Chapter 3

A genome-wide siRNA screen identifies the FA/BRCA pathway as the strongest predictor of cisplatin response in head and neck cancer cells.

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Objective: Patients with advanced head and neck squamous cell carcinoma (HNSCC) are generally treated with cisplatin-containing chemoradiation protocols. Although cisplatin is a low-priced and often used addition to radiation, it causes severe toxicity and not all patients benefit from the treatment. Non-responding HNSCC patients may better be treated with surgery and postoperative radiation, but biomarkers of chemoradiation response, that would allow personalized treatment, are not available.

Methods: We performed an unbiased genome-wide functional genetic screen in vitro to identify genes that influence the response to cisplatin in HNSCC cells.

Results: We identified 269 genes of which siRNA-mediated knockdown seemed to significantly influenced cisplatin response in HNSCC cells. We retested 149 siRNA pools and were able to confirm that 21 genes are important in the cisplatin response in three HNSCC cell lines. The predominant pathway involved in this response is the Fanconi anemia/BRCA pathway, including the SHFM1 gene.

Conclusion: This genome-wide functional analysis identified genes that are important in the response of HNSCC to cisplatin and may guide further biomarker validation. Biomarkers that indicate the activity of the Fanconi anemia/BRCA pathway are the first logical candidates.
INTRODUCTION

Cancer of the head and neck is the eighth most commonly diagnosed cancer worldwide\(^1\). Over 90% of the head and neck cancers are squamous cell carcinomas. Approximately 60% of head and neck squamous cell carcinoma (HNSCC) patients present with advanced stage disease (stage III and IV), which relates to a poor prognosis\(^2\). Treatment options for this group are surgery followed by radiotherapy or cisplatin-containing chemoradiation protocols with salvage surgery if needed. Although chemoradiation is effective\(^3\), not all tumors respond well to this combination of cisplatin and radiotherapy. In addition, cisplatin is an effective and inexpensive addition to radiation protocols, but the major limitation of its use is the severe toxicity. Patients need to be hospitalized for a few days after cisplatin infusion and complications that require extra hospitalization are frequent. This is a large burden for the patient and is associated with high health care costs. As there might be an alternative treatment, surgery with postoperative radiotherapy, response prediction is an important issue. It would be ideal to personalize the application of chemoradiation and to treat only those patients that are likely to respond to the therapy.

Major research efforts aim to find clinical and/or biomolecular markers to predict chemoradiation response of HNSCC. However, the only clinical factor that shows some predictive value for HNSCC chemoradiotherapy outcome proved to be primary tumor volume\(^{4-11}\), and not even all studies could confirm this\(^{12}\).

Using a candidate gene or protein approach several biological and genetic markers have been studied to predict chemoradiation outcome in head and neck cancer patients\(^{13-20}\). Other groups have exploited microarray technology to determine expression profiles that might predict treatment response in head and neck cancer\(^{12,21-28}\), but a predictive profile has not yet been validated.

There are several explanations why these approaches have not led to breakthroughs. First, tumor biopsies may contain mixed cell populations, and particularly the small subpopulations of treatment-resistant cells (e.g. cancer stem cells) will not be detected by expression profiling, whilst they might be highly relevant for treatment outcome\(^{29}\). Second, genes harboring an activating or inactivating mutation, but that are expressed at normal levels, will not be identified as predictors of treatment outcome.

We aim to identify all genes that have an important role in the response to cisplatin. Unveiling these genes might enable us to find biomarkers that can be used to predict cisplatin-based chemoradiation outcome and to personalize HNSCC treatment by only treating those patients that would benefit from the therapy. The application of loss-of-function high-throughput RNA interference screens has become an important genomic tool in research\(^{30-33}\). Especially for the identification of genes that modulate drug response, screens using siRNAs are increasingly used\(^{34-43}\). For example, using this technology Posthuma De Boer et al.\(^{44}\) identified JIP1 as a gene that modulates the sensitivity of osteosarcoma cells to doxorubicine. We hypothesized that a genome-wide siRNA screen could be helpful to identify genes and signaling pathways involved in chemoradiation response in head and neck cancer cells, and we decided to focus on those that influence cisplatin response. Therefore, we employed an arrayed library of 21,121 pools of siRNAs, targeting unique human genes in the NCBI RefSeq database (www.ncbi.nlm.nih.gov/RefSeq and \(^{45}\)) This one-gene-one-well approach allowed us to comprehensively interrogate the cellular response to cisplatin when expression of each individual gene is reduced separately. Results may lead to novel biomarkers that predict which patients will benefit from cisplatin-containing protocols.
MATERIAL AND METHODS

Cell lines, clinical specimens and chemicals
All cancer cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Lonza) containing 5% fetal calf serum (FCS; Lonza) and 2 mM L-glutamine (Lonza). Cells were grown in a humidified atmosphere of 5% CO₂ at 37°C.

Head and neck squamous cell carcinoma (HNSCC) cell lines UM-SCC-11B (a larynx tumor; T2N2A) and UM-SCC-22B (a hypopharynx tumor; T2N1) were obtained from Dr. T Carey (University of Michigan, Ann Arbor, MI, USA)(46). Cell line VU-SCC-120 (formerly known as 93VU120) was established as described previously(47). All HNSCC cell lines were authenticated to the earliest available passages by microsatellite profiling and TP53 mutation analysis(46,48).

Clinical specimens of a panel of 22 HNSCCs and corresponding macroscopically normal mucosa, adjacent to the tumor, were collected according to the Dutch legislations on research with human material. This study was approved by the Institutional Review Board (Medisch Ethische Toetsingscommissie VUmc). Collection was done immediately after surgery in the period 2000-2007 and samples were stored in liquid nitrogen. Inclusion criteria were: squamous cell carcinoma from the oral cavity or oropharynx, and the treatment should consist of surgery with or without post-operative radiotherapy. All normal mucosa samples were judged free from dysplasia by an experienced pathologist. The panel included 16 males and 7 females with an average age of 60.4 ± 10.1 years (range 33-82). The majority of the patients had an history of heavy smoking (≥30 pack-years; 17/23, 74%) and/or heavy alcohol consumption (≥100 unit-years, with 1 unit is equivalent to approximately 15 mL of ethanol; 16/23, 70%). Specimens were obtained from the oral cavity (74%, 17/23) and the oropharynx (26%, 6/23) with the majority showing a moderate degree of differentiation (65%, 15/23), whereas 35% (8/23) was poorly differentiated. Twenty-two percent of the patients had disease stage I/II (5/23) and 78% (18/23) were in stage III/IV.

Cisplatin was obtained from Teva Pharmachemie in a concentration of 1 mg/ml.

IC₅₀ measurements
Cells were plated in 96-well plates and were treated with 18 different concentrations of cisplatin (ranging from 0.01-666 μM) 24 hours later. Plates were incubated for 72 hours at 37°C/5% CO₂. Cell viability was calculated based on fluorescence measurements after 2 hours incubation with 20 μl of a 1:1 dilution of CellTiter Blue (Promega) in cell culture medium. Fluorescence was measured using an Infinite 200 microplate reader (Tecan Group Ltd).

Genome-wide siRNA screen
VU-SCC-120 cells were subjected to high-throughput forward transfection as described before(49). In summary, 1,000 cells were seeded and the next day transfected with 25 nmol of each siRNA SMARTpool derived from the siARRAY Human Genome library (Catalog items G-003500 (Sept05), G-003600 (Sept05), G-004600 (Sept05) and G-005000 (Oct05); Dharmacon, Thermo Fisher Scientific) and 0.03 μl DharmaFECT1 (Dharmacon, Thermo Fisher Scientific). The nontargeting siCONTROL#2 and the PLK1 targeting siRNA SMARTpool were used as negative and positive control for transfection efficiency, respectively. After 24 hours of incubation with the transfection reagents, medium volume was reduced to 40 μl using the Microlab STAR liquid handling station (Hamilton Robotics), and 60 μl fresh medium was added with or without cisplatin to reach final concentrations matching IC₀ (no cisplatin) or IC₂₀ (2 μM) using a Multidrop Combi (Thermo Fisher Scientific). Plates were incubated for 72 hours at 37°C/5% CO₂. Cell viability was measured using CellTiter Blue (Promega) as described.

Deconvolution of siRNA SMARTpools that significantly caused cellular sensitivity to cisplatin was performed using the same procedures, either by hand or using the same automated methods as described above. UM-SCC-22B was transfected with 25 nmol siRNA and 0.10 μl DharmaFECT1.
UM-SCC-11B was transfected with 25 nmol siRNA and 0.065 μl DharmaFECT1. IC$_{20}$ cisplatin values for UM-SCC-11B and UM-SCC-22B were 1.0 μM and 0.05 μM, respectively$^{(63)}$.

**Data analysis of the genome-wide screen**

Many statistical analyses for high-throughput screens have been published, but there seems no preference for a single method. Therefore, the data from our genome-wide high-throughput screen were analyzed with two different statistical methods; Z-score calculation and a limma-based linear regression model.

For the Z-score analysis raw fluorescence values were log10 transformed and per plate the trimmed mean was calculated (trimming factor 0.5). Plate normalization was accomplished using the overall mean per cisplatin condition (IC$_{0}$ or IC$_{20}$) and per replicate. The normalized fluorescent values were back-transformed. Per cisplatin condition, both replicates were normalized towards each other and for each siRNA replicates of each cisplatin condition were averaged. IC$_{0}$/IC$_{20}$ ratios were calculated and used for Z-score calculations.

For the calculations in limma, the data was read into R (version 2.12) and configured using cellHTS2$^{(50)}$. Subsequently, the data was log2 transformed and normalized by correcting for linear effects of “day” and “plate” using a regression model. To estimate the treatment effect, an empirical Bayes linear model accounting for the treatment level$^{(51)}$ was fitted to the normalized data using the BioConductor package limma$^{(52)}$. The resulting p-value list was subsequently corrected for multiple testing using Benjamini-Hochberg’s step-up FDR method$^{(53)}$. This approach yields more power to find associations, in particular in studies with small sample size as the current one$^{(54)}$.

**Deconvolution of siRNA pools**

Gene-specific knockdown was confirmed by investigation of the phenotype caused by each of the four separate siRNAs that make up the siRNA pool. Triplicate experiments were performed as described above and the median of the three IC$_{0}$ as well as the median of the three IC$_{20}$ measurements were calculated. With these medians, p-values against siCONTROL#2 measurements were obtained with the Student’s t-test. We previously analyzed over 1000 siCONTROL#2 measurements and observed that the data follow the normal distribution, allowing t-test analysis (data not shown).

**Quantitative real-time PCR**

Cells were seeded in 96-well plates and transfected as described above. RNA was extracted 96 hours post-transfection using the RNeasy micro kit (Qiagen). cDNA was synthesized from 250 ng of RNA using AMV reverse transcriptase (Promega) and a custom designed reverse primer (Supplementary Table S1). Amplification of the cDNA was performed on the ABI/Prism 7500 Sequence Detector System (Taqman-PCR, Applied Biosystems) with 1x Power SYBR Green PCR Master Mix (Applied Biosystems) and custom designed primers for each gene of interest (Supplementary Table S1). CTR1 expression after siRNA transfection was investigated using Taqman Gene Expression Assay (Hs00977268_g1; Applied Biosystems) according to the manufacturer’s instructions.

For each sample the cycle number at which the amount of amplified target crossed a predetermined threshold (the Ct-value) was determined. To correct for differences in RNA input and quality, beta-glucuronidase (GUSB) was used as a housekeeping gene$^{(55)}$ for each RNA sample. The mRNA expression was calculated relative to that of GUSB using the Delta/Delta Ct method$^{(56)}$. Quantitative RT-PCR reactions without reverse transcriptase were carried out in parallel for each RNA sample to exclude signal caused by contaminating genomic DNA.

**Cell cycle analysis**

Cells were seeded in 25 cm$^{2}$ flasks and transfected 24 hours later in duplicate with siCONTROL#2,
BRCA2 SMARTpool or SHFM1 SMARTpool. After another 24 hours, the cultures were refreshed with either fresh medium or medium containing 500 nM cisplatin. After 72 hours of incubation the cells were harvested and fixed with 70% EtOH during at least three hours. Next, cells were incubated with 0.5 mg/ml RNase A in PBS at 37°C for 30 minutes. The cells were washed and the DNA content was stained with propidium iodide (PI). The cell cycle distribution was analyzed with a BD FACSCalibur flow cytometer (BD Biosciences). Cell cycle analyses were performed using BD CellQuest software (BD Biosciences) and calculations in Excel.

Expression microarray
RNA was isolated with TRIzol (Life Technologies) according to the manufacturer’s instructions. RNA integrity was measured using a RNA nanochip on an Agilent 2100 Bioanalyzer (Agilent Technologies). Synthesis and labeling of cDNA was performed according to manufacturer’s recommendations (Agilent), including quality controls. mRNA expression profiles were determined using a mRNA microarray (4x44K Whole Human Genome Arrays G4112F, (Agilent). Labeling, hybridization and scanning was performed according to the manufacturer’s protocol, using a G2505B scanner and Feature Extraction v9.5 (Agilent). Data analysis was performed as described before(57).

RESULTS
Genome-wide siRNA screen design
Nineteen HNSCC cell lines were subjected to a selection procedure in order to identify the cell line most suitable for genome-wide siRNA application (data not shown). Selection criteria were based on cisplatin sensitivity, growth rate at low density in 96-well plates, no acidification of the medium and reproducibility of transfection. Cell line VU-SCC-120 (formerly 93VU120), a cell line derived from a previously untreated moderately differentiated, advanced stage tongue cancer (T3N1), matched all criteria. The half-maximal inhibitory concentration (IC50) of cisplatin was determined at 4.0 μM for this cell line(63). Since we are interested in siRNAs that increase the response to cisplatin, we decided to screen the siRNA library in the presence of a cisplatin concentration that affected 20% of the cells (IC 20), resulting in a theoretical screening window of 80%. For VU-SCC-120 this meant that the cells were treated with 2.0 μM of cisplatin (Fig. 1A). We used these data to design a robust, high-throughput RNA interference screen for the VU-SCC-120 cell line. The cells were transfected in quadruplicate in separate 96-well plates for duplicate analysis of IC0 (no cisplatin), and IC20 (Fig. 1B). We used the PLK1 siRNA SMARTpool, which resulted in a decrease in cell viability (data not shown), as expected(58), and the nontargeting siCONTROL#2 as positive and negative control, respectively. Cell viability was measured in every plate to evaluate the effect of each siRNA on cell growth (IC0) and the effect of reduced gene expression on cisplatin sensitivity (IC0 vs IC20).

Genome-wide siRNA screen to identify cisplatin susceptibility genes
Raw cell viability values were inspected for transfection induced cell death (siCONTROL#2 vs untransfected cells; 16% cell death) and transfection efficiency (siPLK1 vs siCONTROL#2; 92% cell death), and were found to be acceptable (Z’ factor=0.570). Comparison of the two independent duplicate screens, showed high reproducibility with Spearman’s Rho=0.823. Subsequently, the data were analyzed using two independent statistical methods as described in the Material and Methods section; Z-score analysis and a limma-based linear regression model. Z-score calculations yielded 207 siRNA pools that significantly sensitized VU-SCC-120 cells to cisplatin (Z≥3; p<0.001, Fig. 1C), whereas the linear regression model resulted in 167 hits (raw p<0.05). The hit lists obtained with these two methods shared 105 siRNAs (Fig. 1D and Supplementary Table S2).

We subsequently selected siRNAs for stratification by deconvolution of the siRNA pools into four
separate siRNAs. From the 105 siRNA pools that were found to cause a significant sensitization to cisplatin by both statistical analyses, we were able to re-order the individual siRNAs of 97 pools. In addition, we randomly selected 27 siRNAs that were only identified by Z-score analysis and 25 that were only found by the linear regression model for further analysis. Deconvolution was done in VU-SCC-120, the cell line used for the genome-wide siRNA discovery screen, and two additional HNSCC cell lines, UM-SCC-11B and UM-SCC-22B.

The FA/BRCA pathway predicts cisplatin response in HNSCC.

Figure 1. Functional genetic screen for cisplatin-sensitizing siRNAs. (A) HNSCC cell line VU-SCC-120 was treated with 18 different concentrations cisplatin to determine the IC₅₀ (4.0 μM) and IC₉₀ (2.0 μM) values. (B) Design of high-throughput siRNA screen with VU-SCC-120 cells. White wells contain untransfected control cells, grey wells contain transfection controls (siCONTROL#2 or siPLK1), and black wells contain cells transfected with siRNAs from the genome-wide library. (C) Graphic representation of the Z-score distribution. Black diamonds represent siRNAs from the genome-wide screen. The cut-off for hit list preparation was set at Z≥-3 (p<0.001). None of the nontargeting siCONTROL siRNAs (grey triangles) reached this threshold. (D) Schematic representation of the number of siRNAs that were found to significantly sensitize VU-SCC-120 cells to cisplatin, either by Z-score calculation or a limma-based linear regression model. Comparison of the two hit lists showed that 105 siRNAs were identified by both statistical methods.
We marked an siRNA as “confirmed” in case at least two separate siRNAs significantly (p<0.05, Student’s t-test) sensitized the tumor cells to cisplatin (Supplementary Table S3). The results for VU-SCC-120 showed that both statistical methods that were applied to the genome-wide siRNA screen are of equal strength, since the confirmation rate of the methods is comparable (22% for the Z-score analysis and 24% for the linear regression model). However, the confirmation rate obtained by siRNAs that were identified by both methods proved to be superior (42% confirmed) over either method alone. This observation also holds true for the numbers of siRNAs that were confirmed in at least two cell lines or even in all three cell lines. In total, 53 of the tested 149 primary hits were confirmed in VU-SCC-120 cells, of which 21 were reproducible in all three HNSSC cell lines (Supplementary Table S3).

The FA/BRCA pathway

Network analysis on the results derived from our genome-wide siRNA screen clearly indicated that knockdown of genes involved in DNA repair resulted in significant sensitization to cisplatin. Comprehensive pathway analysis on the complete data set by IPA (Ingenuity systems) revealed that especially members of the Fanconi anemia/BRCA pathway, which is involved in DNA crosslink repair, are implicated in cisplatin response in HNSCC cells (p=2.13x10^{-4}, Fig. 2A). In addition, according to the STRING database (version 9.05) the only cluster that could be found among the siRNAs that were confirmed in all HNSSC cell lines (n=21, Supplementary Table S3), harbored DNA repair genes (Fig. 2B). This indicates that an important determinant for cisplatin sensitivity in HNSCC cells is the proper functioning of DNA repair pathways, and especially the FA/BRCA pathway.

Indeed, close examination of two important players in the FA/BRCA pathway, BRCA1 and BRCA2, showed that knockdown of these genes enhances cisplatin induced cell death (Fig. 2C). Also the split hand/foot malformation type 1 gene (SHFM1 or DSS1), which has been shown to physically interact with BRCA2\(^{59-62}\), could be confirmed as a determinant of cisplatin response with all four separate siRNAs (Fig. 2C), and this could be confirmed in multiple cell lines (Supplementary Fig. S1). Knockdown of mRNA expression by the separate SHFM1 siRNAs was determined by quantitative RT-PCR and correlated with the increase in cisplatin-induced cell death, demonstrating that these are specific effects caused by knockdown of the SHFM1 gene and not due to off-target effects.

Since SHFM1 is known to interact with BRCA2, we questioned whether knockdown of SHFM1 would arrest cells in G2 phase upon cisplatin treatment, like what happens with BRCA2 knockdown cells. We compared cell cycle distributions among cell cultures treated with or without cisplatin (Fig. 3A). Upon BRCA2 knockdown (no cisplatin added), we already detected a significant shift in cell cycle distribution (p<0.05, 2-sided Fisher’s exact test), and this further increased after cisplatin treatment (p<0.001). For the SHFM1 knockdown cells, we found a small, but not significant shift in cell cycle distribution in the cultures that were not treated with cisplatin (p=0.13, 2-sided Fisher’s exact test in comparison with siCONTROL#2 transfected cells.). In contrast, the proportion of cells in each phase of the cell cycle was significantly different after cisplatin incubation (p<0.02). This leads to the hypothesis that SHFM1 might be as valuable as BRCA2 in the DNA repair process after cisplatin treatment.

To investigate if BRCA1, BRCA2 and SHFM1, identified as determinants of cisplatin sensitivity by our genome-wide siRNA screen, could be suitable candidates to serve as markers that predict whether cisplatin-containing chemoradiation is successful in HNSCC patients, we mined microarray mRNA expression profiles made from 22 healthy mucosa/tumor pairs (unpublished data) for these genes. We found that BRCA1, BRCA2 and SHFM1 expression is significantly upregulated (p<0.001) in HNSCC cells compared to the healthy mucosa (1.94x±0.60, 1.27x±0.40, and 1.62x±0.69 fold, respectively) (Fig. 3B). In addition, the range of gene expression varies tremendously among the different tumor samples, leaving open the possibility that variation in gene expression might determine the variation in clinical response to cisplatin.
Resistance to platinum-containing chemoradiation may be a limiting factor in the successful treatment of advanced stage HNSCC. It would be of significant clinical benefit to understand the underlying factors that cause sensitivity and resistance, since this will lead to personalized treatment and might even enhance the efficacy of chemoradiation by modifying relevant genes.

In this study, we identified determinants of response to cisplatin in an unbiased loss-of-function screen.

Figure 2. Functional relationship of the cisplatin sensitizing siRNA-targets.
(A) Comprehensive pathway analysis of all genes present in our genome-wide siRNA library and the associated Z-scores by IPA (Ingenuity) showed that many siRNAs targeting members of the FA/BRCA pathway significantly enhance the cisplatin response in VU-SCC-120. A darker grey is associated with a lower Z-score. (B) String analysis of siRNAs that were confirmed to increase cisplatin sensitivity in all three HNSCC cell lines (n=21) showed that the only cluster that could be identified, harbors DNA repair genes. (C) The siRNA SMARTpools targeting BRCA1 (left), BRCA2 (middle) and SHFM1 (right) were deconvoluted in VU-SCC-120 cells. Dark grey bars represent IC₀ treated cells (no cisplatin), lighter bars correspond to IC₂₀ treated cells. Bars represent cell viability as compared to the IC₀ value of each transfection and error bars show the standard deviation. * p<0.05 compared to siCONTROL IC₂₀ value of the same gene (Student’s t-test).

DISCUSSION

Resistance to platinum-containing chemoradiation may be a limiting factor in the successful treatment of advanced stage HNSCC. It would be of significant clinical benefit to understand the underlying factors that cause sensitivity and resistance, since this will lead to personalized treatment and might even enhance the efficacy of chemoradiation by modifying relevant genes. In this study, we identified determinants of response to cisplatin in an unbiased loss-of-function screen.
In a previous publication, we described that DNA-bound platinum is the most important determinant of cisplatin sensitivity in HNSCC\(^{(63)}\). Here, the results from our genome-wide siRNA screen confirms the importance of the presence and persistence of platinum-DNA adducts in the response to cisplatin treatment. Cisplatin is a DNA crosslinker and genes involved in DNA repair of crosslinks, such as \textit{BRCA1}, \textit{BRCA2} and \textit{ATR}, were identified in our screen. For a good cisplatin response it seems crucial that the cancer cell is unable to repair the induced DNA damage properly. Examination of the functionality of the FA/BRCA pathway prior to cisplatin-containing treatment is an option to upfront predict whether the therapy would succeed, e.g. by screening \textit{BRCA1} and \textit{BRCA2} for mutations. However, mutations in these particular genes are uncommon in HNSCC (COSMIC database; cancer.sanger.ac.uk/cancergenome/projects/cosmic). This highlights the necessity for the identification of biomarkers that disclose whether the FA/BRCA pathway and/or the involved genes are functional. Such biomarkers might be genes that are transcribed by transcriptional co-regulator BRCA1\(^{(64)}\), and possibly BRCA2\(^{(65,66)}\), or genes that are further downstream in the pathway. Such a biomarker approach might help to prevent overtreatment of patients that will not benefit from cisplatin-containing chemoradiation. Furthermore, the combination of chemoradiation and an agent that inhibits proper functioning of homologous recombination might enhance the cisplatin toxicity and eventually lead to higher

**Figure 3. Functional relationship of the cisplatin sensitizing siRNA-targets.**

(A) Cell cycle distribution of VU-SCC-120 cells transfected with siCONTROL, \textit{BRCA2} or \textit{SHFM1} targeting siRNA SMARTpools. Cultures were not treated with cisplatin (0 nM) or treated with 500 nM during 72 hours. Percentages of cells in designated cell cycle phases are given and are schematically represented in the lower panel (* p<0.02, ** p<0.001). Error bars represent the standard deviation. (B) Microarray gene expression data derived from 22 patient-derived pairs of tumor tissue and healthy mucosa were examined for \textit{BRCA1} (upper panel), \textit{BRCA2} (middle panel) and \textit{SHFM1} (lower panel) expression. Thick lines within the box plots represent the median values. The expression of all three genes is significantly higher in the tumor samples than in the normal mucosa (* raw p<0.001, moderated t-test implemented in Limma).
chemoradiation response rates. Unfortunately, with our siRNA approach such biomarkers could not be found.

We identified SHFM1 as an important player in the response to cisplatin treatment. SHFM1 is known to bind to BRCA2 \(^{(60,61)}\) and to stabilize this protein \(^{(59)}\), although this could not be confirmed in every study \(^{(62)}\). SHFM1 has been previously found to sensitize HeLa cells to cisplatin \(^{(35)}\). In that study the authors also focused on members of the FA/BRCA pathway, which indicates that this pathway is not only important in the cisplatin response in SCCs from the head and neck, but also in other squamous cell carcinomas. In addition, a recent study in ovarian cancer showed that SHFM1 has a role in cisplatin resistance \(^{(67)}\) and that SHFM1 levels are elevated in tumor cells as compared to the nontumorigenic neighboring cells. This observation serves as a platform to use SHFM1 expression as a biomarker for ovarian cancer. We found that the SHFM1 expression levels are overall elevated in HNSCC as compared to healthy mucosa (Fig. 3B), and that the level of expression varies tremendously, which implies that SHFM1 may be a very good biomarker for HNSCC.

We noticed that several genes that are known to influence the cisplatin response were not found in our genome-wide siRNA screen. For some of these genes it might have occurred that the siRNA induced gene knockdown was not sufficient, as we determined for \(ATM\) (Supp Fig. S2A). However, the knockdown of \(CTR1\) was significant (Supp Fig. S2B), but we could not find clues for the involvement of this gene in cisplatin response (Supp Fig. S2C), as we could not before \(^{(63)}\). This is surprising since many studies implicated an important role for \(CTR1\) in cisplatin uptake \(^{(68-70)}\). Since these studies were not performed in HNSCC, one might argue that other genes than \(CTR1\) are of greater importance in cisplatin response in HNSCC. Furthermore, it might have occurred that compensatory mechanisms have abolished the effect of loss of expression of a particular gene \(^{(71,72)}\). For example, the functions of \(AKT1\), \(AKT2\) and \(AKT3\) seem partly redundant, implying that knockdown of either one of these genes might not result in a phenotype since the other two genes are able to take over some of the functions of the silenced gene.

On the other hand, we identified novel genes that may influence cisplatin response in HNSCC cells. An example is \(KCNJ2\), a potassium inwardly-rectifying channel, that has a small but significant effect on cisplatin sensitivity and we were able confirm this in all three cell lines used (Supplementary Fig. S3A-B). Also the blocking of the channeling function of \(KCNJ2\), by barium chloride \(^{(73-75)}\), showed that a cell cultures is significantly sensitized to cisplatin (Supplementary Fig. S3C). However, the observed effect is small and not comparable to that of knockdown of for example \(BRCA2\).

Taken together, our data show that the application of an unbiased genome-wide functional genetic screen leads to the identification of unexpected and novel determinants of cisplatin response, which might be exploited as biomarkers for clinical prediction of chemoradiation response. Our data will redirect clinical research towards reliable response predictors for platinum-containing compounds applied in chemoradiation and systemic therapy.

ACKNOWLEDGEMENTS

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Supplementary Fig. S1. Efficiency of siRNA mediated knockdown.
The siRNA SMARTpools targeting BRCA1, BRCA2, and SHFM1 were deconvoluted in UM-SCC-22B (left panel) and UM-SCC-11B (right panel). Black bars represent IC₇₀ treated cells (no cisplatin), grey bars correspond to IC₂₀ treated cells. Bars represent cell viability as compared to the IC₀ value of each transfection and error bars show the standard deviation. * p<0.05 compared to siCONTROL IC₂₀ value of the same gene (Student’s t-test).

Supplementary Fig. S2. ATM and CTR1 were not identified as modulators of cisplatin response in VU-SCC-120.
(A) VU-SCC-120 cells were transfected with the ATM or BRCA1 targeting siRNA SMARTpool. RNA was isolated and used for quantitative real-time PCR. Specific ATM gene expression was determined and RNA isolated from siCONTROL transfected cells was used as a reference (black bar). Bars represent the mean of duplicate measurements and error bars indicate the standard deviation. The level of ATM specific knockdown showed to be insufficient. (B) VU-SCC-120 cells were transfected with the CTR1 targeting siRNA SMARTpool (dark grey bar) and the four separate siRNAs (light grey bars). RNA was isolated and used for quantitative real-time PCR. Specific gene expression was determined and RNA isolated from siCONTROL transfected cells was used as a reference (black bar). * p<0.05 compared to siCONTROL IC₂₀ value of the same gene (Student’s t-test). (C) The siRNA SMARTpool targeting CTR1 was deconvoluted in VU-SCC-120 (left), UM-SCC-22B (middle) and UM-SCC-11B (right) cells. Black bars represent IC₀ treated cells (no cisplatin), lighter bars correspond to IC₂₀ treated cells. Bars represent cell viability as compared to the IC₀ value of each transfection and error bars show the standard deviation. * p<0.05 compared to siCONTROL IC₂₀ value of the same gene (Student’s t-test).
Supplementary Table S1. Primer sequences for quantitative real-time PCR.

<table>
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<tr>
<th>Gene</th>
<th>Forward primer sequence (5’-3’)</th>
<th>Reverse primer sequence (5’-3’)</th>
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<td>ATM</td>
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<td>GGCTGATACATTTGGTTTTGC</td>
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<td>GUSB</td>
<td>GAAAATATGTGGTTGAGAGCTCATT</td>
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Supplementary Fig. S3. KCNJ2 is a mild modulator of cisplatin response in HNSCC cells.

(A) The KCNJ2 siRNA SMARTpool was deconvoluted in VU-SCC-120, UM-SCC-22B and UM-SCC-11B cells. Black bars represent IC_{50} treated cells (no cisplatin), grey bars correspond to IC_{20} treated cells. Bars represent cell viability as compared to the IC_{50} value of every culture and error bars show the standard deviation. *p<0.05 compared to siCONTROL IC_{50} value (Student’s t-test). (B) The KCNJ2 specific knockdown of the siRNAs was tested in VU-SCC-120 cells. (C) Cisplatin response curves were made for VU-SCC-120 (left panel) and UM-SCC-22B (right panel). Cells were incubated during 2 hours with 1 mM of barium chloride dissolved in phosphate- and pyruvate-free DMEM (white squares). Control experiments were incubated only with phosphate- and pyruvate-free DMEM (black squares). Subsequently, all experiments were exposed to 18 different concentrations of cisplatin during 2 hours. The shift in IC_{50} values was calculated and showed to be lower in barium incubated cultures; for VU-SCC-120 from 20.4 μM to 8.4 μM and for UM-SCC-22B from 6.6 μM to 3.4 μM. Error bars represent s.e.m of two independent triplicate assays.
### Supplementary Table S2. List of siRNA SMARTpools that significantly sensitized VU-SCC-120 cells to cisplatin as identified by at least one of two separate statistical analyses.

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Supplementary Table S3. Number of siRNAs that were confirmed to cause the cisplatin sensitizing phenotype after deconvolution of the siRNA pool.

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<td>41 (42.3)</td>
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<td>49 (50.5)</td>
<td>18 (18.6)</td>
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</table>

Numbers between brackets represent percentages.

REFERENCES


64. Mullany PB, Quinn JEHarkin DP. The role of BRCA1 in transcriptional regulation and cell cycle control. Oncogene 2006;25:5854-5863.


