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Transporter Gene Expression in Human Head and Neck Squamous Cell Carcinoma and Associated Epigenetic Regulatory Mechanisms

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The interindividual variability in drug response and the emergence of adverse drug reactions are the main causes of treatment failure in cancer therapy.¹ Recently, membrane transporters have been recognized as an important determinant of drug disposition, thereby affecting chemosensitivity and drug resistance. Moreover, expression levels of transporters have been associated with clinical outcomes in patients with cancer.¹

Two major superfamilies of membrane transporters that influence drug pharmacokinetics are the ATP-binding cassette (ABC) transporters and the transporters belonging to the solute carrier (SLC) family, such as the organic anion transporting polypeptides (OATPs; encoded by the *SLCO*

genes) or the SLC22 organic cation/anion/zwitterion transporters.² The ABC transporters are responsible for the export of diverse substrates, including anticancer drugs, from the cell against a concentration gradient, with ATP hydrolysis providing the driving force.^{3,4} The extrusion of anticancer drugs from the tumor cells often results in multidrug resistance of tumors.³

The OATPs are involved in cellular influx mechanisms. Known substrates of the OATP family include endogenous compounds and a variety of pharmaceuticals, including

anticancer drugs.^{5–7} The OATPs expressed in the liver have contributed to systemic variation in anticancer drug treatments.^{5,6} Moreover, OATPs expressed in tumors may be implicated in cellular uptake of anticancer drugs, thereby increasing the chemosensitivity of tumor cells.¹

The profile and the mechanisms of transporter expression in human head and neck squamous cell carcinoma (HNSCC), one of the most frequent cancers, with an incidence of >200,000 new cases worldwide annually,⁸ have not been studied. Therefore, the first aim of our study was to identify transporters that are differentially expressed in HNSCC compared with adjacent normal control tissue. In addition, the expression of transporter genes was correlated with disease stage and 5-year survival in a small-scale preliminary study.

The second aim of our study was to evaluate mechanisms by which the transporters are regulated in HNSCC. Given that the disruption of the epigenome is a fundamental mechanism in cancer and that the reversal of epigenetic changes represents a potential target of novel treatments in cancer, we focused on epigenetic mechanisms (ie, changes in transporter gene expression that are mediated by DNA methylation).⁹ DNA methylation of the CpG islands within the transporter gene promoters can cause repression of gene expression either directly, by the steric hindrance of methyl-CpG interaction with transcription factors, or indirectly, through the recruitment of methyl-binding proteins that associate with chromatin-modifying proteins to produce a complementary repressive heterochromatin environment.^{10,11} Although gene silencing via aberrant DNA promoter hypermethylation explains some aspects in the tumorigenesis of HNSCC,¹² little is known about the contribution of epigenetics to the regulation of drug transporter expression.¹¹

Most recent studies suggest that transporter genes, such as *ABCB1* (P-glycoprotein), are targets of the transcriptional activator, pregnane X receptor (PXR; alias *NR1I2*).¹³ Several anticancer drugs (eg, taxanes and cisplatin) can activate PXR.^{14,15} Moreover, promoter methylation is involved at least in the regulation of *PXR* in cancer cells,^{16,17} raising the possibility of interactions between epigenetic and nuclear receptor-mediated mechanisms in the regulation of transporter expression in HNSCC.

Therefore, the role of PXR and the epigenetic mechanism of transcriptional regulation of selected SLC and ABC transporters were evaluated in the present study by analyzing the response of human HNSCC cell lines (UMSCC-1 and SCC-15) to the DNA methyltransferase inhibitor, decitabine (5-aza-2'-deoxycytidine), and to the PXR activator, rifampicin, as single or combined treatments. Moreover, the effects of *NR1I2*/PXR knockdown by lentivirus-mediated short-hairpin RNA (shRNA) interference on basal and decitabine-modified transporter expression were evaluated. Promoter CpG methylation was investigated using bisulfite sequencing.

Materials and Methods

Tissue Samples

Human HNSCC tissues and adjacent normal mucosa tissues from patients with HNSCC were attained from the Department of Otorhinolaryngology, Head and Neck Surgery, University of Erlangen-Nuremberg (Erlangen, Germany). A histopathological evaluation was performed on all samples for verification. Patients gave informed consent, and the study was approved by the ethics committee of the University of Erlangen-Nuremberg. None of the patients had received neoadjuvant chemotherapy. The primary tumor and paired macroscopically healthy tissue from the excision border were obtained at surgery. The samples with a normal phenotype were located distant from the macroscopic tumor edge, as judged by the surgeon. Tumor material and adjacent normal mucosa were collected, snap frozen in liquid nitrogen, and stored at -80°C before use. For quality control, cryosections were investigated by a pathologist. Patient details are shown in Table 1.

Cell Culture and Drug Treatments

Human UMSCC-1 cells, derived from an oral cavity carcinoma, were obtained from the Department of Otolaryngology/Head and Neck Surgery, University of Michigan School of Medicine (Ann Arbor, MI).¹⁸ The SCC-15 cell line, derived from a squamous cell carcinoma of the tongue, was obtained from ATCC (Manassas, VA; reference CRL-1623). The cell lines were recently characterized and validated by short tandem repeat profiling in two independent studies.^{19,20} To generate stable UMSCC cells with reduced *NR1I2* mRNA expression, Mission *NR1I2* shRNA lentiviruses (NM_003889; clones TRCN00000211619, TRCN00000211621, TRCN00000211622, and TRCN00000211623; Sigma-Aldrich, St. Louis, MO), with a titer of $>10^6$ transforming units/mL, were used to infect UMSCC-1 cells. Subsequently, stable clones were selected using puromycin (2 $\mu\text{g/mL}$). Control UMSCC cells were infected with nonsilencing control lentiviral particles (SHC002V; Sigma-Aldrich). UMSCC cells were maintained in Dulbecco's modified Eagle's medium and Ham's F12 with GlutaMAX (Invitrogen, Carlsbad, CA), supplemented with 10% fetal bovine serum (Invitrogen), 100 U/mL penicillin (Invitrogen), 100 $\mu\text{g/mL}$ streptomycin (Invitrogen), and 1 mmol/L sodium pyruvate (Invitrogen). SCC-15 cells were maintained in Dulbecco's modified Eagle's medium and Ham's F12 medium containing 1.2 g/L sodium bicarbonate, 2.5 mmol/L L-glutamine, 15 mmol/L HEPES, and 0.5 mmol/L sodium pyruvate, supplemented with 100 U/mL penicillin (Invitrogen), 100 $\mu\text{g/mL}$ streptomycin (Invitrogen), 400 ng/mL hydrocortisone, and 10% fetal bovine serum. For drug treatments, cells were plated on 60-mm dishes and cultured to 75% confluence. Drugs (all from Sigma-Aldrich) were dissolved in dimethyl sulfoxide

Table 1 Characteristics of Patients with HNSCC

Patient no.	Age (years)	Sex	Localization	ICD-10 code	pT class
1	57	Male	Pharynx	C12	T2
2	38	Male	Glottis	C32.0	T3
3	68	Female	Larynx	C32.0	T4
4	65	Male	Larynx	C32.2	Tx
5	58	Male	Larynx	C32.0	T2
6	46	Female	Larynx	C32.0	T4
7	55	Male	Larynx	C32.1	T2
8	77	Male	Floor of mouth	C04.8	T2
9	74	Female	Tonsil	C09.9	T3
10	51	Male	Larynx	C32.1	T3
11	65	Female	Larynx	C32.0	T3
12	44	Male	Tonsil	C05.1	T1
13	82	Male	Tonsil	C09.9	T2
14	63	Male	Floor of mouth	C01	T3
15	63	Male	Floor of mouth	C01	T1
16	51	Male	Larynx	C32.0	Tx
17	51	Male	Supraglottis	C32.1	T2
18	57	Female	Oropharynx	C10.3	T3
19	61	Male	Larynx	C32.1	T1
20	50	Male	Larynx	C32.0	T3
21	57	Male	Oropharynx	C10.9	T4
22	76	Male	Larynx	C32.0	T4
23	68	Male	Larynx	C32.1	T4
24	80	Male	Larynx	C32.0	T4
25	67	Female	Floor of mouth	C02.1	T1
26	44	Male	Floor of mouth	C04.0	T2
27	53	Male	Larynx	C32.0	T4
28	42	Male	Larynx	C32.0	T4
29	57	Male	Oropharynx	C10.9	T3
30	57	Male	Larynx	C32.0	T1
31	74	Male	Larynx	C32.0	T4
32	63	Male	Hypopharynx	C12	T3
33	52	Male	Hypopharynx	C12	T2
34	61	Male	Larynx	C32.0	T4

ICD-10, International Statistical Classification of Diseases and Related Health Problems.

and were used at concentrations as follows: 1 $\mu\text{mol/L}$ decitabine and 25 $\mu\text{mol/L}$ rifampicin. Cells were treated with drugs for 72 hours, with change of medium every 24 hours. Control cells were treated with solvent (dimethyl sulfoxide; 0.1% to 0.2%) only.

RNA Isolation and TaqMan PCR

Total RNA was isolated from human tissue samples (normal and HNSCC tissue samples from 34 patients) and from UMSSC-1 or SCC-15 cells with TRIzol reagent (Invitrogen), according to the manufacturer's protocol. Before TRIzol was added, frozen human tissue samples were grinded in liquid nitrogen with a mortar and pestle. A total of 5 μg of total RNA each was reverse transcribed into cDNA using random primer and Moloney Murine Leukemia Virus Reverse Transcriptase (Promega, Madison, WI) at 37°C for 1 hour.

The PCRs (10 μL) were set up with 1 μL of cDNA (equivalent to 40 ng of input RNA), 5 μL of two times QPCR ROX master mix (Thermo Scientific, Rockford, IL), 0.5 μL of predesigned fluorogenic TaqMan probe and primer mix (Applied Biosystems, Foster City, CA), and 3.5 μL of nuclease-free water. Real-time PCR analysis was performed using the Applied Biosystems ABI PRISM 7900HT Sequence Detection System for 45 cycles, with 3 seconds denaturation at 95°C and 30 seconds annealing/extension at 60°C. Several reference genes were tested, and those showing the highest expression stability were used for normalization. mRNA levels in HNSCC and corresponding normal tissue samples were normalized to *TBT*, *TUBB*, and *HPRT1*. For UMSSC-1 and SCC-15 cells, *GAPDH* and *ACTB* were used for normalization. To evaluate the amplification efficiency, standard curves were generated using a cDNA standard dilution series. Relative mRNA expression levels were calculated with Sequence Detection System software version 2.1 (Applied Biosystems), which allows correction for differences in amplification efficiency and standardization by reference gene expression.

Bisulfite Sequencing

Genomic DNA was isolated from decitabine-treated and untreated (solvent controls) UMSSC-1 cells ($n = 3$ each) and from HNSCC and adjacent normal tissues from two patients using the Genomic Tip 20/G extraction kit (Qiagen, Hilden, Germany). Equal amounts of DNA were pooled by treatment group, and DNA (2 μg) from each pool was bisulfite converted, according to the manufacturer's instruction, using an EpiTect kit (Qiagen). Primers were designed to anneal to non-CpG sequences within CpG islands in *ABCC3* and *SLCO2A1* promoters and, thus, amplify bisulfite-converted genomic DNA regardless of methylation status. For *ABCC3*, a nested PCR with a second primer pair was performed. Primer sequences are listed in Table 2.

Bisulfite-transformed genomic DNA was amplified twice via PCR using Pfu Turbo C_x Hotstart DNA Polymerase (Agilent Technologies, Böblingen, Germany) and Advantage cDNA Polymerase Mix (Clontech, Saint-Germain-en-Laye, France). The first round of amplification with Pfu Turbo C_x Hotstart DNA Polymerase was performed using the following conditions: enzyme activation at 95°C for 2 minutes, denaturation at 95°C for 30 seconds, annealing at temperatures depending on primer melting temperature for 30 seconds, synthesis at 72°C for 1 minute, and final extension at 72°C for 10 minutes. The first 10 cycles were run with an annealing temperature of 2°C, and the subsequent 30 cycles were run with an annealing temperature of 4°C lower than primer melting temperature. The second round of amplification was conducted with Advantage cDNA Polymerase Mix. The amplification conditions were as follows: enzyme activation at 94°C for 1 minute, denaturation at 94°C for 15 seconds, annealing at 52°C for

Table 2 Primers Used for Amplification of Bisulfite-Treated DNA

Name	Sequence	Position (relative to TSS)	Fragment length (bp)
ABCC3_fw_1	5'-GGATTTAGAGGTATTGGGGTAG-3'	-63	573
ABCC3_rev_1	5'-AAAAACCCCTCCATCAAAAACC-3'	509	
ABCC3_fw_2	5'-TTAGAGGTATTGGGGTAGG-3'	-59	405
ABCC3_rev_2	5'-AAACAAAACAAAACAAATCC-3'	345	
SLC02A1_fw	5'-GGATTAAGTAATGGGATAGTGAAG-3'	619	421
SLC02A1_rev	5'-CCTAAAACCATTTCCAACCA-3'	1039	

TSS, transcription start site.

30 seconds, synthesis at 68°C for 1 minute, and final extension at 68°C for 3 minutes. The correct product size and quality of PCR amplicons were controlled by 2% agarose gel electrophoresis. PCR products were cleaned up using Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Freiburg, Germany) to remove any excess nucleotides and primers, and 200 ng each were sequenced directly using the PCR amplification primers. Sequencing data were analyzed using Epigenetic Sequencing Methylation Analysis Software (Epigenomics, Berlin, Germany). The bisulfite sequencing approach allows measurement of DNA methylation with high reproducibility and accuracy.^{21,22}

Statistics

Differences between two groups were tested by the Student's *t*-test for unpaired data. Data were compared with a reference value by the one-sample *t*-test. Differences between more than two groups were tested by one-way analysis of variance and Bonferroni post test. Variables that were not normally distributed (Kolmogorov-Smirnov test) were log transformed before statistical analysis was performed. The χ^2 test was used to analyze associations between transporter expression and pT class or 5-year survival rates. Logistic regression analysis was used to evaluate the association between transporter expression and 5-year survival rates, with pT class as a covariate. Data were analyzed with SPSS for Windows version 16.0 (SPSS Inc., Chicago, IL) and GraphPad Prism version 5.01 (GraphPad Software, San Diego, CA). $P < 0.05$ was required for statistical significance. Data are presented as mean \pm SEM (bar graphs) or as median with lower and upper quartiles and the minimum and maximum of all of the data (box-and-whiskers plots).

Results

Transporter Expression in Human Normal and HNSCC Tumor Tissues

We generated mRNA expression profiles in human HNSCC tissues of those transporters that have been implicated in cancer development or progression, or in the cellular uptake or efflux of anticancer drugs. Nine members of the solute

carrier organic anion transporter family (*SLCO1A2*, *SLCO1B3*, *SLCO1C1*, *SLCO2A1*, *SLCO2B1*, *SLCO3A1*, *SLCO4A1*, *SLCO4C1*, and *SLCO5A1*), two members of the polyspecific solute carrier family 22 (*SLC22A1* and *SLC22A3*), and two members of the multidrug and toxin extrusion transporter family (*SLC47A1* and *SLC47A2*) were analyzed. Furthermore, eight ABC transporters (*ABCB1*, *ABCC1*, *ABCC2*, *ABCC3*, *ABCC4*, *ABCC5*, *ABCC10*, and *ABCG2*) were investigated. Significant differences in expression levels between normal and tumor tissues were observed for 11 transporter genes, as shown in Figure 1A. The expression of *SLCO1A2* and *SLCO1B3* in tumor samples compared with normal tissues was 9.3-fold ($P < 0.001$) and 4.3-fold ($P < 0.001$) higher, respectively. In contrast, mRNA concentrations of *SLCO4A1*, *SLCO3A1*, *SLC47A1*, *SLC22A3*, *ABCC3*, *SLCO2B1*, *SLCO2A1*, *ABCG2*, and *SLCO1C1* were significantly decreased by 36% to 81% in HNSCC samples compared with the respective control samples.

Transporter Expression versus Disease Stage and 5-Year Survival

Because previous reports indicated that certain transporters were involved in cancer etiology and progression, we tested whether transporter expression in tumor tissue correlated with tumor stage or survival of patients with HNSCC. A significant association was observed between mRNA expression of *SLCO2B1* in HNSCC and improved survival during a 5-year period ($P = 0.029$) (Figure 1B). Comparison of tumor samples with high (upper two quartiles) and low (lower two quartiles) *SLCO2B1* mRNA expression (Table 3) revealed a significant association of *SLCO2B1* expression with the pT class ($\chi^2 = 4.394$, $P = 0.036$) and the 5-year survival rate (47% versus 88%; $\chi^2 = 6.585$, $P = 0.010$). The *SLCO2B1* expression level remained a significant predictor of 5-year survival in our study cohort, even when the pT class was considered as a covariate in a logistic regression analysis (odds ratio, 6.5; 95% CI, 1.1 to 38.6; $P = 0.04$).

Epigenetic Regulation of Transporter Expression

Changes in the epigenome, such as aberrant DNA methylation, have been common in cancer.²³ This raised the

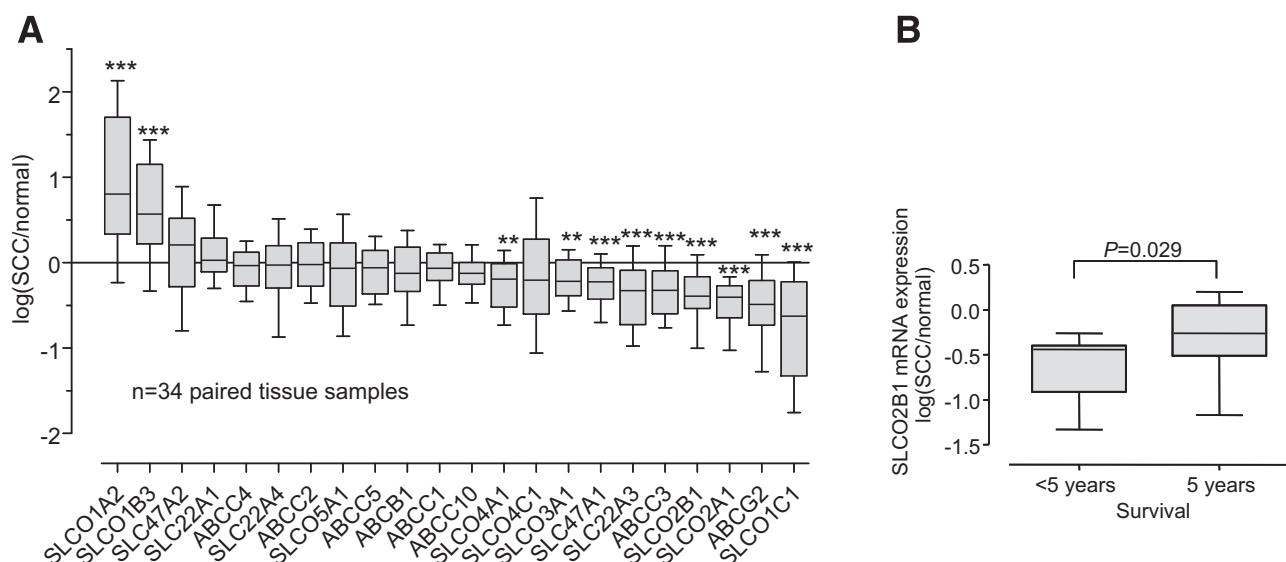


Figure 1 **A:** Transporter mRNA expression in HNSCC tumor samples compared with normal mucosal tissue from the same patients. Transporter mRNA expression was quantified in paired tumor and normal tissue samples from 34 patients with HNSCC by real-time PCR. The expression was normalized to the reference genes *TBT*, *TUBB*, and *HPRT1*. Data are presented as logarithms of fold change of mRNA expression in tumor versus adjacent normal control tissue. Box-whiskers plots show the median, the lower and the upper quartiles, and the minimum and maximum of all of the data (whiskers). **B:** Association between *SLC02B1* mRNA expression in HNSCC tumors and 5-year survival. *SLC02B1* mRNA expression in HNSCC tumor samples relative to normal tissues was stratified according to the 5-year survival. The 5-year overall survival is 67.6% (23 of 34 patients). ** $P < 0.01$, *** $P < 0.001$.

possibility that expression of transporter genes in HNSCC might be affected by epigenetic mechanisms. To prove this hypothesis, the methylation status of promoter CpG islands was investigated. Among the transporters that were differentially expressed between normal and HNSCC tissues, *ABCC3* and *SLC02A1* consisted of promoter CpG islands (defined as a DNA region of at least 200 bp with a C + G content of 50% and an observed CpG versus expected CpG in excess of 0.6). Bisulfite sequencing revealed that promoter CpG islands of *SLC02A1* and *ABCC3* were not methylated and, thus, these genes were not epigenetically silenced in HNSCC tissues (Figures 2, 3).

To investigate the consequences of epigenetic modifications on transporter expression, the effects of DNA methyltransferase inhibition or histone deacetylase inhibition on transporter expression were investigated in the human HNSCC cell lines UMSCC-1 and SCC-15. We primarily focused on transporters that were implicated in multidrug resistance of cancer cells against cytostatic drugs (ie, multidrug resistance 1: *ABCB1*; multidrug resistance-associated proteins: *ABCC1*, *ABCC3*, *ABCC4*, and *ABCC5*; and breast cancer resistance protein: *ABCG2*). We also investigated transporters whose expression was significantly affected in human HNSCC samples relative to control tissues (*SLC02A1*, *SLC02B1*, and *SLC22A3*). In UMSCC-15 cells, DNA methyltransferase inhibition with 1 $\mu\text{mol/L}$ decitabine for 72 hours caused a significant increase in *ABCB1* (17.2-fold, $P < 0.001$), *ABCC3* (20.8-fold, $P < 0.001$), *ABCG2* (2.8-fold, $P < 0.001$), *SLC22A3* (9.8-fold, $P < 0.001$), *SLC02A1* (15.8-fold, $P < 0.001$), and *SLC02B1* (37.3-fold, $P < 0.001$) mRNA expression (Figure 4),

whereas gene expression of other transporters, such as *ABCC1*, *ABCC4*, and *ABCC5*, remained unchanged (data not shown). Qualitatively similar results were obtained in SCC-15 cells (Figure 4).

Role of PXR in the Regulation of Transporter Gene Expression

The effect of decitabine on transporter mRNA expression might be directly due to a reversal of DNA methylation of CpG-rich sequences in the respective gene promoter. Indeed, decitabine treatment moderately reduced CpG island methylation in the *SLC02A1* promoter by $5.1\% \pm 1.3\%$ ($P < 0.01$) (Figure 2). Among the investigated transporters, decitabine treatment caused the most prominent increase in the

Table 3 Association between *SLC02B1* mRNA Expression in Tumors and pT Class and 5-Year Survival Rates in Patients with HNSCC

Variable	Patients with <i>SLC02B1</i> expression in HNSCC	
	Weak ($n = 17$)	Strong ($n = 17$)
pT class		
T1/2	4 (23.5)	9 (60.0)
T3/4	13 (76.5)	6 (40.0)*
Significance	$\chi^2 = 4.39$, $P = 0.036$	
5-Year survival		
Yes	8 (47.1)	15 (88.2)
No	9 (52.9)	2 (11.8)
Significance	$\chi^2 = 6.59$, $P = 0.010$	

*Two patients with pT class Tx were not included.

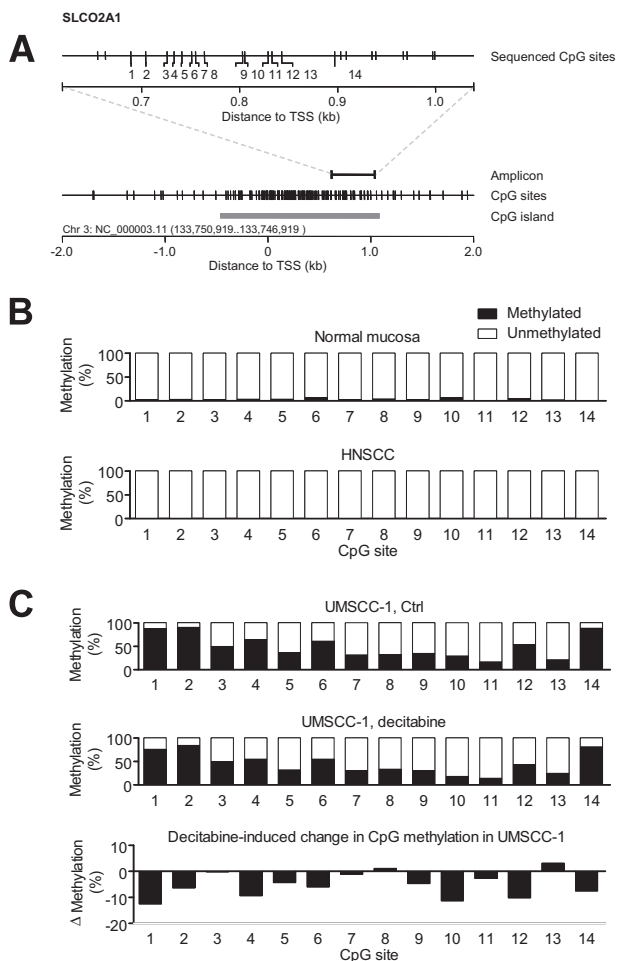


Figure 2 Analysis of CpG island methylation in *SLCO2A1* using bisulfite sequencing. **A:** Position of the CpG island and the sequenced CpG sites relative to the transcription start site (TSS). **B:** Average methylation rate of individual CpG sites in HNSCC tumors and adjacent normal mucosa from two patients with HNSCC. **C:** Average methylation rate of individual CpG sites in vehicle-treated (Ctrl) and decitabine-treated (1 $\mu\text{mol/L}$ for 72 hours) UMSSC-1 cells. Methylation rates of 26 CpG sites were obtained by direct sequencing of PCR amplicons and by analysis of the sequence trace files using Epigenetic Sequencing Methylation Analysis Software.

transcription of *SLCO2B1*, a gene that lacked CpG islands in its promoter region (defined as a DNA region of at least 200 bp with a C + G content of 50% and an observed CpG versus expected CpG in excess of 0.6). Moreover, decitabine treatment increased *ABCC3* transcript expression, although CpG islands in the *ABCC3* promoter were essentially unmethylated in both decitabine-treated and untreated cells (Figure 3). These findings suggested indirect effects of the DNA demethylating agent, decitabine, on transporter mRNA expression.

Because nuclear receptors, such as PXR, were capable of transcriptional activation, particularly of genes involved in drug metabolism and transport, and promoter methylation was involved in the regulation of *NR1I2/PXR* in cancer cells, we hypothesized that nuclear receptors might play a role in the epigenetic regulation of transporter expression in HNSCC cells. Treatment of UMSSC-1 cells with decitabine induced a marked increase in *NR1I2/PXR* (661-fold

versus control; $P < 0.001$) (Figure 5A). Qualitatively similar results were obtained when SCC-15 cells were investigated (12-fold increase versus control; $P < 0.001$). These results supported the notion that the transcriptional regulator, PXR, was strongly induced by modification of the epigenome in HNSCC cells.

The activator of PXR, rifampicin, had no significant effect on *SLCO2A1* and *ABCC3* transcript levels (Figure 5B). This finding might be explained by the relatively low basal expression of *NR1I2/PXR* in UMSSC-1 and SCC-15 cells. However, when *NR1I2/PXR* expression was enhanced by decitabine, cotreatment with rifampicin decreased transporter mRNA expression compared with cells treated with decitabine only (Figure 5B). For example, cotreatment of UMSSC-1 cells with decitabine and rifampicin reduced *SLCO2A1* and *ABCC3* mRNA expression by 78% ($P < 0.001$) and 60% ($P < 0.001$), respectively. These results supported a counterregulatory role of PXR on decitabine-induced transcription of *SLCO2A1* and *ABCC3*.

To further assess the role of *NR1I2* on decitabine-induced increase in transporter mRNA expression, *NR1I2* expression

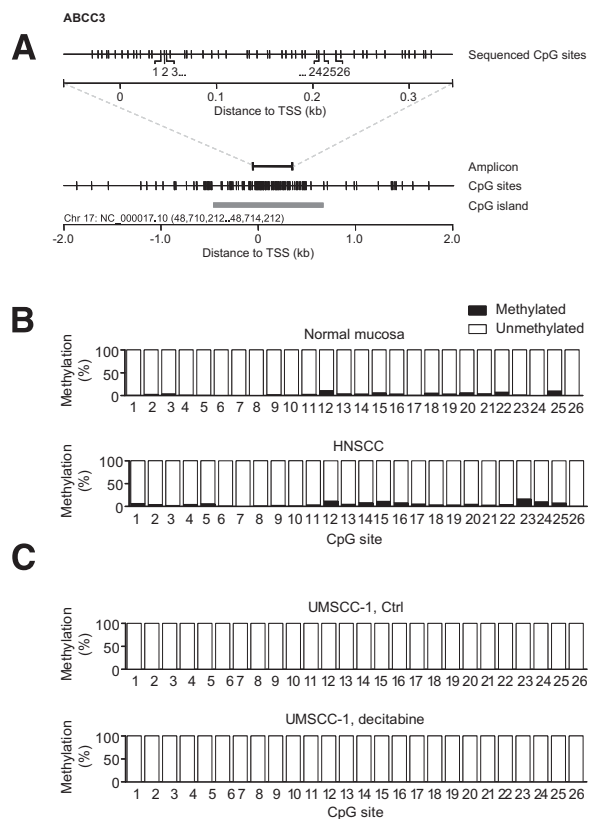


Figure 3 Analysis of CpG island methylation in *ABCC3* using bisulfite sequencing. **A:** Position of the CpG island and the sequenced CpG sites relative to the transcription start site (TSS). **B:** Average methylation rate of individual CpG sites in HNSCC tumors and adjacent normal mucosa from two patients with HNSCC. **C:** Average methylation rate of individual CpG sites in vehicle-treated (Ctrl) and decitabine-treated (1 $\mu\text{mol/L}$ for 72 hours) UMSSC-1 cells. Methylation rates of 14 CpG sites were obtained by direct sequencing of PCR amplicons and by analysis of the sequence trace files using Epigenetic Sequencing Methylation Analysis Software.

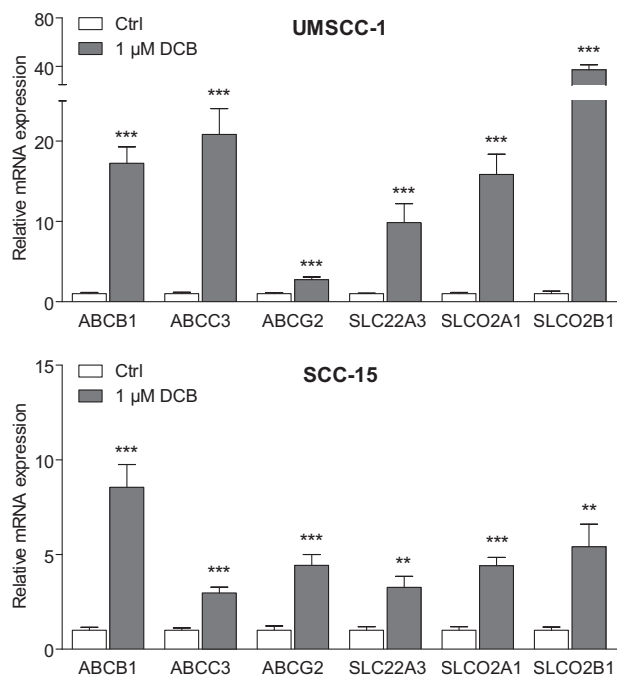


Figure 4 Effect of decitabine (DCB)—induced epigenetic modifications on the mRNA expression of selected *ABC*, *SLC22*, and *SLCO* transporters in UMSCC-1 and SCC-15 cells. Cells were treated with the DNA methyltransferase inhibitor, decitabine (1 μ mol/L), for 72 hours. Control cells (Ctrl) were treated with solvent (dimethyl sulfoxide) only. Transporter mRNA expression was quantified by real-time RT-PCR and normalized to that of the reference genes *ACTB* and *GAPDH*. mRNA expression in the drug-treated cells is presented relative to that in the solvent Ctrl. $n = 11$ to 19 per group. ** $P < 0.01$, *** $P < 0.001$ versus Ctrl.

was stably silenced in UMSCC cells via infection with *NR1I2* shRNA lentiviruses. Two of four tested shRNA constructs silenced *NR1I2* mRNA expression lower than the detection limit (Figure 6A). In cells with effective *NR1I2* knockdown, decitabine treatment still resulted in an increase in *ABCC3* and *SLCO2A1* mRNA expression, but the effect was significantly reduced compared with untransfected cells [*ABCC3*, 6.7- versus 20.8-fold ($P < 0.001$); and *SLCO2A1*, 4.2- versus 15.8-fold ($P < 0.01$)], as shown in Figure 6B. Notably, in cells with *NR1I2* knockdown, basal *ABCC3* mRNA, but not *SLCO2A1* mRNA, levels were significantly reduced compared with parental cells, suggesting that basal *ABCC3* expression in UMSCC depended, in part, on *NR1I2*.

Discussion

This study demonstrates the following about expression of drug transporters: i) expression is markedly changed in the HNSCC tumor tissues compared with normal mucosa, ii) expression might be predictive of the outcome of patients with HNSCC, and iii) expression is affected by novel epigenetic therapies and is further modulated by nuclear receptor—mediated mechanisms.

To our knowledge, this is the first study that systematically compares gene expression profiles of membrane transporters, which are generally implicated in drug disposition or multidrug resistance, in human HNSCC tumors and adjacent normal mucosa. The most prominent changes in the expression profile were observed in transporters of the *SLCO* family, with an increased expression of *SLCO1A2* and *SLCO1B3* and a decreased expression of *SLCO2B1*, *SLCO2A1*, and *SLCO1C1* in HNSCC tumor tissue compared with the normal mucosa.

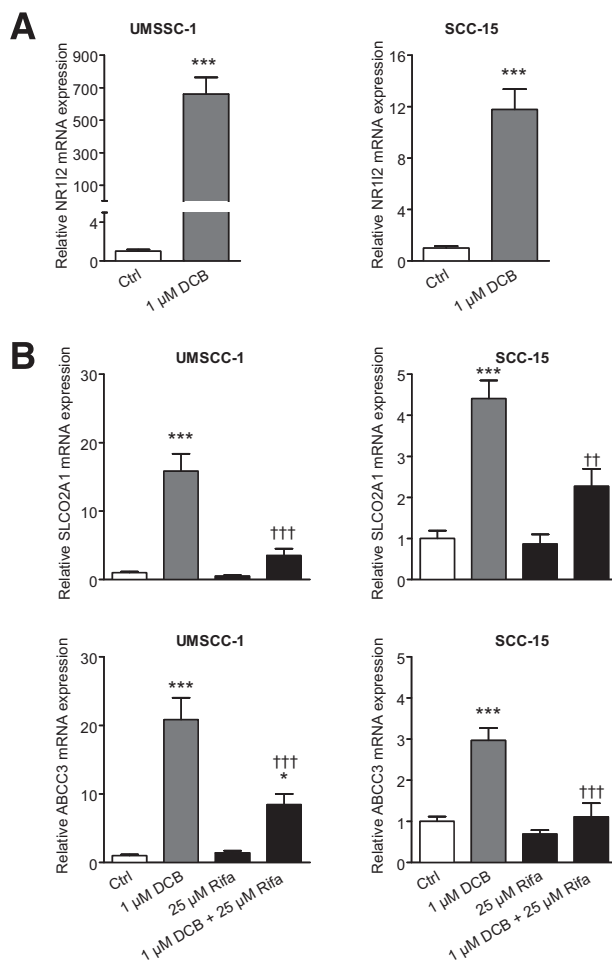


Figure 5 **A:** Effects of decitabine (DCB)—induced epigenetic modification on *NR1I2/PXR* mRNA expression in UMSCC-1 and SCC-15 cells. Cells were treated with 1 μ mol/L DCB for 72 hours. Gene expression of *NR1I2* was quantified by real-time RT-PCR and normalized to the expression of the reference genes *ACTB* and *GAPDH*. mRNA expression in the drug-treated cells is presented relative to that in the solvent control cells (Ctrl). $n = 7$ to 9 per group. **B:** Effects of the PXR ligand/activator rifampicin on *SLCO2A1* and *ABCC3* transcript expression in UMSCC-1 and SCC-15 cells, with or without concomitant DCB treatment. Cells were treated with DCB (1 μ mol/L) and the PXR activator rifampicin (25 μ mol/L), either alone or in combination, for 72 hours. Transporter gene expression was quantified by real-time RT-PCR and normalized to the expression of the reference genes *ACTB* and *GAPDH*. mRNA expression in the drug-treated cells is presented relative to that in the solvent control cells (Ctrl). $n = 7$ to 19 per group. * $P < 0.05$, *** $P < 0.001$ versus Ctrl; ††† $P < 0.01$, †††† $P < 0.001$ versus DCB.

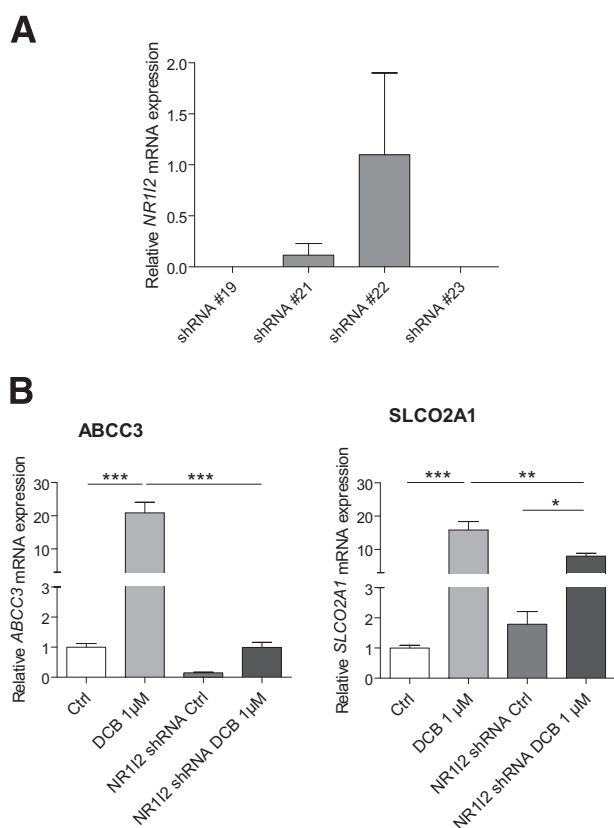


Figure 6 **A:** Quantitative assessment of the *NR1I2*/*PXR* knockdown efficiency. UMSSC cells were stably transfected with four different lentiviral *NR1I2* shRNA constructs (number 19, TRCN00000211619; number 21, TRCN00000211621; number 22, TRCN00000211622; and number 23, TRCN00000211623), and *NR1I2* mRNA expression was quantified by real-time RT-PCR and normalized to the expression of the reference genes *ACTB* and *GAPDH*. mRNA levels are presented as fold change relative to control cells (Ctrl). $n = 4$ independent measurements. **B:** Effect of *NR1I2* silencing on *SLCO2A1* and *ABCC3* transcript expression in UMSSC cells, with or without decitabine (DCB) treatment. Three UMSSC cell lines with efficient *NR1I2*/*PXR* knockdown (*NR1I2* shRNA) were treated with 1 μ mol/L decitabine for 72 hours. Transporter mRNA expression was quantified by real-time RT-PCR and normalized to the expression of the reference genes *ACTB* and *GAPDH*. mRNA expression in the drug-treated cells is presented relative to that in the respective untreated Ctrl. Pooled results from the three *NR1I2* shRNA cell lines are shown. $n = 4$ independent experiments. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

Interestingly, a similar change in the expression of *SLCO1B3* and *SLCO2A1* was found in colorectal cancer. *SLCO1B3* overexpression confers apoptotic resistance to colon cancer cells by altering p53-dependent pathways.²⁴ *SLCO2A1* can facilitate the removal of prostaglandin E2 from the extracellular milieu. In colorectal cancer, decreased *SLCO2A1* expression leads to higher concentrations of prostaglandin E2, which, in turn, mediates cell proliferation, antiapoptotic effects, and neovascularization.²⁵ Taken together, these studies demonstrate that higher *SLCO1B3* and lower *SLCO2A1* expression in colorectal cancer provide a survival advantage to cancer cells. This also might hold true for HNSCC.

We observed a significant association between high *SLCO2B1* mRNA levels in HNSCC tumor tissues and a low

pT class and an improved 5-year survival of patients with HNSCC in a pilot study. In recent studies, expression levels of *SLCO2B1* in liver, prostate, or breast cancer have been linked with patient outcomes, disease stage, or tumor differentiation.^{26–29} Based on these results, *SLCO2B1* has been proposed as a biomarker for risk assessment.^{26–28} However, the specific mechanism by which *SLCO2B1* affects patient outcome remains to be elucidated and most likely depends on the cancer type.

The *SLCO2B1* expression level remained a significant predictor of 5-year survival in our study cohort, even when the pT class was considered as a covariate in a logistic regression analysis (odds ratio, 6.5; 95% CI, 1.1 to 38.6; $P = 0.04$). This result suggests that *SLCO2B1* expression is linked to survival through additional mechanisms, irrespective of the tumor size. This might occur through the interaction of *SLCO2B1* with the chemotherapeutic drugs. Patients in our study received a standard chemotherapy regimen for HNSCC³⁰ that consisted of two or more of the following compounds: cisplatin, docetaxel, fluorouracil, and methotrexate. Although *SLCO2B1* is a well-known uptake transporter for diverse drugs, such as statins, fexofenadine, glibenclamide, or montelukast,^{5,31} it is still unknown whether the standard chemotherapeutic drugs used in HNSCC treatment are substrates of *SLCO2B1*.

In our study, the transcript levels of ABC efflux transporters, which are commonly implicated in multidrug resistance, were unchanged (*ABCC1*, *ABCC2*, *ABCC4*, *ABCC5*, and *ABCC10*) or even reduced (*ABCC3* and *ABCG2*) in HNSCC tumor samples compared with adjacent normal mucosa. Moreover, our data did not reveal a significant association of ABC transporter expression in HNSCC tumor samples with 5-year survival. Tsunoda et al³² found that *ABCG2* expression was an independent prognostic factor in esophageal squamous cell carcinoma. We were not able to replicate this finding in our cohort of patients with HNSCC; however, it is possible that the effects of *ABCG2* expression may have been missed because of the limited power of our study. None of the patients had received neoadjuvant chemotherapy before the tissue samples were obtained. We cannot rule out that the expression of ABC transporters may increase during chemotherapy because multidrug resistance often appears during drug treatment (secondary resistance), and that the ABC transporter levels at that stage may become predictive of outcome.

In the second part of our study, potential mechanisms that influence transporter expression were evaluated in cellular models of HNSCC. Modulation of the epigenome, particularly with the DNA methyltransferase inhibitor decitabine, markedly increased expression of some, but not all, investigated transporters of the *SLCO*, *SLC22*, and *ABC* families in HNSCC cells. Our results extend the findings of two previous studies^{33,34} that demonstrate the induction of *ABCG2* and *SLCO1B3* in cancer cell lines by decitabine. The question of epigenetic modulation of transporter expression may have clinical implications because DNA

methyltransferase inhibitors have already been approved as a treatment for lymphomas and are being tested as a therapeutic option against a variety of human solid tumors, including HNSCC.^{35–37} If these agents are concomitantly administered with classic anticancer drugs, the resultant induction of transporter expression may influence the disposition and effect of these anticancer drugs.⁹ In particular, enhanced expression of *ABCC3* may induce drug resistance of cancer cells and affect efficacy of treatment with *ABCC3* substrates, such as methotrexate, vincristine, doxorubicin, and cisplatin. On the other hand, decitabine-induced restoration of *SLCO2A1* expression in tumor tissue may facilitate the removal from the extracellular milieu of prostaglandins, which were implicated as significant mediators in cancer progression.

Methylation of CpG islands in promoters of transporter genes was investigated in patient tissue samples and an HNSCC-derived tumor cell line. The UMSCC cell line had a different methylation signature than patient samples. Several studies^{38,39} demonstrated marked interpatient variability in promoter methylation at the single-gene level. Thus, the methylation status of the UMSCC cell line may reflect the high methylation status of the original HNSCC tumor sample. Another explanation for the discrepancy between the methylation status of the *SLCO2A1* promoter in the HNSCC tissue and the UMSCC cell line is that cell line methylation is affected by the tissue culture environment.

An important finding of our study was that the presence of CpG islands in the promoters of transporter genes was generally not required for decitabine-mediated induction of their expression. Inhibition of DNA methyltransferase, for example, caused a substantial increase (37-fold) in the transcript levels of *SLCO2B1*, a gene that completely lacks CpG islands in its promoter region. Although a small decrease in the promoter CpG methylation of *SLCO2A1* after decitabine treatment partially explains the induction of *SLCO2A1*, no correlation between *ABCC3* promoter methylation and induction of *ABCC3* mRNA was observed.

Decitabine targets the whole epigenome, including the epigenetic state of transcriptional regulators, such as *NR1I2/PXR*. Given that *PXR* is capable of transcriptional activation of genes involved in drug transport, such as *ABCB1*,⁴⁰ and promoter methylation is involved in the regulation of *NR1I2/PXR* in cancer cells,^{16,17} we hypothesized that nuclear receptors may participate in the epigenetic regulation of transporter expression in HNSCC cells. Consistent with a previous report on colon carcinoma,¹⁶ decitabine markedly induced *NR1I2/PXR* in UMSCC-1 and SCC-15 cell lines. Furthermore, *NR1I2/PXR* knockdown significantly attenuated the inducing effects of decitabine on *ABCC3* and *SLCO2A1* mRNA expression. Surprisingly, cotreatment of these cells with a prototypical *PXR* activator caused a significant repression of *SLCO2A1* and *ABCC3* compared with cells treated with decitabine alone. This finding partially corresponds to observations made by Cheng et al.,⁴¹ who observed a down-regulation of several

Slcos, including *Slco2a1*, in the mouse liver after exposure to prototypical *PXR* ligands. Several anticancer drugs, such as cisplatin, paclitaxel, and cyclophosphamide, are well-known agonists of nuclear receptors.^{14,15,42} This raises the possibility of drug-drug interactions in patients treated with combinations of multiple anticancer drugs, which, in turn, may affect the treatment outcome.⁴³ Taken together, our results demonstrate an interaction between epigenetic and nuclear receptor-mediated mechanisms in the regulation of transporter expression.

To our knowledge, this is the first study to provide a basis for the assessment of the role of uptake and efflux transporters in the accessibility of HNSCC tumors to chemotherapeutic agents. Transporters that are differentially expressed in HNSCC tumors versus normal mucosa were identified. Moreover, the correlation of *SLCO2B1* levels in HNSCC tumors with 5-year survival of patients was demonstrated in a pilot study. Replication of these results in a prospective observational study is necessary. Modulation of the epigenome by the DNA methyltransferase inhibitor, decitabine, induced the expression of several transporters considerably, suggesting the involvement of epigenetic mechanisms in the regulation of transporter expression. These mechanisms do not necessarily involve direct hypomethylation of CpG islands in transporter genes, and the pathway by which transporter mRNA levels (eg, those of *SLCO2B1* and *ABCC3*) are induced by decitabine remains to be elucidated. Finally, interactions between epigenetic and nuclear receptor-mediated mechanisms played a role in the regulation of transporter expression.

References

- Huang Y: Pharmacogenetics/genomics of membrane transporters in cancer chemotherapy. *Cancer Metastasis Rev* 2007, 26:183–201
- Zolk O: Disposition of metformin: variability due to polymorphisms of organic cation transporters. *Ann Med* 2012, 44:119–129
- Szakacs G, Paterson JK, Ludwig JA, Booth-Genthe C, Gottesman MM: Targeting multidrug resistance in cancer. *Nat Rev Drug Discov* 2006, 5:219–234
- Solbach TF, König J, Fromm MF, Zolk O: ATP-binding cassette transporters in the heart. *Trends Cardiovasc Med* 2006, 16:7–15
- Fahrmayr C, Fromm MF, König J: Hepatic OATP and OCT uptake transporters: their role for drug-drug interactions and pharmacogenetic aspects. *Drug Metab Rev* 2010, 42:380–401
- König J, Seithel A, Gradhand U, Fromm MF: Pharmacogenomics of human OATP transporters. *Naunyn Schmiedeberg Arch Pharmacol* 2006, 372:432–443
- Hagenbuch B, Gui C: Xenobiotic transporters of the human organic anion transporting polypeptides (OATP) family. *Xenobiotica* 2008, 38:778–801
- Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM: Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int J Cancer* 2010, 127:2893–2917
- Stewart DJ, Issa JP, Kurzrock R, Nunez MI, Jelinek J, Hong D, Oki Y, Guo Z, Gupta S, Wistuba II: Decitabine effect on tumor global DNA methylation and other parameters in a phase I trial in refractory solid tumors and lymphomas. *Clin Cancer Res* 2009, 15:3881–3888
- Taberlay PC, Jones PA: DNA methylation and cancer. *Prog Drug Res* 2011, 67:1–23

11. Bram EE, Stark M, Raz S, Assaraf YG: Chemotherapeutic drug-induced ABCG2 promoter demethylation as a novel mechanism of acquired multidrug resistance. *Neoplasia* 2009, 11:1359–1370
12. Bennett KL, Karpenko M, Lin MT, Claus R, Arab K, Dychkoff G, Plinkert P, Herpel E, Smiraglia D, Plass C: Frequently methylated tumor suppressor genes in head and neck squamous cell carcinoma. *Cancer Res* 2008, 68:4494–4499
13. Tirona RG: Molecular mechanisms of drug transporter regulation. *Handb Exp Pharmacol* 2011, (201):373–402
14. Harmsen S, Meijerman I, Febus CL, Maas-Bakker RF, Beijnen JH, Schellens JH: PXR-mediated induction of P-glycoprotein by anticancer drugs in a human colon adenocarcinoma-derived cell line. *Cancer Chemother Pharmacol* 2010, 66:765–771
15. Masuyama H, Suwaki N, Tateishi Y, Nakatsukasa H, Segawa T, Hiramatsu Y: The pregnane X receptor regulates gene expression in a ligand- and promoter-selective fashion. *Mol Endocrinol* 2005, 19: 1170–1180
16. Habano W, Gamo T, Terashima J, Sugai T, Otsuka K, Wakabayashi G, Ozawa S: Involvement of promoter methylation in the regulation of Pregnane X receptor in colon cancer cells. *BMC Cancer* 2011, 11:81
17. Misawa A, Inoue J, Sugino Y, Hosoi H, Sugimoto T, Hosoda F, Ohki M, Imoto I, Inazawa J: Methylation-associated silencing of the nuclear receptor 1I2 gene in advanced-type neuroblastomas, identified by bacterial artificial chromosome array-based methylated CpG island amplification. *Cancer Res* 2005, 65:10233–10242
18. Krause CJ, Carey TE, Ott RW, Hurbis C, McClatchey KD, Regezi JA: Human squamous cell carcinoma: establishment and characterization of new permanent cell lines. *Arch Otolaryngol* 1981, 107:703–710
19. Brenner JC, Graham MP, Kumar B, Saunders LM, Kupfer R, Lyons RH, Bradford CR, Carey TE: Genotyping of 73 UM-SCC head and neck squamous cell carcinoma cell lines. *Head Neck* 2010, 32: 417–426
20. Zhao M, Sano D, Pickering CR, Jasser SA, Henderson YC, Clayman GL, Sturgis EM, Ow TJ, Lotan R, Carey TE, Sacks PG, Grandis JR, Sidransky D, Heldin NE, Myers JN: Assembly and initial characterization of a panel of 85 genomically validated cell lines from diverse head and neck tumor sites. *Clin Cancer Res* 2011, 17:7248–7264
21. Eckhardt F, Lewin J, Cortese R, Rakyan VK, Attwood J, Burger M, Burton J, Cox TV, Davies R, Down TA, Haefliger C, Horton R, Howe K, Jackson DK, Kunde J, Koenig C, Liddle J, Niblett D, Otto T, Pettett R, Seemann S, Thompson C, West T, Rogers J, Olek A, Berlin K, Beck S: DNA methylation profiling of human chromosomes 6, 20 and 22. *Nat Genet* 2006, 38:1378–1385
22. Lewin J, Schmitt AO, Adorjan P, Hildmann T, Piepenbrock C: Quantitative DNA methylation analysis based on four-dye trace data from direct sequencing of PCR amplicates. *Bioinformatics* 2004, 20:3005–3012
23. Stefanska B, Huang J, Bhattacharyya B, Suderman M, Hallett M, Han ZG, Szyf M: Definition of the landscape of promoter DNA hypomethylation in liver cancer. *Cancer Res* 2011, 71:5891–5903
24. Lee W, Belkhiari A, Lockhart AC, Merchant N, Glaeser H, Harris EI, Washington MK, Brunt EM, Zaika A, Kim RB, El-Rifai W: Overexpression of OATP1B3 confers apoptotic resistance in colon cancer. *Cancer Res* 2008, 68:10315–10323
25. Holla VR, Backlund MG, Yang P, Newman RA, DuBois RN: Regulation of prostaglandin transporters in colorectal neoplasia. *Cancer Prev Res (Phila)* 2008, 1:93–99
26. Pressler H, Sissung TM, Venzon D, Price DK, Figg WD: Expression of OATP family members in hormone-related cancers: potential markers of progression. *PLoS One* 2011, 6:e20372
27. Yang M, Xie W, Mostaghel E, Nakabayashi M, Werner L, Sun T, Pomerantz M, Freedman M, Ross R, Regan M, Sharifi N, Figg WD, Balk S, Brown M, Taplin ME, Oh WK, Lee GS, Kantoff PW: SLCO2B1 and SLCO1B3 may determine time to progression for patients receiving androgen deprivation therapy for prostate cancer. *J Clin Oncol* 2011, 29:2565–2573
28. Wright JL, Kwon EM, Ostrander EA, Montgomery RB, Lin DW, Vessella R, Stanford JL, Mostaghel EA: Expression of SLCO transport genes in castration-resistant prostate cancer and impact of genetic variation in SLCO1B3 and SLCO2B1 on prostate cancer outcomes. *Cancer Epidemiol Biomarkers Prev* 2011, 20:619–627
29. Al Sarakbi W, Mokbel R, Salhab M, Jiang WG, Reed MJ, Mokbel K: The role of STS and OATP-B mRNA expression in predicting the clinical outcome in human breast cancer. *Anticancer Res* 2006, 26: 4985–4990
30. Furness S, Glenny AM, Worthington HV, Pavitt S, Oliver R, Clarkson JE, Macluskey M, Chan KK, Conway DI: Interventions for the treatment of oral cavity and oropharyngeal cancer: chemotherapy. *Cochrane Database Syst Rev* 2011, (4):CD006386
31. Anderson CM, Thwaites DT: Hijacking solute carriers for proton-coupled drug transport. *Physiology (Bethesda)* 2010, 25:364–377
32. Tsunoda S, Okumura T, Ito T, Kondo K, Ortiz C, Tanaka E, Watanabe G, Itami A, Sakai Y, Shimada Y: ABCG2 expression is an independent unfavorable prognostic factor in esophageal squamous cell carcinoma. *Oncology* 2006, 71:251–258
33. Ichihara S, Kikuchi R, Kusuhara H, Imai S, Maeda K, Sugiyama Y: DNA methylation profiles of organic anion transporting polypeptide 1B3 in cancer cell lines. *Pharm Res* 2010, 27:510–516
34. To KK, Zhan Z, Bates SE: Aberrant promoter methylation of the ABCG2 gene in renal carcinoma. *Mol Cell Biol* 2006, 26: 8572–8585
35. Abele R, Clavel M, Dodion P, Brunsch U, Gundersen S, Smyth J, Renard J, van Glabbeke M, Pinedo HM: The EORTC Early Clinical Trials Cooperative Group experience with 5-aza-2'-deoxycytidine (NSC 127716) in patients with colo-rectal, head and neck, renal carcinomas and malignant melanomas. *Eur J Cancer Clin Oncol* 1987, 23:1921–1924
36. Kim J, Guan J, Chang I, Chen X, Han D, Wang CY: PS-341 and histone deacetylase inhibitor synergistically induce apoptosis in head and neck squamous cell carcinoma cells. *Mol Cancer Ther* 2010, 9: 1977–1984
37. Prystowsky MB, Adomako A, Smith RV, Kawachi N, McKimpson W, Atadja P, Chen Q, Schlecht NF, Parish JL, Childs G, Belbin TJ: The histone deacetylase inhibitor LBH589 inhibits expression of mitotic genes causing G2/M arrest and cell death in head and neck squamous cell carcinoma cell lines. *J Pathol* 2009, 218:467–477
38. Dong SW, Zhang P, Liu YM, Cui YT, Wang S, Liang SJ, He Z, Sun P, Wang YG: Study on RIZ1 gene promoter methylation status in human esophageal squamous cell carcinoma. *World J Gastroenterol* 2012, 18: 576–582
39. Hanada N, Takahata T, Zhou Q, Ye X, Sun R, Itoh J, Ishiguro A, Kijima H, Mimura J, Itoh K, Fukuda S, Saijo Y: Methylation of the KEAP1 gene promoter region in human colorectal cancer. *BMC Cancer* 2012, 12:66
40. Geick A, Eichelbaum M, Burk O: Nuclear receptor response elements mediate induction of intestinal MDR1 by rifampin. *J Biol Chem* 2001, 276:14581–14587
41. Cheng X, Maher J, Dieter MZ, Klaassen CD: Regulation of mouse organic anion-transporting polypeptides (Oatps) in liver by prototypical microsomal enzyme inducers that activate distinct transcription factor pathways. *Drug Metab Dispos* 2005, 33:1276–1282
42. Synold TW, Dussault I, Forman BM: The orphan nuclear receptor SXR coordinately regulates drug metabolism and efflux. *Nat Med* 2001, 7:584–590
43. Harmsen S, Meijerman I, Beijnen JH, Schellens JH: The role of nuclear receptors in pharmacokinetic drug-drug interactions in oncology. *Cancer Treat Rev* 2007, 33:369–380