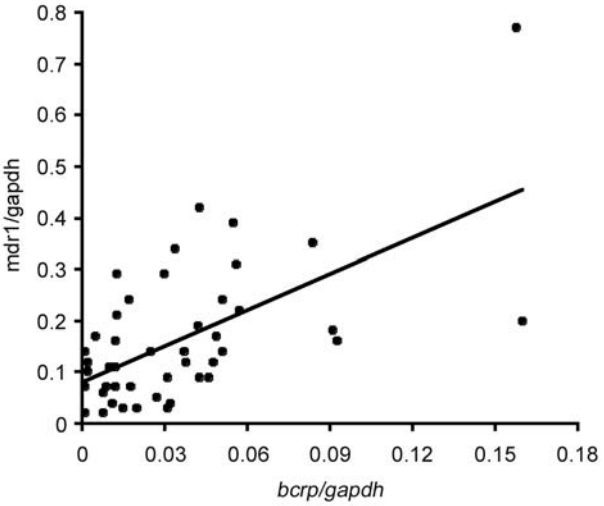

Figure 8. *Co-expression of bcrp and mdr1. The regression analysis reveals r=0.6, p>0.001 (n = 45).*

infection status with human papilloma virus (HPV) and mutations of the p53 tumor suppressor gene (82) were known for some patients of the current study and were used to determine possible interdependencies (65) (Table III).

Gender was unequally distributed in this group [men/women=32 (74%)/11 (26%)] but this correlation is characteristic for this entity. The mean age at the time of diagnosis was 61 years±14 years SD (min.: 37 yrs., max.: 94 yrs.). The mean survival was 36 months. About 3 out of 4 patients were in stage 3 to 4. After 5 years 20% of the patients were surviving. This is in accordance with experiences from other cancer therapy centers. The grading revealed a predominance for moderately-differentiated (diff.) carcinomas [n=27 (75%), highly-diff.: 6 (17%), poorly-diff.: 3 (8%)]. The majority of OSCC were located in the tongue or floor of the mouth (29, 71%). The remaining OSCC were in the cheek 2 (5%), larynx 6 (15%) and oro- or hypopharynx 3 (7%). One tumor recurrence was observed in the lateral neck.

Expression analysis

mdr1: The expression level of the resistance gene *mdr1* was usually low (mean: 0.16±0.02 SEM, n=45). The expression levels in the parallel determined colon carcinomas were clearly higher (0.25±0.03 SEM, n=11, $p<0.01$). The

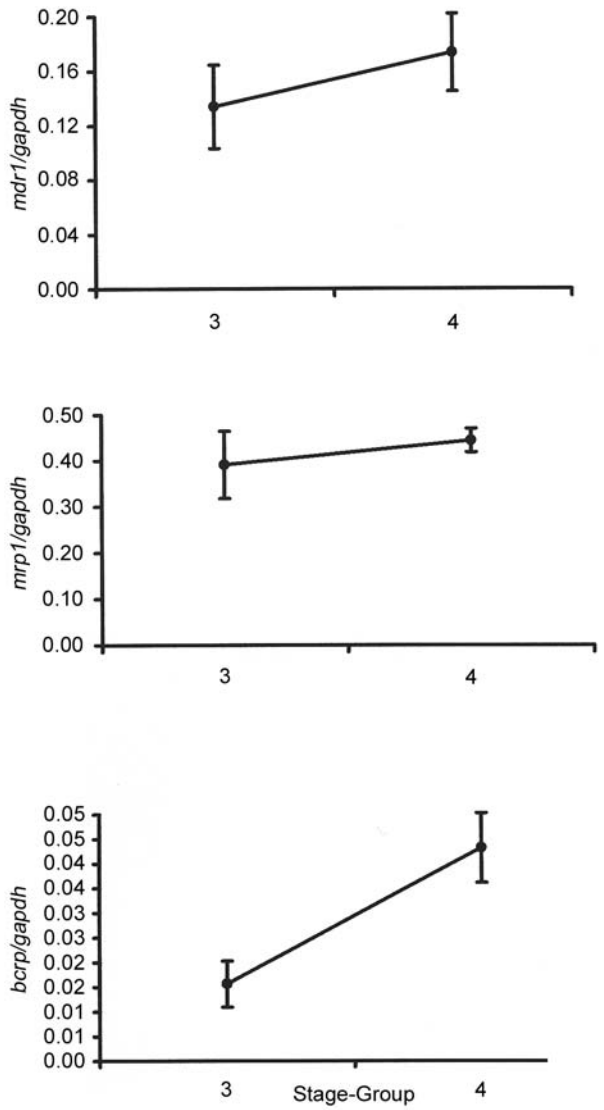

Figure 9. *Expression (mean values±SEM) of the resistance genes mdr1 (top), mrp1 (middle), and bcrp (below) depending on stage group, i.e. stage group 3 and 4 (SG 3; n=8; SG 4; n=29). Stage groups 1 and 2 were not determined due to low number of cases. The differences of bcrp expression depending on stage group are significant (p<0.02 rank sum test).*

expression level in the majority of OSCC was in the range of the cytostatic-sensitive reference cell line K-562. They had to be regarded as *mdr1*-negative. However, 8 tumors overexpressed *mdr1* (18%). The correspondence of immunohistochemical findings and the expression levels was 78%. The distribution pattern of *mdr1* expression is shown on the histogram (Figure 4).

mrp1: The *mrp1* expression was completely different to the *mdr1* expression. Almost all tissues expressed *mrp1* physiologically. The majority of OSCC expressed *mrp1* levels

Table VI. *Frequency of bcrp-positive and bcrp-negative OSCC depending on stage group. The stage groups 1, 2 and 3 were evaluated as one group. A tumor is bcrp-positive if the value exceeds the 95% confidence interval of the mitoxantrone-sensible reference cell line EPG85-257P (p<0.01, Fisher's exact test, n=41).*

	Stage-Group 1-3	Stage-Group 4
bcrp expression positive n (%)	1 (2%)	16 (39%)
bcrp expression negative n (%)	10 (24%)	14 (34%)

equal to the expression of the reference cell line A2780 [OSCC: 0.45±0.024 SEM, n=45; A2780: 0.45±0.016 SEM, n=14). A higher expression level was recorded for 9 tumors (20%) and a lower one for 5 (11%), (Figure 5). The correspondence between *mrp1* immunohistochemistry and *mrp1* gene expression was 70%. The colon carcinoma samples had lower *mrp1* mean values than OSCC (0.34±0.06 SEM, n=11, $p<0.05$, *t*-test).

bcrp: The *bcrp* expression was similar to the *mdr1* expression. The majority of OSCC had expression levels equivalent to the expression level found for the mitoxantrone-sensitive reference cell line EPG85-257P. A higher expression level was detected in 8 tumors (18%), (Figure 6). No OSCC had *bcrp* expression levels equal to or higher than the level determined for the mitoxantrone-resistant variant EPG85-257NOV (mean: 0.22±0.03 SEM, n=11).

Co-expression of resistance genes

mdr1 and mrp1: The expression values of *mdr1* and *mrp1* in OSCC were not correlated (r=0.22, $p=0.14$, n=45). However, the evaluation has to consider the different starting points of the expressions in normal tissues. Whereas *mdr1* is "normally" not expressed, *mrp1* is "normally" expressed at levels of about 0.45. Therefore, an elevated *mdr1* expression could only be determined in cases with normal *mrp1* expression (17 out of 18 cases). On the other hand, the evaluation of cases with deviations from the normal *mrp1* expression (mean±SD) revealed negative *mdr1* expression in 9 out of 10 cases. This evaluation indicates that a normal *mrp1* expression level is a prerequisite for the up-regulation of *mdr1* ($p<0.05$, Fisher's exact test, Table V, Figure 7).

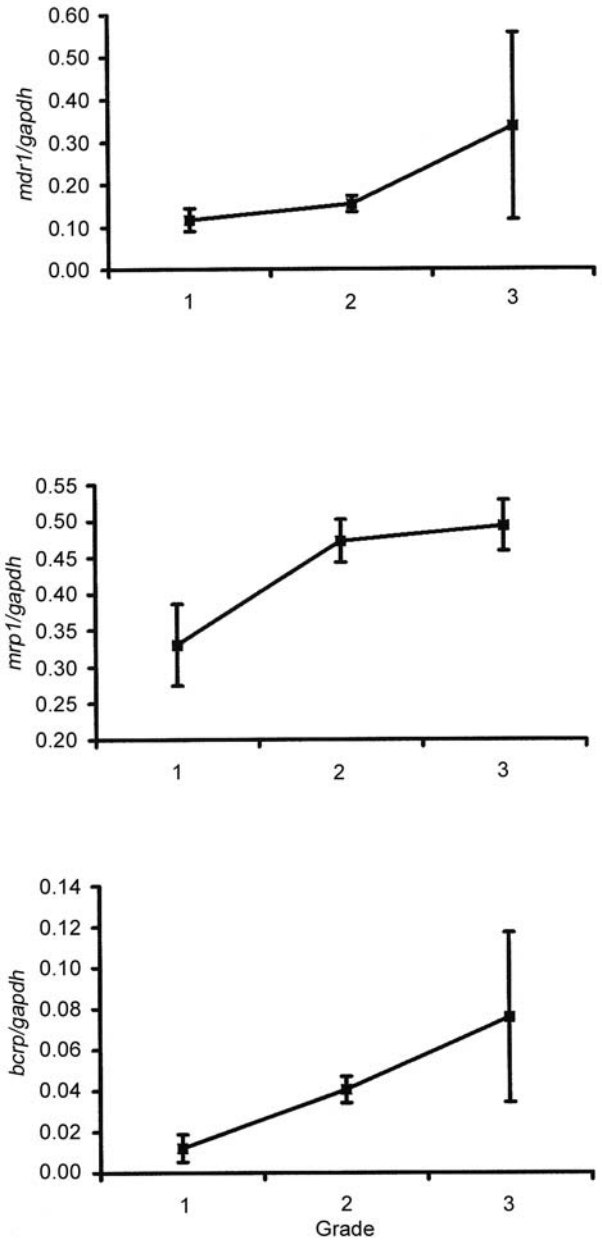

Figure 10. *Mean values±SEM of the resistance gene expressions and grading (Grade 1-3). Top: mdr1, p=0.06 (ANOVA). Middle: mrp1, p=0.1 (ANOVA), mrp1 and G_1 vs. G_{2-3}, p<0.01 (rank sum test), n=36. Bottom: bcrp and grade, p=0.04 (ANOVA), bcrp and G_1 vs. G_{2-3}, p>0.01 (rank sum test), n=36.*

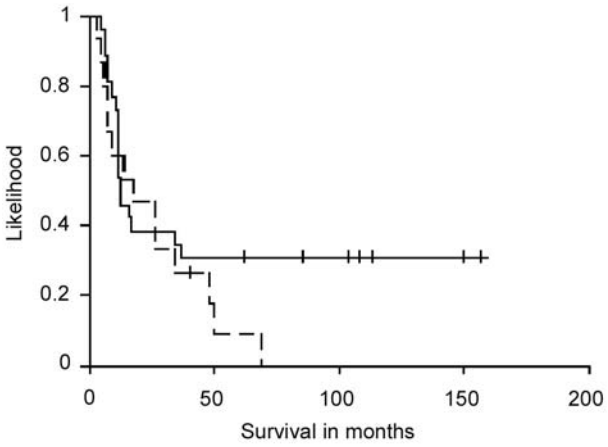

Figure 11. *Kaplan-Meier analysis of patients with OSCC who had alterations of the MDR gene expression (dotted line, n=15) vs. patients with MDR gene expression within the normal range (continuous line, n=26); p=0.24 (log-rank test).*

mdr1 and bcrp: The expression pattern of the genes *mdr1* and *bcrp* were similar in several tumors, indicating common mechanisms of regulation. The expression of *bcrp* correlated with *mdr1* (multiple correlation coefficient r=0.6 ($p<0.01$, n=45)). This correlation coefficient was not altered after omission of one extreme value (Figure 8).

mrp1 and bcrp: The regression analysis showed no correlation between *mrp1* and *bcrp* (r=0.19, $p=0.1$, n=45). Obviously both genes are independently expressed.

Correlation of gene expression patterns and clinical data

T-stage: The expression of the resistance genes was not dependent on age and gender (Levene-test). The data provided no evidence for a dependence of the genes on tumor localisation. The impact of T-stage on the expression was separately analysed. The mean *mdr1* expression increased from T_2- to T_4- carcinoma (T_2: 0.12, n=9; T_3: 0.17, n=10; T4: 0.18, n=20). However, this finding was not significant due to asymmetrically distributed T-stages. The same was found for *bcrp* expression (T_2: 0.03, n=9; T_3: 0.026, n=10; T_4: 0.05, n=20, $p=0.07$). The *mrp1*-expression and T-stage showed no correlation.

N-stage: The calculation of resistance gene expression at nodal stage was based on a two-part subgrouping: N_0 (no lymph nodes affected) *vs.* N_{1-3} (every patient with affected lymph nodes irrespective of site and number). The *mdr1* expression increased slightly from 0.12 (N_0, n=9) to 0.18 (N_{1-3}, n=31). The mean *mrp1* expression in these subgroups remained virtually unchanged (N_0=0.41 *vs.* N_{1-3}=0.44), but the mean *bcrp* expression was doubled from N_0 (0.025) to N_{1-3} (0.40). However, none of these values proved to be significant.

M-stage: The sample characteristics allowed no statistical calculations: 38 out of 41 carcinomas were M_0 staged. The 2 tumors with distant metastases were inhomogenously

expressing the resistance genes, one with no alteration, the second with 300% increase of expression (*mdr1* and *bcrp*). The *mrp1* expression in these cases did not change when comparing M_1 and M_0 cases.

Stage grouping: Stage group 1 and 2 were not analysed due to small sample size (n=3). Figure 9 shows the mean expression value of the resistance gene in comparison to the stage group. The mean *bcrp* expression increased from 0.015 (SG 3) to 0.04 (SG 4). This difference was statistically significant ($p=0.015$, Mann-Whitney *U*-test, rank sum test). Table VI shows the relation of stage group and *bcrp* expression. Carcinomas positive for *bcrp* expression were in stage 4 in 16 of 17 cases. In this comparison, stages 1-3 were grouped together and compared to stage 4 primaries.

Differentiation. The expression of all 3 resistance genes increased with the loss of differentiation. The *mdr1* expression was 0.16 in grade 1 tumors (well-differentiated, n=6) and 0.34 in grade 3 tumors (poorly-differentiated, n=3). The difference was not statistically significant ($p=0.06$). The *mrp1* expression increased from 0.33 (grade 1) to 0.49 (grade 3). Despite an increase of gene expression of about 150% this difference was not significant due to the predominance of moderately-differentiated primaries. However, the six-fold increase of *bcrp* expression (0.012, grade 1, and 0.76, grade 3) proved to be significant ($p<0.01$).

The impact of predisposing factors on MDR expression

HPV infection: Twenty-eight primary carcinoma had been investigated for risk types of human papilloma virus [HPV, type 16 and 18] in an earlier study (65). Sixteen out of these 28 tumors were positive for HPV (57%). A statistical analysis revealed no correlation of *mdr1*, *mrp1* or *bcrp* expression and the HPV infection status (Chi-Square test, ANOVA, rank sum test).

p53 mutation: A correlation between p53 mutations and *mdr1* overexpression for lung carcinoma cell line had been reported and for *mrp1* in non-small cell lung carcinoma (113). P53 mutations had been identified in 23 out of 36 samples (64%) in that study (65). The statistical analysis revealed no correlation between p53 mutation and MDR.

Prognostic impact of the resistance genes

mdr1: Patients expressing *mdr1* survived for 27 months (mean value, 95% confidence interval: 9-45 months, n=5). The 36 patients who were negative for *mdr1* expression survived for 51 months (95% confidence interval: 30 - 71 months, n=36). The survival characteristics (Kaplan-Meier) revealed no differences between the 2 groups ($p=0.67$, log-rank test).

mrp1: The impact of the *mrp1* gene expression on survival was determined for 3 groups (normal expression±1 SD *vs.* lower *vs.* higher expression levels). OSCC patients expressing *mrp1* on normal levels had a mean survival of 46 months (95% confidence interval 26-66 months). Patients expressing lower *mrp1* levels survived for 38 months (95% confidence interval: 0-88 months), and those with higher levels of *mrp1* for 42 months (95% confidence interval: 8-76 months). Alterations of the *mrp1* expression had no impact on the survival of OSCC patients.

bcrp: Patients with *bcrp*-positive tumors had a mean survival of 18 months (95% confidence interval: 6-31 months, n=7). Patients with *bcrp*-negative tumors had a mean survival of 53 months (95% confidence interval: 32-74 months, n=34). These differences were not statistically significant ($p=0.12$, log-rank test). The impact of grading and *bcrp* expression is shown in Figure 10.

Survival of patients with at least one resistance gene out of the normal range: The mean survival of patients with at least one resistance gene outside the normal range was 25 months. The patients with all 3 resistance genes in the normal range at the time of the diagnosis had a mean survival of 53 months. The differences were not statistically significant in the log-rank test due to the lack of survival differences between both groups within the first 2 years (Figure 11). However, a difference in long-term survival could not probably have been determined in the Kaplan-Meier analysis. We found at least one resistance gene expressed out of the range in 15 patients. Only one out of these 15 patients had survived after 5 years and all were dead after 6 years. Out of 26 patients without any alteration of resistance gene expression, 9 had survived after 5 years, and 7 after 6 years. The determination of the long-term survival between these 2 groups revealed an increase in statistically significant differences (5 years: $p=0.067$; 6 years: $p=0.026$, Fisher's exact test).

Discussion

This is the first study to determine, quantitatively, the expression of the MDR genes *mdr1*, *mrp1* and *bcrp* in oral squamous cell carcinoma, using RT-PCR. The alteration of at least one MDR gene is correlated with a poor prognosis in long-term survivors (5 and 6 years after diagnosis). Further on, the gene expressions do not depend on HPV infection or p53 mutation (65).

Expression analysis

mdr1: The *mdr1* expression in different tissues has been described. Predominantly immunohistochemical techniques have been applied, recently extended to RT-PCR. A

physiological *mdr1* expression was found in healthy epithelia of the intestine, the kidneys and in hepatocytes (83, 84). High *mdr1* levels were identified in lymphocytes and mononuclear cells (85). P-glycoprotein is one of the cellular factors that are expressed by cells exposed to stress (25, 26). Therefore, the *mdr1* expression is not static. However, tumors arising from tissues with physiologically higher *mdr1* levels regularly have a higher *mdr1* increase than those from tissues with normally no *mdr1* expression (86). Indeed, high levels of *mdr1* expression were reported for carcinomas of the colon, kidneys, liver and lungs (87, 88). A low or irregular *mdr1* expression was found in acute lymphatic leukemia (88), breast carcinoma (87) and ovarian carcinoma (89, 90).

Whether or not *mdr1* is expressed in healthy oral mucosa is controversial. Possibly, these contradictions depend on the population of the study. Uemeatsu *et al.* (91) identified no *mdr1* expression in oral mucosa of Japanese; Muzio *et al.* (92) revealed oral *mdr1* expression in 66% of southern Europeans. It is well known that oral cancer can be correlated to certain environmental factors in the majority of patients, in particular to smoking and drinking habits (93-96). Therefore, the expression of resistance genes as a cellular response to permanent exposure to noxae is plausible. However, the current data are inconclusive. The *mdr1* expression in OSCC patients range between 34% [Japanese (91)], 62% [Chinese (97)], and 80% [southern European (92)]. All 3 reports were based on immunohistochemical analysis.

In this study the *mdr1*-positive tumors accounted for 18% of the study group. This finding is lower than previously reported. The calibration of the measurement to the reference gene *gapdh* and the reference cell lines allowed a quantification of the gene expression. The *mdr1* expression was judged to be negative if the value was equivalent to or higher than the value determined for the cytostatic-sensible cell line K562, but lower than the level that had to be expected in *mdr1*-expressing tissues (98). According to this definition 18% of OSCC were *mdr1*-positive. The comparison of the *mdr1* levels in OSCC with the known high levels in colon carcinoma, using the same detecting technique, identified that OSCC expressed *mdr1* more rarely and at lower levels than carcinomas of the gastrointestinal tract.

mrp1: A basal *mrp1* expression was found in almost all tissues (99, 100). Both acute and chronic leukemia have been associated with high *mrp1* levels (55-57, 101) identified in squamous cell carcinoma of the esophagus *mrp1* in 100% and verified immunohistochemically. In the present study, *mrp1* expression was identified in almost all cases. In accordance with the current literature, the *mrp1* expression is different from the expression of *bcrp* and *mdr1* in tumors. The normal expression level of *mrp1* in healthy tissues is high. Using the quantifying expression analysis provided by the RT-PCR of this study, only 10% of tumors had lower than normal *mrp1* levels and 20% had higher. Comparing the simultaneously determined expression levels of OSCC and colon carcinoma, the *mrp1* level in colon cancer was generally higher. Up to now Tsuzuki *et al.* (102) are the only investigators who studied *mrp1* expression in OSCC, using immunohistochemistry. These authors revealed *mrp1* expression in 30% of their cases. The discrepancy between their and our findings remains unclear.

The resistance gene *bcrp* has been studied only recently. The majority of studies deal with the localisation and function of the *bcrp* product (58, 59, 103, 104). Up to now a physiological expression of *bcrp* was reported for the placenta (105-107) and low levels for liver and intestine (58, 104). Acute myeloid leukemia is associated with *bcrp* expression in 33% of patients (58, 104). In our study OSCC expressed an elevated *bcrp* level in 18% of cases. The normal expression range was defined by the levels determined in the cell line EPG85-257P. The expression pattern of *bcrp* was similar to that found in *mdr1*. In one patient with advanced stage carcinoma, extremely high levels were measured for *bcrp* and *mrp1*. These levels were not caused by variations of the detection system: the tumor had normal expression of the reference gene *gapdh* and the reference gene expressed *mrp1* at normal levels. Possibly, the extreme values of *bcrp* could be caused by the advanced stage of the disease.

Co-expression of resistance genes. Extensive analysis of the current literature provided no information on a correlation between *mdr1* and *mrp1* expression. This assessment was substantiated by our results. Both genes were differentially expressed. However, a connection between both genes might probably exist. In OSCC the expression of *mdr1* could only be up-regulated when *mrp1* was expressed in the normal range. Low *mrp1* levels were expressed in almost all tissues (99, 100). Deviations from normal *mrp1* expression levels obviously correlated with a loss of the OSCC to up-regulate *mdr1*.

A correlation was found for *bcrp* and *mdr1* expression ($r=0.61$; $p<0.01$; $n=45$). Ross *et al.* (58, 104) revealed a similar *bcrp*/*mdr1* correlation in patients with acute myeloid leukemia ($r=0.66$). The presence of a common regulation for both genes could not be proven by our study. Both genes were positively correlated to an increase in stage group and grading. Therefore, indirect correlations could not be excluded. A correlation analysis of both genes based on a larger study group should be recommended.

No correlation was found between *mrp1* and *bcrp* expression in OSCC. To our knowledge a comparable investigation has yet not been undertaken.

Correlation of RT-PCR findings and patient data. No correlation was found for MDR expression and age or gender. This finding supports recent investigations of Park *et al.* (67) on osteosarcoma patients and Tsuzuki *et al.* (102) on OSCC. Both groups found no correlation for *mrp1* and *mdr* expression and these parameters.

Weinstein *et al.* (75) postulated that *mdr1* expression could be an indicator of tumor aggressiveness in colon cancer. Further studies revealed this hypothesis for several entities (104). The TNM classification, the stage group, the grading and the survival times of patients are indirect parameters to estimate tumor aggressiveness. Our analysis was impaired due to inhomogeneous group distribution. The mean values of *mdr1* and *bcrp* increased with higher tumor stage, nodal stage and evidence of distant metastases. However, these findings were not statistically significant. The mean *mrp1* levels remained surprisingly constant.

Taking into consideration the tumor parameters, as they were categorized in the stage group, the figure was similar. Both *mdr1* and *bcrp* expression levels increased from stage 3 to 4. This correlation was significant for *bcrp* only ($p<0.05$). The comprehensive analysis revealed that in stage 4 cancer at least one gene expression was up-regulated ($p<0.05$). Other authors reported even higher correlations based on more homogeneously distributed patient groups. Uemeatsu *et al.* (91) revealed a three-fold higher expression of *mdr1* in stage 4 compared to stage 3. Jain *et al.* (108) also found a higher *mdr1* expression comparing stage 3 and 4 OSCC. Both studies were based on an earlier report of Kelly *et al.* (109). Gan *et al.* (110) and Rabkin *et al.* (111) reported a correlation of increased *mdr1* expression and regional tumor spread to lymph nodes in oral and pharyngeal carcinoma. Similar findings are known for gastric cancer (112) and chronic lymphatic leukemia (55).

Tsuzuki *et al.* (102) revealed an increased *mrp1* expression in OSCC correlated to advanced stage. The *mrp1* expression was three times higher in stage 4 compared to stage 1. In our study the *mrp1* levels were higher than the values reported by Tsuzuki *et al.* (102), irrespective of stage. This might be the reason for our finding that no differences were detected in *mrp1* expression correlated to stage group.

The correlation of tumor de-differentiation and the expression of resistance genes are presently not clear. An increase of loss of cellular differentiation was correlated with resistance gene expression in this study (*mdr1*: $p=0.06$; *mrp1*: $p<0.05$; *bcrp*: $p<0.05$). Gan *et al.* (110) identified a positive correlation of loss of differentiation and *mdr1* expression. However, Xie *et al.* (97) revealed an inverse relationship, *i.e.* an increased *mdr1* expression, in well-differentiated OSCC, while others found no correlation at all (67, 92).

Impact of predisposing factors. It is presently not known whether HPV infections could act as stress factors that induce the expression of resistance genes. In this study no impact of HPV infection was found, neither any correlation between expression rates nor on the survival rates of the OSCC patients.

Mutations of the tumor suppressor gene p53 can influence the expression of p-glycoprotein (63, 113). Correlations between p53 mutations and *mdr1* over-expression were noted for the lung carcinoma cell line H358 (113), for osteosarcoma (67) and embryonic fibroblasts (63); and further on for p53 mutations and *mrp1* in non-small cell lung carcinoma (101). However, for OSCC no correlations between *mdr1* expression and p53 mutations were identified in the study of Ng *et al.* (19). This result is in accordance with ours. Indeed, we identified no correlation between p53 mutations and any of the resistance genes under study.

Prognostic impact. At present no study has investigated the impact of *mdr1* on the prognosis of OSCC patients. A worse prognosis for patients expressing *mdr1* was determined for acute myeloid leukemia (114-116), acute lymphatic leukemia (117, 118) and osteosarcoma (67). Contradictory findings were reported for neuroblastoma (47, 119, 120). In our study patients with *mdr1* expression survived for about half as long as those with no *mdr1* expression (27 months *vs.* 51 months). However, this mean difference of survival was not statistically significant and, therefore, needs to be evaluated in larger studies.

Elevated *mrp1* expression correlates with a poor prognosis for patients with acute and chronic leukemias (55, 57). This correlation was denied for OSCC (102).

The prognostic impact of *bcrp* has yet not been investigated. The mean survival time of patients who express *bcrp* is shorter than those without *bcrp* expression (18 months *vs.* 53 months). However, this difference was not statistically significant. It is likely that the large subgroup of *bcrp*-expressing patients (34/41 patients) did not render possible a statistical proof of the difference. The impact of *bcrp* expression on the parameter "survival time" was similar to that found for *mdr1*. Both parameters correlated with stage group and grade.

The synopsis on the survival times of the OSCC patients and the expression profiles of the resistance genes revealed that patients expressing at least one gene were all dead after 6 years (n=15), whereas 8 without this finding survived (n=26; $p=0.026$). Therefore, the up-regulation of resistance genes seems to diminish the likelihood of surviving OSCC. This assumption has to be proved in studies with larger sample size.

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