Identification of cisplatin-resistant factor by integration of transcriptomic and proteomic data using head and neck carcinoma cell lines

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ABSTRACT

Cisplatin is an important drug for the treatment of head and neck squamous cell carcinoma (HNSCC). Determining chemoresistant factors prior to treatment will lead to great benefits for clinicians and patients. Here, we evaluated chemoresistant factors by integrating proteomic and transcriptomic data using HNSCC cell lines to identify a more precise chemoresistant factor in HNSCC. We used four HNSCC cell lines: cisplatin-sensitive, acquired cisplatin resistance, naturally cisplatin-resistant, and acquired 5-FU resistance. Proteomic analysis was performed using iTRAQ, tandem mass spectrometry, and liquid chromatography-electrospray ionization-tandem mass spectrometry. Transcriptomic analysis was performed using microarrays. By integrating these independent data, common factors were addressed and functional analysis was performed using small interfering RNAs (siRNAs) to change the chemosensitivity. Using iTRAQ analysis, 7 proteins were identified as specific for cisplatin chemoresistance factors. Transcriptomic analysis revealed hundreds of potential candidate factors. By combining and integrating these data, S100A2 was identified as a potential cisplatin-specific chemoresistance factor. Functional analysis with siRNA revealed that the expression of S100A2 was reduced and cisplatin sensitivity recovered in the acquired and naturally cisplatin-resistant cell lines, but not in the cisplatin-sensitive cell lines. S100A2 was identified as a cisplatin-specific chemoresistance factor by integrating the transcriptomic and proteomic results obtained using HNSCC cell lines. This is a novel technique that allows for a precise identification, also known as a comprehensive analysis. Our findings indicate that these proteins could be used as biomarkers of HNSCC treatments, providing physicians with new treatment strategies for patients with HNSCC, showing chemoresistance.

Keywords: S100A2, transcriptomics, proteomics, cisplatin resistant factor, head and neck squamous cell carcinoma

Abbreviations:
HNSCC: Head and neck squamous cell carcinoma
INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) is now the sixth most common type of cancer worldwide. Over 600,000 new patients are diagnosed each year across the world, with about 350,000 fatalities. The standard treatments for HNSCC include surgery, radiation, and chemotherapy.

Chemotherapy is an important part of the treatment strategy, and cisplatin (cis-diamminedichloroplatinum; CDDP) and 5-fluorouracil (5-FU) are currently the most effective chemotherapeutic agents. However, patients can be resistant to both of these two drugs. In chemoresistant patients, CDDP-based treatment is ineffective and does not elicit a clinical response, but still induces adverse effects. In addition, the cost effectiveness of treatment using chemotherapeutic agents in these cases is very low. Although the identification of true chemoresistance factors prior to treatment would improve the treatment strategies for head and neck cancer, this remains a challenge due to the sheer volume of candidate factors that are related to CDDP and other drugs.

We previously reported on the identification of chemoresistance factors for head and neck carcinoma by protein expression analysis in HNSCC cell lines using isobaric tags and the relative and absolute quantitation (iTRAQ) method. However, we hypothesized that more accurate results could be obtained by performing a comprehensive analysis using the same cell lines. In addition, the cost effectiveness of treatment using chemotherapeutic agents in these cases is very low. Although the identification of true chemoresistance factors prior to treatment would improve the treatment strategies for head and neck cancer, this remains a challenge due to the sheer volume of candidate factors that are related to CDDP and other drugs.

We previously reported on the identification of chemoresistance factors for head and neck carcinoma by protein expression analysis in HNSCC cell lines using isobaric tags and the relative and absolute quantitation (iTRAQ) method. However, we hypothesized that more accurate results could be obtained by performing a comprehensive analysis using the same cell lines. In the present study, we conducted a microarray analysis using the same cell lines. As a type of transcriptomic analysis, microarray analysis can be used to identify many candidate chemoresistance factors. In this study, we combined microarray analysis with independent gross data from mRNAs and proteins in an attempt to identify potential factors more precisely. The resulting data may be used to elucidate true candidate chemoresistance factors for HNSCC, in particular for CDDP. In this study, we also performed western blotting of the candidate proteins and conducted a functional analysis via knockdown using small interfering RNA (siRNA). The aim of this study was to identify the true CDDP-resistance factors to provide a new therapeutic strategy for the treatment of head and neck cancer.

MATERIALS AND METHODS

Cell lines

Four HNSCC cell lines were used in this study, namely UM-SCC-23, UM-SCCC-81B, UM-SCC-23-CDDPR, and UM-SCC-23/WR. The CDDP-sensitive cell line UM-SCC-23 and the naturally CDDP-resistant cell line UM-SCC-81B were provided by Dr Thomas E. Carey, University of Michigan (Ann Arbor, MI). We developed the acquired CDDP resistance cell line UM-SCC-23-CDDPR by repeated exposure to CDDP. The acquired 5-FU resistance cell line UM-SCC-23/WR was kindly gifted by Dr. Kei Ijichi, Nagoya City University (Nagoya, Japan). Cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) (IBL, Tokyo, Japan), 1% penicillin-streptomycin as Pen Strep Solution with 10,000 units penicillin, and 10 mg streptomycin/mL in 0.9% NaCl (Sigma-Aldrich, St Louis, MO) in a humidified
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atmosphere of 5% CO2 at 37°C. The doubling time was the same across all cell lines.

Cell viability assay
Cells were plated in 96-well flat-bottom plates (Falcon 3072; Becton Dickinson, Franklin Lakes, NJ) at a density of $2.0 \times 10^4$ cells/well and allowed to adhere for 24 h. The medium was then replaced with fresh medium supplemented with CDDP (Nippon Kayaku, Tokyo, Japan) at various concentrations (0, 1.56, 3.12, 6.25, 12.5, and 25 µg/mL) and incubated for 48 h using a WST-1 assay kit (Cell Counting Kit-8; Dojindo, Tokyo, Japan). WST-1 reagent (10 µL/well) was added to the cells and cultured for 4 h. The resulting coloration value was measured using a 96-well plate reader at a wavelength of 450 nm (SoftMax Pro 6.2.1; Molecular Devices, Sunnyvale, CA).

iTRAQ labeling and liquid chromatography-tandem mass spectrometry analysis
Samples were processed individually using the iTRAQ method. iTRAQ-tagged reagents were labeled for each sample. Samples were then mixed and measured as one sample, using liquid chromatography (LC)-mass spectrometry (MS), as previously reported. The mass spectra measured using LC-MS after labeling with iTRAQ were detected as a single peak, and the proportion of each protein expressed was determined by analyzing the mass spectrum data obtained by cleaving. Protein identification was subsequently performed based on the peptide sequences. Thus, the extracted proteins were labeled with iTRAQ reagents and analyzed by tandem mass spectrometry (MS/MS) to identify CDDP-resistance. Thereafter, MS and MS/MS data searches were carried out using Protein Pilot software.

Microarray analysis
The experimental procedure for the cDNA microarray analysis was based on the manufacturer’s protocol (Agilent Technologies, CA). In brief, total RNA was extracted from four cell lines using Nucleospin RNA with DNase treatment (TaKaRa Bio, Shiga, Japan). Next, 200 ng of total RNA was used for cDNA synthesis, and cRNA labeling with cyanine 3 (Cy3) dye was performed using the Agilent Low Input Quick Amp Labeling Kit (Agilent Technologies). The Cy3-labeled cRNA was purified, fragmented, and hybridized on a Human Gene Expression 4x44K v2 Microarray Chip containing 27,958 Entrez Gene RNAs, using a Gene Expression Hybridization kit (Agilent Technologies). The microarray slide was scanned with an Agilent DNA microarray scanner (Agilent Microarray Scanner-G2505C; Agilent Technologies). Feature Extraction software (version 11.0.1.1, Agilent Technologies) was used to extract and analyze the signal intensities, which were further normalized, as previously described. The background signals were normalized, and the microarray expression data were rank-ordered according to the expression levels using Agilent GeneSpring software (Agilent Technologies) as follows: upregulated and downregulated genes in the UM-SCC23-CDDPR, UM-SCC-23/WR, and UM-SCC-81 cells qualified in comparison with the UM-SCC-23 parental cells as follows: (i) a > 10-fold increase or <–10-fold decrease; (ii) a >5.0-fold increase or <–5.0-fold decrease; (iii) a >2.0-fold increase or <–2.0-fold decrease. In each setting, the CDDP-specific factors were counted.

Western blotting
After centrifugation at 10,000 × g for 10 min, the cells were lysed and passed through a 0.45-mm filter. The extracted proteins were run on 20.0% polyacrylamide gels and transferred to a nitrocellulose membrane (Immobilon-P; Millipore, Billerica, MA) after electrophoresis. Membranes were incubated with blocking reagents (ECL Prime Blocking Reagent; GE Healthcare Ltd., Little Chalfont, UK). The primary antibodies were rabbit polyclonal anti-human S100A2
and rabbit polyclonal anti-human GAPDH (Cell Signaling Technology). The secondary antibodies were peroxidase-conjugated anti-rabbit IgG (WestVision Peroxidase Polymer Anti-Rabbit IgG for Western Blot Detection; Vector Laboratories, CA). After incubation with the secondary antibodies, an ECL™ Prime Western Blotting System (GE Healthcare Ltd.) was used to detect bands via a luminous detector (LAS 4000; GE Healthcare Ltd.).

**Transfection of siRNAs**

siRNAs against S100A2 (4392420; Thermo Fisher Scientific, Waltham, MA, USA) were used for transfection as follows: sense (5’-3’), CGAGAUAGGUUGCUGACUUt and antisense, AAGUCAGCAACCUAUCUGAt.

Cells were plated in 24-well plates and transfected with 6 pmol of S100A2 and negative control (Stealth RNAi Negative Control high GC Duplex; Invitrogen, Carlsbad, CA) using the corresponding transfection reagents (Lipofectamine RNAiMAX Transfection Reagent; Thermo Fisher Scientific). The next day, the cells were re-plated in 96-well plates and allowed to attach overnight. Cells were then incubated with or without each drug for an additional 96 h. Lastly, cell growth was measured using the WST-1 assay.

**Statistical analysis**

The results of siRNA transfection of UM-SCC-23, UM-SCC-23-CDDPR, and UM-SCC-81B cells were analyzed using a linear mixed model in the mixed procedure of SAS 9.4 software (SAS Institute Inc., Cary, NC). The model included the group, dose, and interaction between the groups and the dose as fixed effects, while the date of the experiment was denoted as a random effect. We assumed a compound symmetry correlation structure within the date of the experiment. All tests were two-sided, and the significance level was set at 0.05.

**RESULTS**

**Sensitivity of CDDP and 5-FU for each cell line**

We investigated the sensitivity of the UM-SCC-23, UM-SCC-23-CDDPR, and UMSCC-81B cell lines to CDDP using the WST-1 assay. As shown in Figure 1, UM-SCC-23 was a more CDDP-sensitive cell line than the other cell lines (Figure 1). This result indicates that UM-SCC-23-CDDPR is an acquired CDDP resistance line and that UM-SCC-81B is a naturally CDDP-resistant cell line. The acquired 5-FU resistance cell line, UM-SCC-23/WR, is 1.4 times more resistant to 5-FU than the UM-SCC-23 line.7

**iTRAQ labeling and LC-MS/MS analysis**

As reported in our previous study, 2,173 proteins were identified by comparing their levels of expression in different cell lines using iTRAQ.2 Using ProteinPilot Software (AB Sciex, Framingham, MA), iTRAQ-based detection and relative quantification were obtained. Based on the mass spectra and MS/MS spectra, the proteins that show the highest expression only in CDDP-resistant cell lines are likely to be CDDP-specific factors (Figure 2). A total of 7 proteins were classified as CDDP-specific resistant proteins, demonstrated by a decrease in UM-SCC-23 (CDDP-sensitive) and UM-SCC-23/WR (5-FU-resistant) cells, and an increase in UM-SCC23-CDDPR and UM-SCC-81B (CDDP-resistant) lines (p < 0.05) (Table 1).

A total of 2,173 proteins were identified by comparing their expression in different cell lines using iTRAQ. The reproducible rate or critical false discovery rate was 1.0%, indicating that the proteins were highly sensitive biomarkers for HNSCC cell lines. Statistical calculations
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Fig. 1  Characterization of cell lines. Assessment of cell survival after treatment with CDDP, according to the WST-1 assay.
Blue: UM-SCC-23, brown: UM-SCC-23-CDDPR, gray: UM-SCC-81B.

Fig. 2  iTRAQ method for protein analysis showing the representative results of the mass spectra.
Left: Expression of a protein in the cisplatin-sensitive cell line UM-SCC-23.
Right: CDDP-resistant strains CC-23-CDDPR and UM-SCC-81B.
The bar on the far right denotes the expression of 5-FU resistant UM-SCC-23/WR. This protein was highly expressed in cisplatin-resistant cell lines, and may be a cisplatin-specific factor. A total of 7 proteins were found to follow this pattern.
for iTRAQ-based detection and relative quantification were then performed using the Paragon algorithm in ProteinPilot (AB Sciex). Proteins with decreased levels of expression in UM-SCC-23 (CDDP-sensitive) and UM-SCC-23/WR (5-FU-resistant) cells and increased levels of expression in UM-SCC23-CDDPR and UM-SCC-81B (CDDP-resistant) cells were regarded as CDDP-specific resistant. A total of 7 proteins were placed in this group (p < 0.05). %Cov denotes the percentage of matching amino acids from the identified peptides with a confidence greater than 0 divided by the total number of amino acids in the sequence. N denotes the rank of the specified protein relative to all other proteins in the list of detected proteins.

| Accession # | protein name                  | % Cov | N  |
|-------------|-------------------------------|-------|----|
| sp|P35579 | Myosin-9 | 32.7 | 1  |
| sp|P06733 | α-enolase | 77.7 | 24 |
| sp|P02538 | Keratin, type II cytoskeletal 6A | 74.4 | 54 |
| sp|P09211 | Glutathione S-transferase P | 70 | 97 |
| sp|Q9NZM1 | Myoferlin | 54 | 80 |
| sp|P29034 | Protein S100-A2 | 56.1 | 298 |
| sp|P23526 | Adenosylhomocysteinase | 39.4 | 142 |

Microarray analysis and combined and integrated iTRAQ results

Based on the microarray analysis results, we initially analyzed the differences in the expression of >5-fold and >10-fold increases using Gene Spring software. However, by integrating the iTRAQ results, we did not identify any common factors associated with the >5-fold and >10-fold higher microarray differences in expression. Therefore, we analyzed the >2-fold higher expression levels using UM-SCC-23 cells as a control in Gene Spring software. As a result, we identified 321 factors associated with CDDP-specific chemoresistance. Finally, we combined and integrated the iTRAQ and microarray data showing >2-fold higher expression levels, and identified one common protein as a chemoresistance factor, namely as a CDDP-specific factor: S100A2 (Figure 3).

Verification by western blotting

We performed western blotting analysis to confirm the accuracy of iTRAQ in identifying S100A2 as a CDDP-specific resistant protein. As shown in Figure 4, the expression patterns were identical between the two analyses. Each expression pattern of the proteins corresponded to the iTRAQ results for CDDP-resistant proteins (Figure 4). There was no expression in the S100A2, UM-SCC-23, or UM-SCC-23/WR (5-FU-resistant) cells, while expression was observed in UM-SCC-23-CDDPR and UMSCC-81B cells. This indicates the presence of a CDDP-resistant protein.

Transfection of siRNAs and effect

We conducted a functional analysis of S100A2 as a CDDP-specific factor using siRNA. The knockdown effects were analyzed by western blotting. As shown in Figure 5, in UM-SCC-23, the negative control and siRNA knockdown samples did not show S100A2 expression (Figure 5). In UM-SCC-23CDDPR and UM-SCC-81B, the negative control expressed S100A2 as shown in Figure 5, while the siRNA knockdown samples were found to have lower levels of S100A2 expression. We then evaluated the chemosensitivity of the cells to CDDP after the siRNA
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knockdown of S100A2.

**Fig. 3** Results of microarray analysis and iTRAQ analysis. Microarray results showing the differences in expression levels by >2-fold. A total of 321 factors showed cisplatin-specific chemoresistance. At the protein level, iTRAQ analysis identified 7 factors showing cisplatin-specific chemoresistance. Using iTRAQ and microarray combined data, one protein, S100A2, was found to be a cisplatin-specific factor.

**Fig. 4** Validation of iTRAQ results by western blotting for the detection of S100A2. Results were confirmed by western blotting, and the same results as those for ProteinPilot were obtained. The density of each band was normalized to that of GAPDH (internal loading control).
The results of the linear mixed effect model for each cell line treated with CDDP are shown in Table 2. Regarding the UM-SCC-23 (CDDP-sensitive) line, by comparing the negative control and S100A2 siRNA did not affect expression in the CDDP-sensitive cell line, UM-SCC-23. However, the resistant cell lines, UM-SCC-23-CDDPR and UM-SCC-81B, initially showed high levels of S100A2 expression. The effect of S100A2 knockdown was determined in both cell lines. The density of each band was measured and normalized to that of GAPDH (internal loading control) using Image Quant TL (version 7.0).

**Table 2.** Linear mixed effect model of the relation between cell line and group, dose and interaction (see Figure 6).

| response          | variables               | Coefficient | 95% CI      | p-value |
|-------------------|-------------------------|-------------|-------------|---------|
| UM-SCC-23         | Negative Control        | 0           |             |         |
|                   | group Si S100A2         | –0.056      | –0.159      | 0.048   | 0.2816  |
|                   | dose                    | 0.0313      | 0.025       | 0.038   | <.0001  |
|                   | interaction             | 0.0028      | –0.007      | 0.012   | 0.5572  |
| UM-SCC-23-CDDPR   | Negative Control        | 0           |             |         |
|                   | group Si S100A2         | –0.117      | –0.291      | 0.057   | 0.181   |
|                   | dose                    | 0.03        | 0.019       | 0.042   | <.0001  |
|                   | interaction             | 0.004       | –0.012      | 0.02    | 0.5792  |
| UM-SCC-81B        | Negative Control        | 0           |             |         |
|                   | group Si S100A2         | –0.152      | –3.01       | –0.004  | 0.045   |
|                   | dose                    | 0.0282      | 0.019       | 0.038   | <.0001  |
|                   | interaction             | 0.004       | –0.009      | 0.018   | 0.5109  |

**Association of cell line treated with CDDP with group, dose and interaction**

The results of the linear mixed effect model for each cell line treated with CDDP are shown in Table 2. Regarding the UM-SCC-23 (CDDP-sensitive) line, by comparing the negative control and
the cells after the knockdown siRNA of S100A2, the objective variables were found to decrease significantly as the concentration decreased, while no interaction was observed between the group and the concentration \((p = 0.2816, p = 0.1810)\) (Figure 6A). No significant differences were observed in the UM-SCC-23-CDDPR (acquired CDDP resistance) line \((p = 0.1810)\) (Figure 6B). Furthermore, the coefficient of the UM-SCC-81B (naturally CDDP-resistant) line after S100A2 siRNA knockdown compared to the negative control group was significantly reduced \((0.152; p = 0.0450)\) (Figure 6C).
Chemotherapy plays a very important role in the treatment of head and neck cancer. The head and neck area has important implications in practical functions, including chewing, tasting, and talking, but also serve an aesthetic purpose. Thus, a treatment that is able to preserve function while eliminating disease is needed. Although CDDP-based chemotherapy is currently the main treatment for HNSCC, there is a proportion of patients with HNSCC that do not respond well to this treatment in clinical settings. As head and neck oncologists, we wanted to explore the mechanisms behind CDDP-resistance, the so-called CDDP resistant factors. Patients who are CDDP-resistant often experience adverse effects when treated with chemotherapy drugs, resulting in very low cost-effectiveness, which is both undesirable and places the patients at a disadvantage. As such, from a social point of view, the use of chemotherapy in chemoresistant patients is inefficient. Thus, the identification of the chemoresistance factors in patients with head and neck cancer is fundamental to determine patient sensitivity to chemotherapeutic drugs before the administration of treatment.

A number of studies on CDDP and 5-FU chemosensitivity have been reported to date. Borsellino reported that endogenous interleukin 6 is a resistance factor for CDDP, while Bradford reported that a P53 mutation correlates with CDDP sensitivity in HNSCC lines. Xu reported that Xrcc3 induces CDDP resistance. In terms of 5-FU sensitivity factors, Yamamoto revealed that CEACAM1 re-expresses and promotes hollow spheroid formation, which is a predictor for 5-FU sensitivity. However, the true resistance factors related to CDDP and 5-FU have not yet been identified.

It remains problematic to compare clinical samples between sensitive and resistant groups of patients. In clinical settings, it is often difficult to differentiate between cases with good sensitivity.
responses and those with poor responses to resistance. In addition, the comparison of samples from each different cancer patient can further complicate the identification of candidate factors, resulting in samples with many phenotypes. Therefore, this type of sample collection is unsuitable for the simple and precise identification of chemosensitivity factors. As such, we believe that a simple study design, such as an in vitro study, could be used to identify chemoresistant factors more precisely. In this study, we designed our experiments using HNSCC cell lines.

Cells sensitive to CDDP, namely UM-SCC-23 cells, and CDDP-resistant cells, namely UM-SCC23-CDDPR, were used in this study. The cell lines were prepared by the repeated administration of anti-cancer drugs. However, the latter cell line was an acquired resistance line, and was unsuitable for the identification of true resistance factors. Therefore, we also included a natural CDDP-resistant cell line, UM-SCC-81B, in our study. We then compared these resistant cell lines to identify the true resistance factors, as well as establishing a 5-FU-resistant cell line, UM-SCC-23/WR.

Indeed, in a previous study, we detected CDDP-specific factors using proteomic analysis with the iTRAQ method and found that this method was very useful for detecting precise factors. We identified candidate proteins by determining their sensitivity via iTRAQ analysis, resulting in the identification of 7 proteins that were specifically resistant to CDDP. However, many candidates proteins were obtained using this method. Thus, we established a new strategy by combining transcriptomics using microarray analysis to detect precise factors, in addition to proteomic analysis by iTRAQ, based on the fact that the integration of transcriptomics and proteomics had the potential to identify the true significant factors from the viewpoint of the central dogma of molecular biology.

For microarray analysis, we examined the differences in the 5- and 10-fold expression levels between the chemosensitive control strain UM-SCC-23 and the chemoresistant strains 23R, 81B, and FU-W, which were 1267, 464, and 244, respectively. However, the results of our proteomics analysis indicated that there were no common factors between CDDP-specific resistant factors. Thus, we verified the 2-fold differences in the factors common to the sensitive control strain and identified S100A2 as a factor for CDDP-specific resistance. Based on protein expression analysis, we found that S100A2 was highly expressed only in the acquired resistance UM-SCC-23-CDDPR and naturally CDDP-resistant UMSSC-81B cell lines, and not in UM-SCC-23 or UM-SCC-23/WR, which had no association with CDDP resistance. This study is the first to report on the identification of a factor for CDDP-specific resistance using transcriptome and proteome analysis.

CDDP is the main chemotherapeutic drug used for the treatment of HNSCC. To address the issue of chemoresistance in patients with head and neck cancer, we conducted a functional analysis on the CDDP-specific factors using transfection with siRNA. As a result, the knockdown of S100A2 was found to lead to a reduction in protein expression and a recovery of the chemosensitivity of CDDP-resistant cell lines. We found natural and acquired chemoresistance, as well as a significant difference in the sensitivity reacquisition of naturally resistant cell lines after S100A2 knockdown compared to the acquired resistance cells. In the acquired resistance cell line, UM-SCC-23CDDPR, the western blotting results for the negative control and siRNA knockdown showed a slight difference in terms of the levels of S100A2 expression. This may be due to the lack of a significant difference in the survival rate after S100A2 in UM-SCC-23CDDPR cells. The significant differences found in terms of the naturally resistant cells indicate that S100A2 can be used to identify factors for CDDP sensitivity prior to the administration of chemotherapy in patients with HNSCC.

The S100 proteins consist of over 20 structurally similar subunits, which are not functionally similar to each other. This family of proteins regulates cellular responses by functioning as both intracellular Ca2+ sensors and extracellular factors. The biology of most S100 proteins is
complicated and has multi-factor roles. These proteins contribute to cell growth, tumorigenesis, angiogenesis, and immune avoidance.\textsuperscript{12}

The S100A2 protein, a member of the S100 protein family, has been reported to play a role in cancer. In a previous study, Lee suggested that S100A2 plays a potentially important role in carcinogenesis.\textsuperscript{13} On the other hand, S100A2 has also been identified as a potential tumor-suppressor candidate. Almadori and Zha reported on the function of S100A2 as a putative tumor suppressor in laryngeal squamous cell carcinoma.\textsuperscript{14-15} Similarly, Wolf reported that the S100A2 gene potentially exhibits cancer suppressing activity, but may be related with cancer cell proliferation.\textsuperscript{16} Kumar investigated the mechanism by which S100A2 affects cancer by studying the subcellular localization of S100A2. However, its mechanism has not yet been fully elucidated.\textsuperscript{17}

There are currently no reports on the resistance of S100A2 to CDDP. However, a complex mechanism is likely to underlie how S100A2 affects CDDP resistance. This study is the first to identify S100A2 as a CDDP-specific chemoresistance factor, based on transcriptomic and proteomic data, which was generated via comprehensive analysis. Although we were unable to determine the mechanism by which S100A2 affects CDDP resistance, this mechanism could be elucidated through further examination. Since S100A2 is a highly accurate marker, it is a good candidate for use as a biomarker for CDDP resistance in patients with HNSCC.

The use of S100A2 as a biomarker for the detection of CDDP resistance could help improve the process of selecting an effective treatment strategy for patients with HNSCC. In a clinical setting, the levels of S100A2 expression in patients could be used as a benchmark for the selection of alternative treatments to CDDP-based chemotherapy, including surgery, radiation therapy, or other types of therapeutic drugs. These findings would benefit both, surgical oncologists as well as patients with cancer currently seeking treatment.

CONCLUSION

We identified a CDDP-specific chemoresistance factor, S100A2, by comprehensively analyzing transcriptomic and proteomic data using HNSCC cell lines. S100A2 has the potential to be used as a biomarker to determine CDDP resistance in patients with HNSCC prior to therapeutic treatment. As a result, our findings provide a basis for the improvement of therapeutic strategies for the treatment of head and neck cancer in chemoresistant patients.

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CONFLICT OF INTEREST

The authors declare no competing interests.

REFERENCES

1. Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of cancer
Identification of CDDP-resistant factor

in 2008: GLOBOCAN 2008. *Int J Cancer*. 2010;127:2893–2917.

2. Nishimura K, Tsuchiya Y, Okamoto H, et al. Identification of chemoresistant factors by protein expression analysis with iTRAQ for head and neck carcinoma. *Br J Cancer*. 2014;111:799–806.

3. Brenner JC, Graham MP, Kumar B, et al. Genotyping of 73 UM-SCC head and neck squamous cell carcinoma cell lines. *Head Neck*. 2010;32:417–426.

4. Adachi M, Ijichi K, Hasegawa Y, et al. Hypersensitivity to cisplatin after hRev3 mRNA knockdown in head and neck squamous cell carcinoma cells. *Mol Med Rep*. 2008;1(5):695–698.

5. Ijichi K, Adachi M, Hasegawa Y, et al. Pretreatment with 5-FU enhances cisplatin cytotoxicity in head and neck squamous cell carcinoma cells. *Cancer Chemother Pharmacol*. 2008;62:745–752.

6. Mizuno S, Hanamura I, Ota A, et al. Overexpression of salivary-type amylase reduces the sensitivity to bortezomib in multiple myeloma cells. *Int J Hematol*. 2015;102:569–578.

7. Murata S, Adachi M, Kioi M, et al. Etodolac improves 5-FU sensitivity of head and neck cancer cells through inhibition of thymidylate synthase. *Anticancer Res*. 2011;31:2893–2898.

8. Borsellino N, Belldegrun A, Bonavida B. Endogenous interleukin 6 is a resistance factor for cis-diaminedichloroplatinum and etoposide-mediated cytotoxicity of human prostate carcinoma cell lines. *Cancer Res*. 1995;55:4633–4639.

9. Bradford CR, Zhu S, Ogawa H, et al. PS3 mutation correlates with cisplatin sensitivity in head and neck squamous cell carcinoma lines. *Head Neck*. 2003;25:654–661.

10. Xu ZY, Loignon M, Han FY, Panasci L, Aloyz R. Xrcc3 induces cisplatin resistance by stimulation of Rad51-related recombinational repair, S-phase checkpoint activation, and reduced apoptosis. *J Pharmacol Exp Ther*. 2005;314:495–505.

11. Yamamoto N, Yokoyama S, Ieda J, et al. CEACAM1 and hollow spheroid formation modulate the chemosensitivity of colorectal cancer to 5-fluorouracil. *Cancer Chemother Pharmacol*. 2015;75:421–430.

12. Bresnick AR, Weber DJ, Zimmer DB. S100 proteins in cancer. *Nat Rev Cancer*. 2015;15:96–109.

13. Lee J, Wysocki PT, Topaloglu O, et al. Epigenetic silencing of S100A2 in bladder and head and neck cancers. *Oncosience*. 2015;2(4):410–418.

14. Almadori G, Bussu F, Galli J, et al. Diminished expression of S100A2, a putative tumour suppressor, is an independent predictive factor of neck node relapse in laryngeal squamous cell carcinoma. *J Otolaryngol Head Neck Surg*. 2009;38:16–22.

15. Zha C, Jiang XH, Peng SF. iTRAQ-based quantitative proteomic analysis on S100 calcium binding protein A2 in metastasis of laryngeal cancer. *PLoS ONE*. 2015;10:e0122322.

16. Wolf S, Haase-Kohn C, Pietzsch J. S100A2 in cancerogenesis: a friend or a foe? *Amino Acids*. 2011;41:849–861.

17. Kumar M, Srivastava G, Kaur J, et al. Prognostic significance of cytoplasmic S100A2 overexpression in oral cancer patients. *J Transl Med*. 2015;13:8.