Increased interleukin-6 expression is associated with poor prognosis and acquired cisplatin resistance in head and neck squamous cell carcinoma

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Abstract. Increased expression of interleukin 6 (IL-6) is associated with poor prognosis and chemoresistance in many different carcinomas, but its role in head and neck squamous cell carcinoma (HNSCC) is still unsettled. Analyzing tumorous mRNA expression data from 399 HNSCC patients revealed that high IL-6 expression predicted poor prognosis. Similar tendency was observed in platinum treated patients, suggesting an IL-6 associated cisplatin resistance. IL-6 increase was also found in two in-house acquired cisplatin-resistant HNSCC cell lines (both basaloid and conventional squamous cell carcinoma) by using microarray analysis. However, although the in-house acquired cisplatin-resistant cell lines had higher basal and markedly increased cisplatin-induced IL-6 expression, IL-6 did not mediate the cisplatin resistance as neither exogenous IL-6 nor IL-6R/gp130 inhibitors affected cisplatin sensitivity. Moreover, the IL-6/STAT3 pathway was impaired in the resistant cell lines, partly due to decreased IL-6R expression. Thus, high IL-6 expression correlated to poor prognosis and acquired cisplatin resistance, but it did not mediate cisplatin resistance in the HNSCC cell lines.

Introduction

Approximately 644,000 head and neck squamous cell carcinoma (HNSCC) cases are diagnosed worldwide each year (1) and although surgery, radiotherapy and chemotherapy improve disease control, its 5-year survival rate remains ~60% (2), partly because acquired chemoresistance limits its efficiency.

Interleukin (IL)-6 is a multifunctional cytokine produced by various cell types involved in a wide range of biological activities, including cellular growth and apoptosis (3,4). In general, IL-6 binds to the non-signaling alpha-receptor (IL-6R/CD126) that dimerizes with the membrane bound signaling transducer receptor, gp130 and activates receptor-associated kinases. The intracellular signaling pathways induce phosphorylation of the transcription factor: signal transducer and activator of transcription 3 (STAT3). The cytoplasmic phosphorylated STAT3 subsequently translocates to the nucleus where it regulates genes involved in apoptosis (e.g., Bcl-x$_L$, XIAP and Fas). In addition, IL-6 initiates the PI3K/AKT and RAS/RAF/MEK/ERK pathways, which regulate cell proliferation (5).

Increased serum IL-6 levels predict poor prognosis in several carcinoma types including colorectal, ovarian, pancreatic, mammary and gastric carcinomas, which has been related to IL-6-induced tumor cell proliferation, apoptosis inhibition and tumor angiogenesis (6). However, to what extent IL-6 influences HNSCC prognosis remains controversial due to conflicting results obtained by different methods. Whereas positive IL-6 mRNA *in situ* hybridization signals are associated with favorable prognosis (7), tumorous IL-6 immunoreactivity and IL-6 serum levels have been associated with poor prognosis (8-11).

IL-6 may induce cisplatin resistance in oral carcinomas similar to that reported in ovarian, lung and prostate carcinoma cell lines, where IL-6 increases expression of anti-apoptotic factors such as Bcl-2, Bcl-xL and cIAP-2 and/or induces cell proliferation (12-14). Moreover, IL-6 gene knock-down reverses cisplatin resistance in esophageal carcinoma cell lines (15) and increased IL-6 production is associated with resistance to other chemotherapy drugs, such as fluorouracil, doxorubicin and VP-16 (6,10). Finally, a single *in vitro* cisplatin challenge induces high IL-6 mRNA levels in surviving HNSCC cells and increases their tumor potential in a xenograft murine model (16), suggesting that IL-6 participates in rescuing cells from cisplatin-induced apoptosis.

The aim of the current study was to evaluate whether increased cancerous IL-6 mRNA expression had a prognostic value in HNSCC, and whether IL-6 influenced cisplatin resistance. We used high-throughput RNA-sequencing and clinical data of 399 HNSCC patients in the cancer genomic atlas database (TCGA, http://cancergenome.nih.gov/) and

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investigated how IL-6 gene expression was related to patient prognosis in general and in patient subgroups. In order to examine IL-6 induced cisplatin resistance, we furthermore tested five HNSCC cell lines, including two in-house acquired cisplatin-resistant cell lines of both basaloid and conventional HNSCC types, for cisplatin sensitivity and IL-6 expression.

Materials and methods

Clinical data and RNA expression analysis. Clinical data and mRNA expression profiles from 498 HNSCC patients were collected from the TCGA database: (https://tcga-data.nci.nih.gov/tcgafiles/ftp_auth/distro_ftpusers/anonymous/tumor/hnsc/bcr/biotab/clin/). All patients, diagnosed and treated during 1997-2014, were followed up until September 30, 2014. For detailed tumor sample acquisition, see reference (17). Briefly, biospecimens were collected from diagnosed patients with HNSCC at the time of surgical resection. The patients had received no prior treatment for their disease including chemotherapy or radiotherapy. Cases were staged according to the American Joint Committee on Cancer (AJCC), Seventh Edition. mRNA expression profiles were estimated by normalizing raw counts of mapped RNA-sequences reads to human reference genes, and mRNA levels measured as fragments per kilobase per million mapped reads (FPKM). Patients without follow-up data or who died within two months were excluded, and finally 399 patients, 284 (71%) men and 115 (29%) women, median 61 years (range 19-90 years) were included.

Cell lines and cell culture. Three human HNSCC lines were used in the study. PE/CA-PJ49 clone E10 (male, 55 years) were established from tongue tissue; PE/CA-PJ34 clone C12 (male, 60 years) and PE/CA-PJ41 clone D2 (female, 68 years) were derived from the oral cavity and the oral squamous epithelium, respectively. The cell lines (a kind gift from Dr A. Berndt and Dr H. Kosmehl, Friedrich-Schiller University, Germany) were cultured under standard condition as previously described (18).

Establishing the cisplatin-resistant C12 (C12cis) and D2 (D2cis) HNSCC cell lines. Two primary cisplatin sensitive HNSCC cell lines, the basaloid squamous cell carcinoma (BSCC) C12 and the conventional squamous cell carcinoma (CSCC) D2 cell lines, were cultured to acquire cisplatin resistance. Cells were initially treated with their 50% inhibitory concentration (IC$_{50}$) (3 μM) of cisplatin (Sigma-Aldrich, St. Louis, MO, USA) at 80% confluence. The conditioned medium was discarded and fresh medium was added after 24-h incubation. The cells were then treated with gradually increasing concentrations of cisplatin ranging from 3 to 10 μM at weekly intervals for eight months. The parental C12 and D2 cells were cultured in parallel using cisplatin-free medium.

Cell viability assay. Cells were seeded (4x10^3 cells/well) in 96-well microtiter plates (Nunc, Wiesbaden-Biebrich, Germany) in 100 μl IMDM with 10% FBS in quintuplicate. After 24 h, culture medium was changed to IMDM with 10% FBS and different concentrations of drugs or inhibitors. For drug IC$_{50}$ detection, cells were treated with different dosages of cisplatin, 5-FU or docetaxel directly. Cells were also cultured in the presence of human recombinant IL-6 or human IL-6R/gp130 neutralizing antibody (all from R&D Systems, Minneapolis, MN, USA) for 24 h, followed by 5 μM cisplatin treatment to examine changes in drug resistance. Cells were further grown for 72 h, before incubated in 50 μl XTT labeling mixture (Roche Molecular Biochemicals, Mannheim, Germany) for 4 h, and then scanned at 450 nm. IC$_{50}$ was calculated using GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA, USA).

RNA isolation and microarray analysis. C12, C12cis, D2 and D2cis cells were lysed and total RNA was extracted using RNeasy kit (Qiagen, USA), and concentrations were measured using the NanoDrop 2000c spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The integrity of samples was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Microarray was performed by the Department of Tumor Biology, Institute for Cancer Research, Norwegian Radium Hospital. Briefly, 500 ng of total RNA for each individual sample was used with the Illumina TotalPrep Amplification kit (Ambion) to make biotin-labelled, amplified cRNA. Thereafter, 750 ng cRNA was hybridized to HumanHT-12 v4 Expression BeadChip (Illumina) enabling profiling of >48,000 transcripts. The Illumina arrays were scanned with the BeadArray reader, and data extraction and initial quality control were performed in GenomeStudio version 2011.1 using Gene Expression module v.1.9.0 (Illumina). Raw data were log2 transformed and analyzed using lumi package in R (version 3.2.2).

Quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR). After RNA isolation, complementary DNA (cDNA) was synthesized by RT-RTCK-05 kit (Eurogentec, Berlin, Germany) and stored at -20˚C. A standard real-time PCR reaction with SYBR Green Real Master Mix (Eppendorf, Hamburg, Germany) was performed in duplicates using Mx3005p (Agilent Technologies) under the following conditions: 95˚C for 2 min followed by 40 cycles of 95˚C for 20 sec, 60˚C for 1 min and 68˚C for 30 sec. The primers used were: IL-6 forward, 5'-GCA-GAA-AAA-GGC-AAA-GAA-TC-3' and reverse, 5'-CTA-CAT-TTG-CCG-AAG-AGC-3'; IL-6 X1 isoform forward, 5'-TCC-TCA-TTC-CCT-CAA-CTT-GG-3' and reverse, 5'-GCA-GAA-GAG-AGC-CAA-CCA-AC-3'; and IL-6R forward, 5'-CTG-GAA-AGC-ATT-CAT-GCT-ACC-3' and reverse, 5'-GAC-TGT-TCT-GAA-ACT-TCC-TC-3' (all designed by Sigma-Aldrich); TATA box binding protein (TBP): forward, 5'-CGT-GGC-TCT-CTT-ATC-CTC-ATG-A-3' and reverse, 5'-GCC-CGA-AAC-GCC-GAA-TAT-A-3' (designed by Eurogentec). Dissociation curves ensured product uniformity. Expression data were normalized to the housekeeping gene TBP. The relative expression levels of the genes of interest were calculated using the 2$^{-\Delta\Delta Ct}$ method.

Western blotting. Cells were pre-treated with 10 ng/ml human recombinant IL-6, 40 μg/ml human IL-6R neutralizing antibody or 60 μg/ml human gp130 neutralizing antibody for 24 h, following by 5 μM cisplatin treatment for 6 h. For the stimulation assay, cells were treated with 1 or 10 ng/ml IL-6 for 30 min. Cells were harvested and lysed in CelLytic M Cell Lysis reagent (Sigma-Aldrich) with protease and phosphatase inhibitor cocktails (Pierce Biotechnology, Rockford, IL, USA). Protein
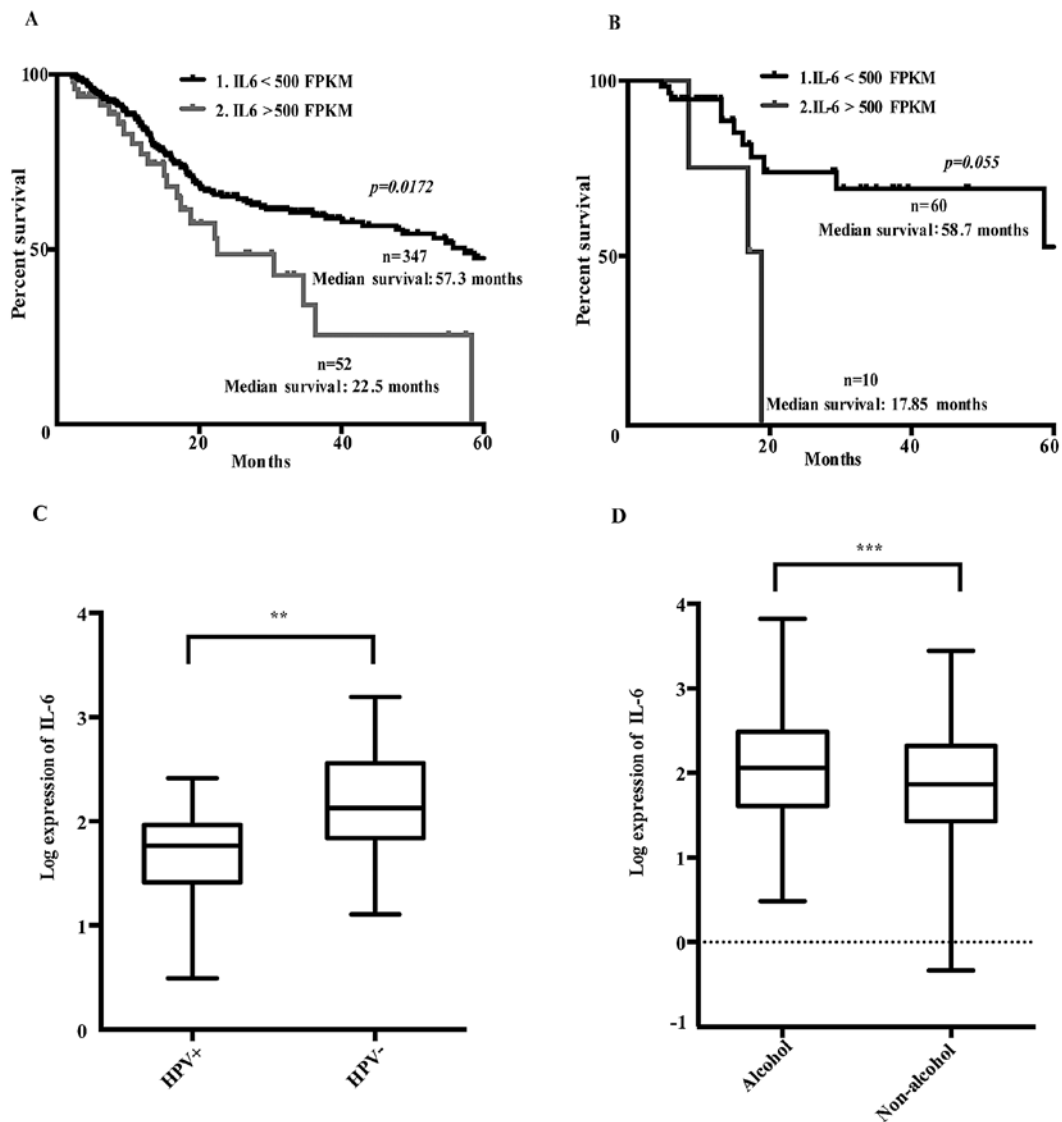
Figure 1. IL-6 mRNA expression in the HNSCC patients in the TCGA database. (A and B) High IL-6 mRNA levels (>500 FPKM) predicted poor prognosis in all patients (A) and in cisplatin/carboplatin treated patients (B, Kaplan-Meier curve). The patients were censored at loss to follow-up, defined as the last date of contact or at 5 years after diagnosis. IL-6 mRNA levels measured as fragments per kilobase per million mapped reads (FPKM). Censoring samples are marked as '⊥' (Log rank test). (C) HPV positive (+) patients had lower IL-6 mRNA levels than HPV negative (-) patients. (D) Patients with alcohol history had increased IL-6 mRNA levels. Boxplots are log2 transformed IL-6 mRNA expression levels (C and D). Horizontal lines indicate median; upper and lower boxes are the 75 and 25% quartiles, respectively; whiskers indicate the min and max values ([***]p<0.001; [**]p<0.01; Wilcoxon rank-sum test).

concentrations were determined (Bio-Rad, Munich, Germany), and 50 μg proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted onto PVDF membranes (Bio-Rad). After blocked with 5% BSA for 1 h, the membranes were incubated with primary antibody to human anti-phospho-STAT3(Tyr705) (rabbit polyclonal, 1:1,000; Cell Signaling Technology, Beverly, MA, USA), anti-STAT3 (mouse monoclonal, 1:5,000; R&D Systems) and anti-GAPDH (mouse monoclonal, 1:1,000; Abcam, Cambridge, UK) overnight at 4°C. The blots were then washed three times and incubated with alkaline phosphatase-conjugated anti-rabbit IgG (1:10,000; Sigma-Aldrich) or mouse IgG (1:10,000; Dako, Glostrup, Denmark) antibodies at room temperature for 1 h, then washed three times and visualized with ECF substrate in a scanner (Storm) (both from GE Healthcare, Uppsala, Sweden).

Enzyme-linked immunosorbent assay (ELISA). Cells were seeded in duplicates in 96-well-plate at a density of $4x10^3$ cells/well and cultured in 200 μl medium with 10% FBS. The supernatant was harvested and stored frozen (-70°C) prior to use. The IL-6 concentration was determined in quadruplicates by Human IL-6 ELISA (R&D Systems).

Statistics. Statistical analysis was performed using GraphPad Prism 6.0. The survival distributions were compared with the log-rank test (Kaplan-Meier method). Normally distributed data were shown as mean ± SD, and group differences were analyzed using paired Student's t-test; data that were not normally distributed were shown as median ± SD, and group differences were analyzed using Wilcoxon rank-sum test. For all *in vitro* assays, data are shown of at least three experiments. p<0.05 were considered as significant.
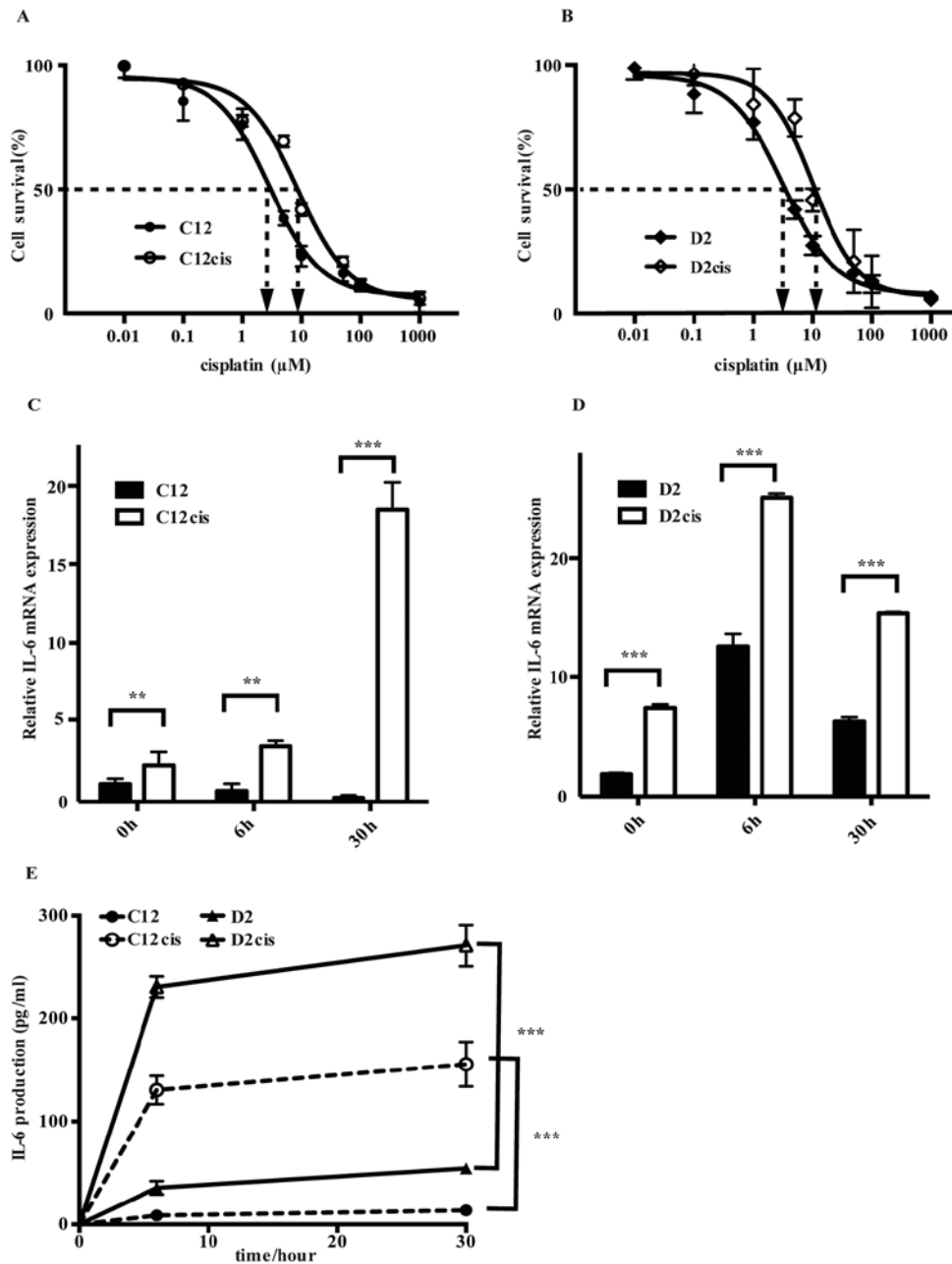
Figure 2. IL-6 mRNA expression and protein production in HNSCC cell lines. (A and B) Cisplatin resistance in C12cis (A) and D2cis (B) cells measured by the XTT cell viability assay. The cisplatin IC_{50} concentrations increased 3.06 and 3.20 times (the factor of resistance) in the acquired cisplatin resistant cell lines (right arrows) compared to the parental cell lines (left arrows). All data were normalized to untreated controls ($p<0.05$; n=3, paired Student's t-test). (C and D) The cisplatin resistant C12cis (C) and D2cis (D) cell lines had increased IL-6 mRNA levels compare to parental cells. IL-6 expression levels were quantified using qRT-PCR before, 6 and 30 h after receiving 3 μM cisplatin. Gene expression was normalized to the housekeeping gene TBP ([**]$p<0.01$; [***]$p<0.001$; n=4, paired Student's t-test). (E) The cisplatin resistant C12cis (C) and D2cis (D) cell lines had increased basal IL-6 protein production compared to parental cells (measured by ELISA). After allowing cells to attach overnight, new medium was added and supernatant was collected at 0, 6 and 30 h ([***]$p<0.001$; n=3, paired Student's t-test).

Results

High IL-6 expression predicts poor prognosis. Dividing patients in high (>500 FPKM) and low (<500 FPKM) IL-6 expression levels revealed that the high IL-6 expressing group had a significantly reduced 5-year survival rate (Fig. 1A).

Further investigation of 70 cis-/carboplatin treated patients showed that those with high IL-6 mRNA expression levels tended to have a lower 5-year survival rate, suggesting reduced response to platinum-based treatment (Fig. 1B). Thus, an increased IL-6 gene expression in the HNSCC tumors was related to poor prognosis and presumably also to cisplatin resistance, similar to that previously reported in ovarian carcinomas (6).

Patients with human papillomavirus infection (HPV[+]) had lower IL-6 expression levels than those without HPV (Fig. 1C). Moreover, IL-6 mRNA expression levels were correlated to alcohol consumption history (Fig. 1D), but not to any other clinical parameters or high risk factors such as tumor sites, pathological or histological grading or smoking history.

Table I. Characterization of HNSCC cell lines.[a]

	Diameter (μM)[c]	Doubling time time (h)[d]	IC$_{50}$ for cisplatin (μM)	IC$_{50}$ for 5-FU (μM)	IC$_{50}$ for docetaxel (nM)
C12	13.70±2.78	35±2,41	2.8±0,21	1.2±0,31	1.4±0,29
C12cis	13.59±2.90	31±1,57[b]	8.7±0,43[b]	2.5±0,54[b]	2.6±0,23[b]
D2	15.08±2.23	32±1,72	3.2±0,76	5.2±0,81	0.9±0,14
D2cis	14.45±2.26	41±2,21[b]	10.3±0,84[b]	7.9±0,47[b]	2.2±0,33[b]

[a]Data are shown as the mean ± SD, n=3. [b]Represents statistic significance compared to parent cell line. [c]Cell diameter was examined by Multisizer 4 Coulter Counter (Fullerton, CA, USA). [d]Cells were plated into 96-well plates (4x10^3 cells/well) and cultured at 37˚C. Absorbance was examined by XTT array on day 1 and 4. DT was calculated using the following equation: DT (h) = (log2 x t)/(logA$_t$ - logA$_0$), where t = time in culture (h), A$_t$ = final absorbance, A$_0$ = original absorbance.

Characterization of the cisplatin-resistant C12 (C12cis) and D2 (D2cis) HNSCC cell lines. To examine whether IL-6 signaling is involved in cisplatin resistance in HNSCC, we established cisplatin-resistant sublines from C12 and D2 cells, as described in Materials and Methods. The IC$_{50}$ values for cisplatin treated cells (i.e., C12cis and D2cis) were more than three times higher than in the parental cells (Fig. 2A and B), and it was unaltered after two months of cisplatin-free culturing, revealing stable phenotypic changes.

Interestingly, cisplatin treatment induced cross-resistance for two other common drugs used in HNSCC treatment (19), fluorouracil (5-FU) and docetaxel by increasing their IC$_{50}$ values by 50-100% (Table I).

Differentially expressed genes in C12cis and D2cis cell lines. We compared the mRNA expression profiles of the cisplatin-resistant cell lines (C12cis and D2cis) and parental cell lines (C12 and D2) by using microarray analysis, and found that 137 genes were differentially expressed (increased or decreased 100%) in C12cis cell lines and 141 genes in D2cis cell lines (Fig. 3). Among them, only 3 genes, OXTR (oxytocin receptor), CPVL (probable a serine carboxypeptidase) and IL-6 were found upregulated in both cell lines (Table II). Since IL-6 is associated with poor prognosis and cisplatin resistance in several different carcinomas, we hypothesized that IL-6 mediated cisplatin resistance in current cell lines.

Acquired cisplatin-resistant cells express more IL-6. Whereas cisplatin exposure decreased IL-6 mRNA expression in the parental cisplatin sensitive C12 cell line, it increased markedly, reaching 71 times higher expression in the resistant C12cis cell line than former 30 h after cisplatin treatment (Fig. 2C). Similarly, although IL-6 mRNA expression increased after cisplatin treatment in both the sensitive D2- and the resistant D2cis cell lines, the basal and cisplatin induced IL-6 mRNA expression was two times higher in the latter (Fig. 2D). Importantly, the IL-6 mRNA increase was accompanied with high IL-6 production in both the acquired resistant cell lines (Fig. 2E).

Cisplatin resistance is not affected by IL-6 receptor inhibitors or exogenous IL-6. Since IL-6 was highly upregulated in the cisplatin-resistant cell lines, we examined whether cisplatin resistance could be blocked by IL-6 receptor inhibitors. Interestingly, neither IL-6R nor gp130 neutralizing antibody altered cisplatin resistance in the acquired cisplatin-resistant cell lines or the intrinsic cisplatin-resistant cell line E10 (Fig. 4A). Moreover, STAT3 was constantly phosphorylated in these cell lines, with no decrease after anti-IL-6R/gp130 inhibition (Fig. 4C). Despite cisplatin induced IL-6 production, p-STAT3$_{Tyr705}$ was not further increased in the resistant cell lines after cisplatin treatment, suggesting IL-6 independent STAT3 activation. Further experiment revealed that the IL-6/STAT3 pathway was diminished in the resistant cell lines (Fig. 4E), as exogenous IL-6 induced less STAT3$_{Tyr705}$ phosphorylation in the cisplatin-resistant than in the cisplatin- sensitive parental cell lines.

Furthermore, exogenous IL-6 did not increase cisplatin resistance in the sensitive cell lines regardless of different dosages (10-100 ng/ml; Fig. 4B), despite *de novo* STAT3$_{Tyr705}$ phosphorylation which could be blocked by IL-6R inhibitor (Fig. 4D).

Expression of IL-6 splicing variants and its receptor. Since IL-6/STAT3 pathway was impaired in the acquired cisplatin-resistant cell lines, we examined the expression of IL-6 isoforms with putative antagonistic effects, and IL-6 receptor expression in the cell lines.

The expression levels of IL-6 splicing variants (based on NCBI RefSeq gene annotation - release 69) were evaluated using qRT-PCR. Only one alternative transcript variant (X1, accession: XM_005249745) was expressed and at similar, low levels in both the parental and the cisplatin-resistant cell lines (expression ratio of IL-6 X1 isoform/wild type = 1/20), which was also confirmed by analyzing the IL-6 isoform expression levels in patient samples from the TCGA database. Thus, no known competitive IL-6 isoforms were expressed in our HNSCC cell lines.

However, unlike IL-6, the mRNA and protein levels of its receptor were downregulated in both C12cis and D2cis cells in comparison with the parental cells (Fig. 4F and G).

Discussion

Analysis of 399 HNSCC patients revealed that patients with high tumorous IL-6 mRNA expression (>500 FPKM) had a
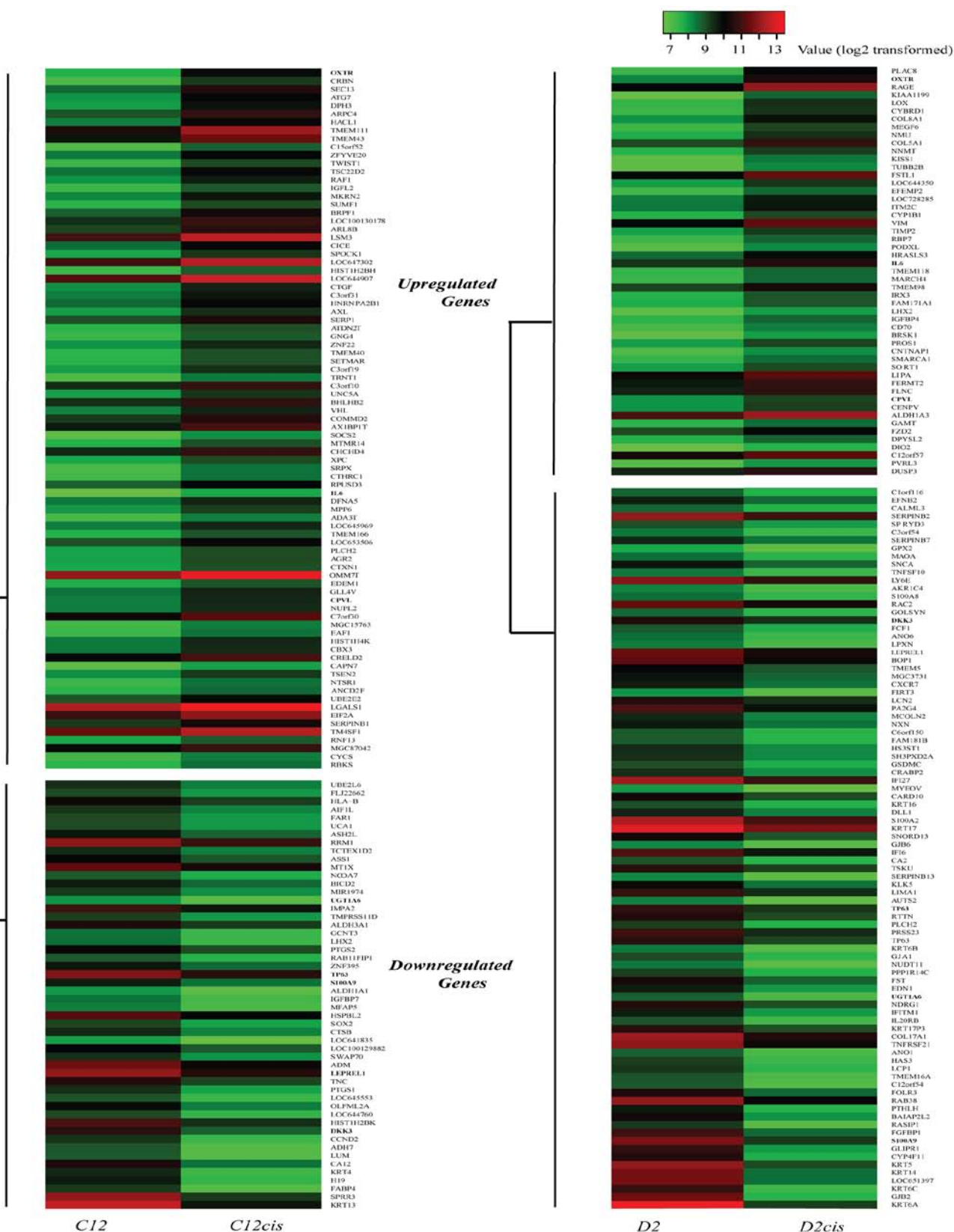
Figure 3. Gene expression heatmap: cisplatin resistance-related genes in cisplatin-sensitive and acquired resistant cell lines. In total, 137 genes were more than 100% differentially expressed in C12cis cell lines compared to C12 cell line (85 upregulated and 52 downregulated, left figure) and 141 genes were more than 100% differentially expressed in D2cis cell lines compared to D2 cell line (51 upregulated and 90 downregulated, right figure). Among them, only 8 genes were found in both cell lines (names in bold). The columns correspond to the gene symbol names; the rows correspond to cell lines. Gene expression levels were depicted as color variation from red (high expression) to green (low expression) according to color bar scale.

significant reduced 5-year survival. However, this association became less statistically evident when median IL-6 expres-

sion level (~100 FPKM) was used as discriminator. Similar results were obtained by separate *de novo* analysis of two

Table II. Expression levels of genes exhibiting >2-fold expression changes in both C12cis and D2cis cell lines.

Gene symbol	Full name	Fold change in C12cis/C12[a]	Fold change in D2cis/D2[a]
Upregulated			
OXTR	Oxytocin receptor	2.19	2.13
IL6	Interleukin 6	1.20	1.24
CPVL	Probable serine carboxypeptidase	1.12	1.07
Downregulated			
DKK3	Dickkopf-related protein 3	-1.84	-1.11
LEPREL1	Prolyl 3-hydroxylase 2	-1.40	-1.16
S100A9	S100 calcium-binding protein A9	-1.16	-2.46
TP63	Transformation-related protein 63	-1.16	-1.47
UGT1A6	UDP-glucuronosyltransferase 1-6	-1.09	-1.61

[a]Data were log2 transformed.

other databases using SurvExpress (20), which showed that IL-6 expression tended to be associated with poor prognosis (bordline non-significant) as median expression was automatically used as discriminator. Thus, the association between IL-6 expression and poor prognosis may be more intriguing in HNSCC than reported for other carcinoma types (6), which may be reflected in previous studies (7,9,11). Besides, although Chen *et al* (8) observed the association between increased IL-6 expression and poor prognosis in male patients only, a case-control study design revealed no gender specific differences in the current HNSCC patients (not shown), and the gender differences may have been due to less advanced disease in Chen *et al* female patients. An IL-6 associated poor prognosis would explain why serum and salivary IL-6 levels were increased in more aggressive HNSCC grades (11) with higher recurrence rates (9,21).

Although the mechanism for why high IL-6 expression is associated with poor prognosis is not fully understood, IL-6 is known to inhibit cellular apoptosis and induce epithelial-mesenchymal transition (EMT) (22), both of which increase drug resistance, cellular invasiveness and metastatic potential (6,23). In particular, the IL-6 induced anti-apoptotic effects may prevent cisplatin treated, DNA-damaged cells to undergo apoptosis, which actually may facilitate the development of mutation-induced drug resistance (12,13,15). It is, therefore, intriguing that HNSCC cells which survived a single cisplatin dosage *in vitro*, had increased IL-6 expression and increased tumor forming capacity in a xenograft mouse model (16), suggesting that cisplatin induced IL-6 expression is an important factor to reduce cisplatin sensitivity. Survival analysis of the 70 patients who had been treated with cisplatin or carboplatin, further suggested an IL-6 associated reduction of cisplatin cytotoxicity, as IL-6 was associated with poor prognosis in these patients as well. However, although IL-6 may reduce cisplatin cytotoxicity and increase cell proliferation in prostate (13,24,25) and ovarian carcinomas (26), neither anti-IL-6 receptor antibodies nor exogenous IL-6 affected cell proliferation or cisplatin toxicity. In fact, the cisplatin-resistant cell line D2cis, despite having higher IL-6 expression, grew slower than the parental, cisplatin sensitive cell line D2 (Table I).

IL-6 may generally suppress cisplatin-induced apoptosis through STAT3 induced upregulation of anti-apoptotic factors (27,28). Both IL-6R and p-STAT3 are highly expressed in OSCC patients with poor response to chemoradiotherapy, suggesting that activation of IL-6/STAT3 signaling may be involved in modulation of chemosensitivity to anticancer drugs (10). In our study, p-STAT3 was observed in unstimulated cisplatin-resistant cell lines (C12cis, D2cis and E10). However, this was independent of IL-6/IL6R/gp130 signaling despite increased IL-6 production in the resistant cell lines, as p-STAT3 was not reduced after IL-6 receptor inhibition. Besides, higher IL-6 dosages, which induced p-STAT3 phosphorylation in sensitive cell lines, did not induce cisplatin resistance in the parental cell lines nor increase expression of any of the apoptosis inhibitors, illustrating that cisplatin resistance was not mediated through IL-6/STAT3 activation. Further investigation in six additional HNSCC cell lines revealed, moreover, that neither IL-6 gene expression nor protein production correlated to cisplatin resistance (p>0.05, data not shown). Although this is in contrast to cisplatin resistance mechanisms in other carcinomas, similar results have been noted in myeloma and lymphoma cell lines: although chemotherapy resistance correlated to IL-6 secretion, IL-6 blocking antibodies did not reverse the resistance (29).

A few alternative spliced IL-6 variants exist in human, some of which have antagonistic activities and a tissue-specific expression pattern, similar to IL-4 (30). For example, lung tissue, renal tissue, renal carcinomas and fibroblasts produce three IL-6 inhibitory variants lacking either exon 4 (IL-6δ4), exon 2 (IL-6δ2) or both exons (IL-6δ2δ4) (31,32). Human fetal tissues express these variants in a tissue-specific manner (33). However, there was no IL-6δ2 or IL-6δ4 expression either in patient samples or in our cell lines. Only one variant, IL-6X1 was detected in HNSCC patients and cell lines. Although its function is still unknown, the mRNA level was 5% of the wild-type IL-6, reducing the possibility that it had any significant inhibitory function. Thus, the impaired IL-6/STAT3
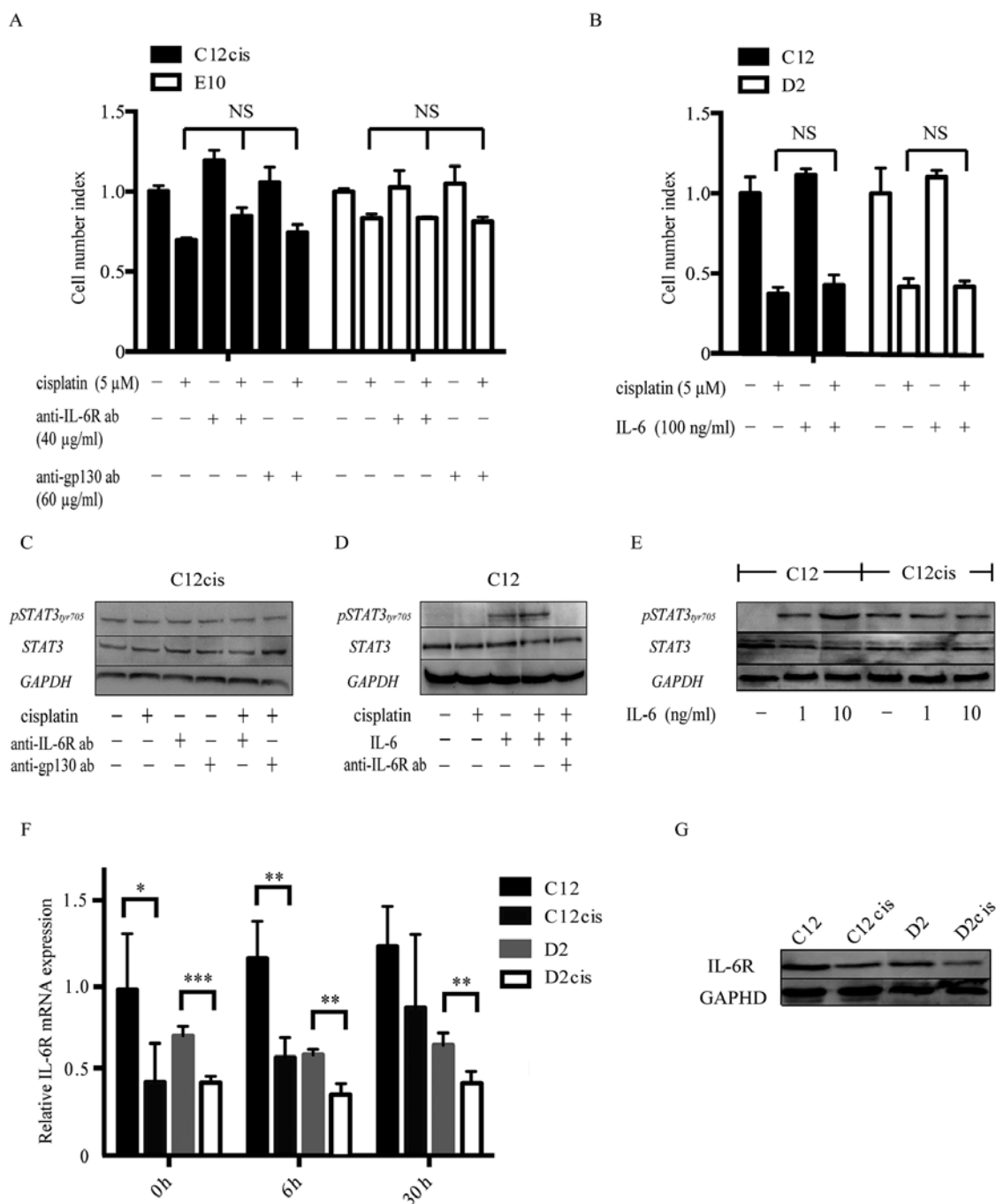
Figure 4. Cisplatin resistance in HNSCC cell lines was independent of IL-6/gp130/STAT3 signaling pathway. (A and B) Neither exogenous IL-6 nor IL-6 pathway inhibitors affected cisplatin resistance in HNSCC cell lines. Cells were grown in the presence (+) or absence (-) of human IL-6 receptor neutralizing antibody (40 μg/ml; A), human gp130 neutralizing antibody (60 μg/ml; A) or human recombinant IL-6 (100 ng/ml; B), for 24 h and then treated with (+) or without (-) 5 μM cisplatin. Cells without any treatment were set as control. NS, not significant ($p > 0.05$; n=5, paired Student's t-test). (C and D) Effect of cisplatin (C and D), exogenous IL-6 (D), anti-IL-6 antibody (C and D) and anti-gp130 antibody (C) on the acquired cisplatin resistant C12cis (C) cell line and sensitive C12 cell line (D). Whereas IL-6 induced p-STAT3$_{Tyr705}$ in C12 cell line through IL-6R, unstimulated C12cis cells had p-STAT3$_{Tyr705}$ that was unchanged after IL-6 receptor inhibition. (E) IL-6/STAT3 pathway was reduced in the acquired cisplatin-resistant cell lines. (F and G) Acquired cisplatin-resistant cell lines had decreased IL-6R mRNA and protein levels. The mRNA expression levels were normalized to housekeeping gene TBP (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$; n=4, paired Student's t-test).

pathway may rather have been due to decreased IL-6R expression in the resistant cell lines (Fig. 4F and G).

Dysregulated microRNA (34) expression is common in various malignancies where miRNAs regulate cell proliferation, apoptosis and invasion by controlling downstream target genes (35). Some inhibitory miRNAs (i.e., miR-200b, miR-200c, miR-203 and miR-205), were negatively associated

with IL-6 mRNA expression in the current HNSCC patients ($p < 0.001$, not shown), suggesting that IL-6 overexpression may result from demethylation of the IL-6 promoter triggered by heat shock factor protein 1 (HSF1) due to reduction of miR-200c (36). Similar phenomena were observed in tongue and lung carcinoma where reduced miR-200b and miR-200c expression inhibited cell proliferation, apoptosis and cisplatin

cytotoxicity (37,38). The reduced miR-205 expression, which may induce increased IL-6 expression, was associated with poor prognosis in head and neck cancer (39). miRNA regulates several downstreams gene targets, e.g., miR-203 may affect expression of more than 100 genes involved in EMT and other cellular processes (40). The IL-6 associated poor prognosis may therefore either be an epiphenomenon due to miRNA induced co-regulation of genes more directly related to cancer survival and/or cisplatin resistance, or involved in paracrine signaling, inducing tumor surrounding fibroblasts to become cancer-associated fibroblasts (CAFs) which support carcinomas growth, survival, and metastatic potential (41).

Cisplatin cytotoxicity is mediated by several transcription factors and downstream pathways, such as RAS/RAF/MEK/ERK and PI3K/AKT and may involve many different signal transduction pathways and gene regulatory networks (42). In comparison with previous studies in other cancer types, IL-6 signaling pathway appeared not to be critical for cisplatin resistance in the current HNSCC cell lines. Additionally, the acquired cisplatin-resistant cell lines tended to gain cross-resistance to two other chemotherapy drugs, 5-FU and docetaxel, suggesting induction of anti-apoptotic proteins (43). Although the conventional cisplatin-resistant SCC cell line D2cis had an increased expression of the cellular Inhibitor of Apoptosis-1 and -2 (c-IAP1, c-IAP2), there was no increased expression of any apoptosis inhibitors in the cisplatin-resistant BSCC cell line C12cis (microarray data, confirmed by qRT-PCR, not shown). This is in contrast to previous reports on HNSCC, which in particular focused on the importance of increased XIAP expression for cisplatin resistance (44). Thus, further investigation is needed to reveal which major mechanisms BSCC and conventional SCC may use to overcome cisplatin cytotoxicity.

Patients with HPV infection had lower IL-6 mRNA levels (this study), and the HPV-positive HNCSS patients had noticeably better prognosis as shown by Cancer Genome Atlas Network (17). Such IL-6 regulation is probably mediated by E6 and E7 proteins, and may contribute to maintenance of the viral genome and to escape the immune activity in HPV-related cancers (45).

In conclusion, high tumor IL-6 transcription levels were associated with poor prognosis and acquired cisplatin resistance in HNSCC, but IL-6 did not itself mediate cisplatin resistance. Thus, inhibiting IL-6 signaling may not reduce cisplatin resistance in HNSCC.

Acknowledgements

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