Reduced Cisplatin Sensitivity of Head and Neck Squamous Cell Carcinoma Cell Lines Correlates with Mutations Affecting the COOH-Terminal Nuclear Localization Signal of p53

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Abstract Purpose: Head and neck squamous cell carcinomas (HNSCC) are the most frequent malignancies of the upper aerodigestive tract. Cisplatin resistance is a major problem in the treatment of a large number of HNSCC cancer patients. In this study, nine randomly selected HNSCC cell lines were investigated regarding expression, presence of mutations, nucleocytoplasmic distribution of p53, and sensitivity to cisplatin.

Experimental Design: Protein expression was evaluated by Western blot analysis. The whole open reading frame of p53 was determined by reverse transcription-PCR sequencing. Nucleocytoplasmic distribution was evaluated by confocal laser scanning microscopy. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide viability assay was used to test for cisplatin sensitivity.

Results: p53 mutations were found in all nine investigated HNSCC cell lines. Nuclear p53 signal was detected in six cell lines, whereas three cell lines exhibited total loss of nuclear p53 signal. Nuclear signal depended on the presence or absence of the COOH-terminal nuclear localization signal in p53. Cisplatin sensitivity was highly reduced in the group with loss of nuclear p53 signal compared with those with detectable nuclear signal. Transfection of wild-type and mutant p53 into a rat embryonic cell system showed highly reduced activity of the nuclear localization signal mutant p53 protein.

Conclusion: Taken together, these data suggest that “loss of nuclear p53 signal” correlates with cisplatin resistance in HNSCC. If these results can be validated on a larger number of tumor samples, including fresh tumor tissues, it potentially could help in sparing a subgroup of HNSCC patients the side effects associated with unnecessary chemotherapy by identifying cisplatin nonresponders before chemotherapy induction.

Head and neck squamous cell carcinomas (HNSCC) account for the vast majority (>90%) of malignancies found in the upper aerodigestive tract. They are characterized by their early predominantly lymphatic metastatic spread (1, 2). For over 30 years now, there was no significant improvement of the patient survival time regardless of new developments in surgical techniques as well as in the chemo(radio)therapy treatment scheme of this tumor entity. Despite successful surgical removal of the primary tumor, which can be achieved in most cases, a high number of patients already developed regional lymph node or distant metastases at the time of diagnosis. The treatment scheme of this head and neck cancer, therefore, frequently includes additional surgery to remove tumor-related lymph nodes (neck dissection) and postoperative chemo(radio)therapy or in the case surgery is not possible because of advanced tumor stage for example, primary chemo(radio)therapy. Postoperative survival of patients with regional lymph node or distant metastases then largely depends on the success of chemo(radio)therapy. Frequently, however, patients relapse after chemo(radio)therapy. Then, after having exhausted the full dose of local radiotherapy, chemotherapy typically remains the only therapy option that can be offered to the patient. However, many patients do not respond to chemotherapy and their tumors grow in the presence of this therapy. Cisplatin is the major compound used in HNSCC chemotherapy treatment. Resistance to cisplatin was found to correlate with p53 mutation status; however, the mechanisms underlying cisplatin resistance are still unclear. Therefore, being able to identify nonresponders before a scheduled chemotherapy would greatly help in performing a more selective therapy and could spare these patients the side effects associated with unnecessary chemotherapy by identifying cisplatin nonresponders before chemotherapy induction.
patients the additional side effects associated with ineffective and thereby unnecessary chemotherapy.

p53 is the most widely studied tumor suppressor protein. It was discovered initially in SV40-transformed cell lines, where it was suspected to act as an oncogene (3). Later, it became clear that wild-type (wt) p53 is a major protector against tumor development and is instead a tumor suppressor. Damage to DNA results in p53 activation, cell cycle arrest, and repair of the damage due to activation of repair enzymes such as GADD45 (growth arrest and DNA damage-inducible protein). If repair fails, p53 induces transcription of proapoptotic genes, resulting in death of the damaged cell (4). Ten years after its discovery, it was found that mutations in p53 are common events in many tumors (5). Since then, several thousand mutations in the p53 gene were discovered in the majority of carcinogen-related human cancers (International Agency for Research on Cancer TP53 database, http://www-p53.iarc.fr/index.html). Mutated p53 often is incapable of proper functioning and is unable to induce apoptosis, which result in uncontrolled progression through cell cycle and tumor development. The overwhelming majority of p53 mutations (95.1%) are located in the central region, whereas only 1.7% are in the NH2-terminal and 3.2% in the COOH-terminal region of the protein, respectively. Most (93.6%) of all the mutations are point mutations, whereas only 6.4% represent frameshift mutations or deletions (4). Translocation from the cytosol to the nucleus is essential for the p53 transcriptional function (6–8). This depends on the presence of an intact COOH-terminal nuclear localization signal that was initially described by Addison et al. (9) and was mapped to amino acid residues 305 to 322 in the COOH terminus of the protein (10–12). Additionally, p53 localization depends on a nuclear export signal (amino acids 340–351) that is located within the tetramerization domain of the protein (amino acids 326–355) and enables the protein to shuttle back to the cytoplasm (10, 13, 14). Very little is known about the cytoplasmic function of p53. p53 mutations are found in over 50% of HNSCC cancers and are commonly associated with tobacco and alcohol use (15). p53 mutation status is linked to worse prognosis (16) and to cisplatin sensitivity or resistance (17); however, the exact mechanism underlying this correlation still remains unclear.

In this study, nine HNSCC cell lines were characterized regarding their p53 mutation status by full-length sequencing of the p53 transcript, structural analysis, and nucleocytoplasmic distribution of the mutant proteins. These data were correlated to cisplatin sensitivity of the respective cell lines.

### Materials and Methods

**Cell culture and transfection of cell lines.** The squamous cell carcinoma cell lines UM-SCC-3, UM-SCC-4, UM-SCC-22B, UM-SCC-27 and UT-SCC-24A and UT-SCC-26A were as previously described by T.E. Carey (University of Michigan, Ann Arbor, MI) and R. Grénman (University of Turku, Turku, Finland), respectively (18). The UM-BSCC-745, UMB-SCC-864, and UMB-SCC-969 (University of Marburg) cell lines were derived from tumors of the oropharynx, tongue, and pharynx, respectively (Table 1). Control keratinocytes were derived from normal mucosa during a scheduled tonsillectomy after informed consent of the patient. Cells were grown in DMEM supplemented with 10% FCS in the presence of penicillin and streptomycin.

**Reverse transcription-PCR, subcloning, and sequencing.** Total RNA of all cell lines was prepared with the RNeasy MIDI kit (Qiagen GmbH, Hilden, Germany) according to the instructions of the manufacturer. Reverse transcription was done in the presence of an oligo(dT) or p53-specific primer (5′-CTCCCCACACAAACACCGG-3′; located in the 3′ untranslated region) to generate cDNA covering the whole open reading frame of p53. PCR amplification [1: 95°C, 15 minutes; 2: 95°C, 1 minute; 3: 45°C, 1 minute; 4: 72°C, 3 minutes; 5: 35 cycles (2–4); 6: 72°C, 15 min] was done to recover the entire coding region of p53, using the p53-specific primers 5′-GGGACGCGTGCCTTTCCACGCGG-3′, 5′-CCGGTCTCCTCCACAGACGAGG-3′, 5′-CCGGTCTCCTCCACAGACGAGG-3′, 5′-GCTGGTGGAGTATTTGGATGAC-3′, and 5′-AGTGGGGGACACAAAGTGGAGG-3′ according to the published human wt p53 sequence (Genbank accession nos. M60950 and X02469). PCR products were gel purified (Qiagen) and subcloned into the pBluescript KS+ vector (Stratagene, La Jolla, CA), which was previously cut with EcoRV (New England Biolabs, Inc., Beverly, MA) and incubated with dITP in the presence of Taq polymerase (Perkin-Elmer, Norwalk, CT) to generate T overhangs at the 3′ end of the vector, utilizing T4-DNA ligase (New England Biolabs). Positive (white) clones were screened for the right insert by double digestion with EcoRI and HindIII (New England Biolabs). DNA sequencing was done by a commercial provider (Seqlab, Göttingen, Germany). The p53 mutant with loss of nuclear signal due to a premature stop as found in UT-SCC-26A was subcloned into the HindIII/EcoRI site of the pcDNA3.1(+) vector (Invitrogen, Carlsbad, CA).

**Antibodies.** The anti-p53 mouse monoclonal antibody BP53-12 (Sigma-Aldrich Corp., Saint Louis, MO), which recognizes an NH2-terminal epitope (amino acids 37–45) of p53 that was present in all of the

### Table 1. HNSCC cell lines used in the study

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<tr>
<th>Name</th>
<th>Origin of primary</th>
<th>Origin of specimen</th>
<th>TNM</th>
<th>Grading</th>
<th>Age (y)</th>
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<td>Male</td>
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<tr>
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<td>G2</td>
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</tr>
<tr>
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<td>G2</td>
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<td>Male</td>
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<td>G2</td>
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<tr>
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<td>Lymph node metastasis</td>
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<td>G2</td>
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Abbreviation: TNM, tumor-node-metastasis.
the investigated cell lines, was used in all studies. Antibodies recognizing p21WAF1/CIP1 (C19, goat polyclonal), Bcl-xL (N5, mouse monoclonal), and Bax (N-20, rabbit polyclonal) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The secondary FITC- or horseradish peroxidase–coupled antibodies were from Santa Cruz Biotechnology and Calbiochem (Calbiochem-Novabiochem, La Jolla, CA).

**Cisplatin treatment, SDS-PAGE, and Western blot analysis.** HNSCC cell lines were treated with 25 μmol/L cisplatin for 24 hours. For total protein lysates, cells were harvested with a cell scraper, washed in ice cold PBS, and lysed in RIPA buffer. Lysates were sonicated and centrifuged at 12,000 × g. Supernatants were used as total cell lysates. SDS-PAGE and Western blot analysis were done under standard conditions (19) using 35 μg of whole cell lysate protein per lane. In short, after protein transfer, nitrocellulose membranes were blocked with 3% milk/PBS and incubated with BP53-12 (1:1,000) for 3 hours. Membranes were washed three times in 3% milk/PBS and then incubated with a horseradish peroxidase–coupled secondary anti-mouse antibody (1:1,000) for 1 hour. Membranes were washed and bands were visualized on X-ray film (Agfa, Cologne, Germany) using the enhanced chemiluminescence method (Amersham, Buckinghamshire, United Kingdom).

**Immunocytochemistry.** Cells were grown on cover slides in six-well tissue culture plates (Greiner Bio-One GmbH, Frickenhausen, Germany) until 50% confluent, fixed with methanol (−20°C), and stained with the anti-p53 antibody (1:250) in the presence of 3% bovine serum albumin/0.3% NP40/PBS for 1 hour. Subsequently, the cells were washed and incubated with the secondary FITC-coupled anti-mouse antibody (1:250) for an additional 45 minutes. To visualize the nucleus, cells were stained for 10 minutes with 4',6-diamidino-2-phenylindole (1 μg/mL final concentration in H2O). Negative controls (secondary antibody alone) did not show any significant background staining. The resulting FITC signal was visualized by confocal laser scanning microscopy (Leica TCS SP2, Leica Microsystems AG, Wetzlar, Germany; Olympus Deutschland GmbH, Hamburg, Germany) to assess the cytosolic and nuclear distribution of p53.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide viability assay. Cells were plated at 3 × 10^3 cells/well in duplicates into 96-well tissue culture plates. After 24 hours, cells were treated with cisplatin at a concentration range of 1 to 100 μmol/L for 48 hours. Cells were then incubated for 30 minutes with 1 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and lyzed with DMSO. Absorption was measured at 550 nm using an immunoreader (Anthos Labtec HT2, Vienna, Austria). For comparison of cell lines, the absorption of untreated cells was normalized to 1. At least three independent experiments were done with each cell line. The SD varied between 0.05 and 0.2 depending on the cell line and cisplatin concentration.

Luciferase assay. Primary rat embryonic cells were cultured in DMEM/10% FCS. Cells (5 × 10^4) were seeded into 24-well plates and transfected on the next day with 1 μg DNA using ExGen 500 (Fermentas, Inc., Hanover, MD) according to the instructions of the manufacturer. Reporter plasmid p53CON (kindly provided by W. Wright), wild-type p53 (kindly provided by M. Oren, Weizmann Institute of Science, Rehovot, Israel), and diverse p53 mutants (kindly provided by H. Kovar, Vienna, Austria) were cotransfected in a 1:1 ratio. The basic vector pGUS.PA8 was used as negative control. The temperature-sensitive p53 mutant A138V was shifted to 32°C for 12 hours before harvesting. Lysates were analyzed 24 hours after transfection. Firefly luciferase was detected by Luciferase Reporter System (Promega, Mannheim, Germany) according to the instructions of the manufacturer and measured in a Luminoskan RS (Labsystems Oy, Helsinki, Finland).

**Results**

Western blot analysis was done on whole cell lysates from the nine HNSCC cell lines (Table 1) and control keratinocytes to assess the expression levels of p53. The HNSCC cell lines UM-SCC-3, UM-SCC-22B, UM-SCC-27, and UM-SCC-969 expressed a protein of the same size as predicted for the wt p53 protein (Fig. 1A and B), whereas the cell lines UM-SCC-4, UM-SCC-864, UM-SCC-745, UT-SCC-24A, and UT-SCC-26A exhibited smaller-sized proteins (Fig. 1B and C). Significantly lower expression levels were found in the UM-SCC-4, UT-SCC-24A, and UT-SCC-26A cell lines, respectively (Fig. 1B and C). The cell lines UM-SCC-3, UM-SCC-22B, UM-SCC-27, UM-SCC-745, UM-SCC-864, and UM-SCC-969 exhibited expression levels comparable with or higher than the wt protein (Fig. 1A-C). An additional protein of the predicted size for wt p53 was consistently observed in the UT-SCC-24A cell line and exhibited expression levels equivalent to the smaller-sized protein (Fig. 1B). Actin was used as an internal control to verify same protein input of the different cell lines (not shown). Immunofluorescence staining was done as described in Materials and Methods and the nucleocytoplasmic distribution of the p53 protein was assessed by confocal laser scanning microscopy (Fig. 1A-C). Nuclear localization was observed for the wt p53 protein (Fig. 1A) and in the UM-SCC-3, UM-SCC-22B, UM-SCC-27, UM-SCC-745, and UM-SCC-969 cell lines (Fig. 1B), whereas the UM-SCC-4, UM-SCC-864, and UT-SCC-26A cell lines exhibited a total lack of nuclear signal with cytoplasmic sequestration of the protein (Fig. 1C). The UT-SCC-24A cell line exhibited an intermediate phenotype with dominant cytoplasmic p53 signal and less intense but clear nuclear signal of the protein (Fig. 1B). A single nucleotide polymorphism in exon 4 of TP53, which is found in the general population, predicts for two different residues at amino acid position 72 (20, 21). The amino acid at position 72 is either proline or arginine. Because there are two alleles, one can find P72/P72, P72/R72, or R72/R72. The codon 72 polymorphism status of p53 is shown in Fig. 1. All HNSCC cell lines exhibited the R72/R72 (CCG/CCG) polymorphism (Figs. 1B and C and 2A). The control keratinocytes shown in Fig. 1A exhibited both alleles, showing the P72/R72 (CCC/CCG) polymorphism. However, other keratinocytes also exhibited the P72/P72 (CCC/CCC) or R72/R72 (CCG/CCG) polymorphism (not shown), reflecting the variability in the general population.

The whole open reading frame of p53 was retrieved by reverse transcription-PCR sequencing as described in Materials and Methods (Fig. 2A). All nine HNSCC cell lines exhibited p53 mutations. Single point mutations were found in UM-SCC-3, UM-SCC-4, UM-SCC-22B, UM-SCC-27, UM-SCC-969, UM-SCC-745, and UT-SCC-26A, respectively (Figs. 1B and C and 2A). Mutations in UM-SCC-3, UM-SCC-22B, UM-SCC-27, and UM-SCC-969 were found to result in single amino acid substitutions (Figs. 1B and 2A), whereas mutations in UM-SCC-4, UM-SCC-864, and UT-SCC-26A led to the appearance of a premature stop codon, resulting in truncated proteins lacking 181, 45, or 158 COOH-terminal amino acids, respectively (Figs. 1B and 2A). The single amino acid substitutions included two arginine mutations, which were found in UM-SCC-3 (R248Q) and UM-SCC-27 (R273C) as well as two tyrosine mutations in UM-SCC-22B (Y220C) and UM-SCC-969 (Y205H), respectively (Figs. 1B and 2A and B). Structural analysis according to published data by Cho et al. (22) showed that all four single amino acid substitutions to be located in the central core domain of the protein, which is required for proper binding to DNA (Fig. 2B). Frameshift mutations were found in UT-SCC-24A and UM-SCC-864, predicting for proteins...
Fig. 1. Nucleocytoplasmic distribution, expression, and codon 72 polymorphism of p53. 

A, control keratinocytes show intact nuclear localization and expression of a regular-sized p53 protein and exhibit the R72/R72 codon 72 polymorphism. B, HNSCC cell lines with intact nuclear signal are four cell lines with single amino acid substitutions (UM-SCC-3, UM-SCC-22B, UM-SCC-27, UMB-SCC-969), one cell line with a C-terminal truncation due to a premature stop (UMB-SCC-745), and a cell line expressing a wt and a mutated p53 protein (UT-SCC-24A). Except for UT-SCC-24A, having reduced protein expression, all cell lines exhibit high p53 expression levels similar with or higher than in the control keratinocytes. C, three HNSCC cell lines, one with a frameshift mutation (UMB-SCC-864) and two with a premature stop (UM-SCC-4, UT-SCC-26A), exhibit total loss of nuclear p53 signal with cytoplasmic sequestration of the protein. UMB-SCC-864 exhibits high p53 expression levels, whereas UM-SCC-4 and UT-SCC-26A show reduced p53 levels in the Western blot analysis. B and C, all HNSCC cell lines exhibit the R72/R72 codon 72 polymorphism. Actin controls were used to verify same protein input (not shown). Confocal laser scanning microscopy, original magnification, ×100.
truncated by 150 and 50 amino acids, respectively (Figs. 1B and C and 2A). Except for the UT-SCC-24A cell line, which had a wt p53 transcript as well as the mutated version (Fig. 2A), all of the HNSCC cell lines we studied exhibited loss of heterozygosity.

Because cisplatin is a major component in the chemotherapy treatment scheme of HNSCC tumors (23), we investigated sensitivity against this agent in the patient-derived HNSCC cell lines. Cell viability in the presence of 1 to 100 μmol/L cisplatin after 48 hours was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and ID50 calculation (Fig. 3A). ID50 values of control keratinocytes expressing wt p53 were 10.01 ± 2.99 μmol/L, whereas HNSCC cell lines with mutant p53 but intact nuclear signal had values ranging from 8.47 ± 3.45 to 29.00 ± 2.59 μmol/L cisplatin. In sharp contrast, ID50 values of the three HNSCC cell lines with loss of nuclear p53 signal and cytoplasmic sequestration of the mutant p53 protein ranged from 26.24 ± 4.78 to 78.42 ± 9.74 μmol/L cisplatin (Fig. 3A). As a second approach, we compared the dose response of all cell lines (data not shown). Hence, we subdivided this panel of HNSCC cell lines carrying diverse p53 mutations into two groups, namely cell lines with nuclear p53 signal and such with loss of nuclear p53 signal and calculated the average curves (Fig. 3B). UT-SCC-24A, which was the only HNSCC cell line expressing a nuclear localization signal–deficient p53 mutant and wt p53 protein (Figs. 1B and 2A), responded to cisplatin-like control keratinocytes. This cell line had cytoplasmically sequestered p53 with still intact nuclear p53 signal, likely representing the wt protein, and, therefore, was counted to the “mutants with nuclear p53 signal” group (Fig. 3). UM-SCC-4, UMB-SCC-864, and UT-SCC-26A, which are expressing p53 mutants with total loss of nuclear signal (Fig. 3B, ▲), exhibit decreased sensitivity over the used cisplatin concentration range. The average curve of the other six cell lines with nuclear p53 signal was comparable with the dose-response curve of control keratinocytes, expressing wt p53 (Fig. 3B, ▲).

It was observed that HNSCC cell lines exhibit diverse sensitivities against cisplatin (17). The variety in ID50 values among the group of cell lines with mutated p53 in this report is similar to our observation. We compare two highly cisplatin-resistant HNSCC cell lines, UMB-SCC-864 and UT-SCC-26A, with six cisplatin-sensitive HNSCC cell lines and normal keratinocytes for their expression levels of p21WAF1/CIP1 as a target of p53, Bcl-xL as an antiapoptotic, and Bax as a proapoptotic factor upon cisplatin treatment (Fig. 4). The basal p21WAF1/CIP1 expression level is high in UM-SCC-22B and in UM-SCC-27. All other cell lines exhibit low levels of p21WAF1/CIP1 comparable with normal keratinocytes (Fig. 4, top). p21WAF1/CIP1 was induced upon cisplatin treatment in normal keratinocytes and all cell lines that express p53 mutants with nuclear localization. The most prominent p21WAF1/CIP1
up-regulation occurred in UT-SCC-24A, which express both wt and mutated p53. In both cell lines with loss of nuclear localization signal mutants, p21\(^{\text{WAF1/CIP1}}\) is hardly detectable and is not induced upon cisplatin treatment. Bcl-x\(_L\) level (Fig. 4, middle) from normal keratinocytes are comparable with those in UM-SCC-3, UMB-SCC-969, UMB-SCC-745, and UT-SCC-24A and is lower in the other cell lines. Both cisplatin-resistant cell lines have similarly low expression levels of Bcl-x\(_L\) comparable with the cisplatin-sensitive cell lines UM-SCC-22B and UM-SCC-27. Also, Bcl-x\(_L\) regulation after cisplatin treatment is heterogeneous regarding p53 mutations. Generally, Bax levels are higher in HNSCC cell lines than in normal keratinocytes (Fig. 4, bottom). Upon cisplatin treatment, proapoptotic Bax is up-regulated in almost every cell line, except in the cisplatin-resistant cell lines with loss of nuclear localization signal p53 mutations. Taken together, a common feature of the cisplatin-insensitive cell lines UMB-SCC-864 and UT-SCC-26A is the low and not inducible level of p21\(^{\text{WAF1/CIP1}}\), up-regulation of Bcl-x\(_L\), and simultaneous down-regulation of Bax upon treatment with cisplatin.

To test the transcriptional activity of one p53 mutant with low expression of nuclear localization signal derived from UT-SCC-26A, we did luciferase assays and compared it with the R273C mutant and the temperature-sensitive mutant A138V. Due to low transfection efficiency in human cell lines, we did these assays in a well-characterized primary rat embryonic cell system (19, 24–26). Endogenous wt p53 expression is low (data not shown) and, hence, transcriptional activity from a p53 consensus luciferase construct (p53CON) is marginal (Fig. 5). Overexpression of a temperature-sensitive p53 mutant, A138V (27), leads to luciferase expression also at the permissive temperature of 37°C. Transcriptional activity is enhanced 2-fold upon conformational change to wt at 32°C. It is reported that the R273C p53 mutant exhibits transcriptional activity for the MDR1 promoter (28), but not for the p21\(^{\text{WAF1/CIP1}}\) promoter in Saos-2 cells (28, 29). On the other hand, the mutant R273H retains the ability to transactivate p53CON (30). In this rat embryonic cell assay, the p53 mutant derived from UT-SCC-26A with premature stop, loss of nuclear localization signal, and strict cytoplasmic localization exhibited no transcriptional activation in this assay (Fig. 5).

### Discussion

**p53 protein expression.** p53 overexpression is frequently observed in malignancies and interpreted as mutation and inactivation of this tumor suppressor (31). We found mutations in p53 in all of the tested HNSCC cell lines, whereas three of them, UM-SCC-4, UT-SCC-24A, and UT-SCC-26A, had reduced protein expression in the Western blot analysis. The other six

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Fig. 4. p21\(^{\text{WAF1/CIP1}},\) Bcl-x\(_L\), and Bax expression upon cisplatin treatment. Normal keratinocytes and HNSCC cell lines were treated for 24 hours with 25 \(\mu\)mol/L cisplatin and total protein lysates were analyzed by immunoblotting. The two cell lines with total loss of nuclear p53 signal are marked with an asterisk.
Nuclear Localization of p53 and Cisplatin Sensitivity

Codon 72 polymorphism. In this study, all investigated HNSCC cell lines exhibited the R72/R72 codon 72 polymorphism, which is consistent with frequent observations of this polymorphism in tumors. However, no association has been found between p53 polymorphism and risk of developing squamous cell carcinomas of the head and neck (32). On the other hand, advanced squamous cell carcinomas of the head and neck were found to be less susceptible to chemo(radio)therapy (33). Total loss of p53 nuclear localization was observed in the UM-SCC-4, UMB-SCC-864, and UT-SCC-26A cell lines and is consistent with the p53 sequence analysis that documented loss of the COOH-terminal nuclear localization signal (amino acids 305-322) either due to the appearance of a premature stop codon (UM-SCC-4, UT-SCC-26A) or frameshift mutations (UMB-SCC-864). The UT-SCC-24A cell line exhibited a phenotype that is very different from the other cell lines because it had a predominantly cytoplasmic p53 signal, which is in sharp contrast to the other cell lines that either exhibited a dominant nuclear localization or total loss of nuclear signal (Fig. 1). This phenotype could be explained by the presence of an intact wt allele, because UT-SCC-24A was the only cell line harboring two transcripts and expressing two different p53 proteins, a wt and a mutated one, in which the mutated version lacks the COOH-terminal nuclear localization signal (Figs. 1B and 2A).

In this study, all investigated HNSCC cell lines exhibited the R72/R72 codon 72 polymorphism, which is consistent with frequent observations of this polymorphism in tumors. However, no association has been found between p53 polymorphism and risk of developing squamous cell carcinomas of the head and neck (32). On the other hand, advanced squamous cell carcinomas of the head and neck were found to be less susceptible to chemo(radio)therapy when the mutant p53 also carries the R72/R72 polymorphism. This is apparently due to enhanced inhibition of p73, which is required for treatment-induced apoptosis (33). However, there still are many inconsistent reports regarding the significance of p53 polymorphism in the pathogenesis and therapy of cancer.

Structural analysis. The p53 protein consists of four domains, which are the NH2-terminal acidic transactivation domain, the core (DNA binding) domain, the tetramerization domain, and the regulatory domain as well as one SH3 ligand sequence (Fig. 2A; ref. 34). Full-length sequencing of the whole open reading frame was required for correct determination of the mutation status in TP53 of all tested HNSCC cell lines (35). All point mutations that resulted in a single amino acid substitution were found in the core domain that is required for DNA binding (Fig. 2A and B). The four single amino acid substitutions (Y205H, Y220C, R248Q, R273C) can be separated into two functional classes. The first class represents mutations involving the DNA contact region of the core domain and includes the R248Q and R273C mutations. Reportedly, these mutations do not affect the conformation of the p53 protein (36). The second class represents mutations in the β-sandwich region of the core domain and includes the Y220C and Y205H mutations. Y205H and Y220C may destabilize the β-sandwich region of p53. Y220C is a well-known mutation and has been previously described (34). Mutations at amino acid position 220 alter the free energy of unfolding in water, ΔG, by -1 kcal/mol. H205 loops back to its preceding residues and may alter the stability and conformation of the preceding helix (P177-S183) if mutated. The arginine mutations R248Q and R273C affect the binding of p53 to DNA (Fig. 2B). R248Q and R273C, which most often is R273H, are the most commonly found p53 mutations (International Agency for Research on Cancer TP53 database, http://www-p53.iarc.fr/index.html) and are affecting DNA binding of the protein (Fig. 2B). According to recent reports, p53 function in some of these mutants can be rescued by the newly developed compounds CP-31398 and CP-257042 (34).

With the exception of UMB-SCC-745, all sequences that are truncated or frameshifted affect the core domain and result in loss of the COOH-terminal nuclear localization signal. These sequences are deleterious to the p53 molecule in total and will likely lead to malfunction. Notably all p53 mutants shorter in size than the p53 mutant found in UMB-SCC-864, which are the p53 mutants found in the UM-SCC-4, UT-SCC-24A, and UT-SCC-26A cell lines, exhibited severely lowered p53 expression levels (Fig. 1B and C), reflecting the reduced stability of these mutant proteins. In sharp contrast, the truncated p53 proteins found in UMB-SCC-745 and UMB-SCC-864 exhibit similar to or higher p53 protein expression levels than the wt protein (Fig. 1B and C). The p53 mutant found in UMB-SCC-745 had an intact core domain and nuclear localization signal (Fig. 2A).

Sensitivity to cisplatin and nuclear p53 signal. Generally, alterations of the p53 gene have been shown to contribute to tumorigenesis and drug resistance by modulating apoptotic response (37) and enhancing sublethal damage repair capacity (38). In a recent study it was shown, that HNSCC cell lines differ in their sensitivity against cisplatin depending on their p53 status (17). The statistical spread of cisplatin ID50 values of cell lines with mutant p53 is similar to ID50 values we determined here. In contrast, the other cell lines that either exhibit a dominant nuclear localization or total loss of nuclear signal (Fig. 1) showed that nuclear localization of p53 is a prerequisite for the function of the protein. The nuclear localization signal of p53 was mapped to amino acid residues 305 to 322 (9–12). This bipartite nuclear localization signal is similar to other nuclear localization signal sequences, such as those found in the SV40 T-antigen, interleukin-1, nucleoplasmin, and retinoblastoma proteins (9, 10). Total loss of p53 nuclear localization was observed in the UM-SCC-4, UMB-SCC-864, and UT-SCC-26A cell lines and is consistent with the p53 sequence analysis that documented loss of the COOH-terminal nuclear localization signal (amino acids 305-322) either due to the appearance of a premature stop codon (UM-SCC-4, UT-SCC-26A) or frameshift mutations (UMB-SCC-864). The UT-SCC-24A cell line exhibited a phenotype that is very different from the other cell lines because it had a predominantly cytoplasmic p53 signal, which is in sharp contrast to the other cell lines that either exhibited a dominant nuclear localization or total loss of nuclear signal (Fig. 1). This phenotype could be explained by the presence of an intact wt allele, because UT-SCC-24A was the only cell line harboring two transcripts and expressing two different p53 proteins, a wt and a mutated one, in which the mutated version lacks the COOH-terminal nuclear localization signal (Figs. 1B and 2A).

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Fig. 6. Transcriptional activity of p53 mutants in rat embryonic cells. Primary rat embryonic cells were transfected with p53CON-luciferase reporter alone or together with diverse p53 mutants. The temperature-sensitive p53 mutant A138V was shifted to 32°C for 12 hours before analysis to achieve p53 wt conformation and activity. Columns, mean; bars, SD.
signal were virtually resistant against cisplatin treatment, whereas HNSCC cell lines with nuclear p53 signal were as sensitive as control keratinocytes (Fig. 3A and B). Interestingly, a human papillomavirus–immortalized embryonic rat cell line that is characterized by lack of nuclear p53 signal exhibits resistance to cisplatin-induced apoptosis as observed for the three human HNSCC cell lines with loss of nuclear signal. This suggests that this mechanism is conserved between different species and tissues of origin. These observations therefore strongly point to “intact nuclear p53 signal” as a prerequisite for cisplatin-inducible apoptosis in HNSCC cancer. Single amino acid substitutions or short COOH-terminal deletions, not affecting the nuclear localization signal of p53, seem to be less disturbing for apoptosis induction by this agent. Naturally, one will also find p53 mutants with intact nuclear signal that are highly resistant to cisplatin, for example, as a result of severe mutations (e.g., deletions) affecting the NH2-terminal transactivation domain. However, such mutations would be less obvious than mutations affecting the nuclear localization signal because for the latter it would only be required to know if p53 is in the nucleus or exclusively cytoplasmic.

**Conclusion.** This study found loss of the COOH-terminally located nuclear localization signal in p53 to be a common event in the investigated nine HNSCC cell lines because three of the cell lines exhibited a total loss of nuclear p53 signal due to mutations affecting the nuclear localization signal. Total loss of nuclear p53 signal resulted in a dramatic decrease in cisplatin sensitivity. If the results presented here can be validated on a larger number of tumor samples, it could impact the chemotherapy treatment scheme of HNSCC cancer. Recognizing a large number of cisplatin nonresponders before therapy induction could help in sparing these patients the side effects associated with unnecessary (i.e., ineffective) chemotherapy treatment. However, further studies on a larger number of samples, which have to include fresh tumor tissues, are required to assess the impact nuclear localization signal mutations have in the development and progress of HNSCC tumors as well as resistance to chemotherapy.

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Reduced Cisplatin Sensitivity of Head and Neck Squamous Cell Carcinoma Cell Lines Correlates with Mutations Affecting the COOH-Terminal Nuclear Localization Signal of p53

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