



RESEARCH PAPER

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# Mass spectrometry-based proteomic analysis to characterize cisplatin induced early signaling events in head and neck squamous cell carcinoma

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## ABSTRACT

Cisplatin is the commonly used chemotherapeutic drug in treatment of various cancers. However, development of resistance towards cisplatin results in tumor recurrence. Here, we aim to understand the mechanisms of action of cisplatin and emergence of resistance to cisplatin using mass spectrometry-based proteomic approach. A panel of head and neck squamous cell carcinoma (HNSCC) cell lines were treated with cisplatin at respective IC<sub>50</sub> for 24 h and label-free mass spectrometry analysis was carried out. Proteomic analysis of A253, FaDu, Det562 and CAL27 cell lines upon cisplatin treatment resulted in the identification of 5,060, 4,816, 4,537 and 4,142 proteins, respectively. Bioinformatics analysis of differentially regulated proteins revealed proteins implicated in DNA damage bypass pathway, translation and mRNA splicing to be enriched. Further, proteins associated with cisplatin resistance exhibited alterations following short-term cisplatin exposure. Among these, class III tubulin protein (TUBB3) was found to be upregulated in cisplatin-treated cells compared to untreated cells. Western blot analysis confirmed the elevated expression of TUBB3 in cells treated with cisplatin for 24 h, and also in cisplatin resistant HNSCC cell lines. This study delineates the early signaling events that enable HNSCC cells to counteract the cytotoxic effects of cisplatin and facilitate the development of resistance.

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Mass spectrometry; drug resistance; cisplatin; head and neck cancer; proteomics


## Introduction

The discovery of anticancer properties of cisplatin was a breakthrough in tumor treatment. Cisplatin was the first platinum drug to be approved by the FDA as early as 1978 in treatment of testicular, ovarian and bladder cancer.<sup>1</sup> Platinum-based drugs including cisplatin, carboplatin, and oxaliplatin still remains a backbone in treatment of various cancers. Cisplatin continues to be choice of drug in the treatment of 20 different tumor types including head and neck cancer, lung cancer, and ovarian cancers.<sup>2</sup> Primary target of cisplatin is DNA and binding of cisplatin to DNA results in the formation of DNA adducts. This further activates DNA damage response and apoptotic pathways eventually leading to cell death. Although cisplatin induced molecular aberrations in cells are studied, the activation of specific signaling pathways upon cisplatin treatment are not completely understood, especially the early signaling events. Identifying these early molecular changes will provide clue on the factors that determine the fate of damaged cell toward apoptosis or cell cycle.<sup>3</sup> Understanding these signaling pathways will further pave way to delineate the mechanisms of drug resistance employed by cells and to develop a targeted therapy.

Towards this, Ehardt HA, *et al.*, employed a targeted mass spectrometry-based proteomic approach to determine the immediate response of pancreatic cancer cells to six drugs

and their combinations used in pancreatic cancer treatment.<sup>4</sup> This study showed that the downregulated proteins Jupiter microtubule associated homolog 1 (JPT1), p21 (RAC1) activated kinase 1 (PAK1) and sperm associated antigen 5 (SPAG5) could serve as early indicators of drug efficacy, whereas the upregulated proteins, 14-3-3 and kallikrein related peptidase 2 (KLK2) play a role as potential early drug response markers and could be used in targeted therapy. Musiani D, *et al.*, carried out proteomic analysis of ovarian cancer cells treated with cisplatin for 6 and 24 h. Pim-2 proto-oncogene, serine/threonine kinase (PIM2), a kinase was found to be upregulated in cells treated with cisplatin and overexpression of PIM2 was able to rescue cells from undergoing apoptosis via regulation of BAD. This study has shown that PIM2 to be involved in regulating apoptosis, cell survival, and DNA damage in response to cisplatin, thus proving that PIM2 can be used as druggable target to improve platinum sensitivity.<sup>5</sup> In a recent study, the authors employed proteomics as a tool to understand the mechanisms of action of over 800 compounds by examining the changes in proteome of HCT116 cell line upon 24 h treatment.<sup>6</sup> Mitchell *et al.* and Frenjo *et al.* recently published an activity landscape of 125 cell lines from NCI60 and CRC65 cohort analyzing early proteomics response to over more than 200 drugs identifying markers of drug sensitivity, resistance, and common mode of action.<sup>7,8</sup> However, these repositories lack information pertaining to HNSCC.

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In India, HNSCC is one of the top-most common cancers and oral squamous cell carcinoma (OSCC), a sub-type of HNSCC is the 2<sup>nd</sup> most common cancer.<sup>9</sup> Alcohol consumption, tobacco use (both smoke and smokeless form), and infection by human papilloma virus (HPV) are the major risk factors in the development of oral cancer. Surgery, radiation therapy and chemotherapy are the treatment options available for oral cancer patients. Cisplatin (cis-diaminedichloroplatinum, CDDP) is the most commonly used chemotherapeutic drug for OSCC. However, development of chemoresistance either inherited or acquired has limited the use and efficacy of cisplatin in oral cancer treatment. Several studies have been carried out to understand the mechanisms of cisplatin resistance in head and neck cancer.<sup>1,10</sup> However, there are only a few studies reporting the immediate response of cells to cisplatin. In this study, we employed label-free quantitative mass spectrometry-based approach to understand the immediate protein alterations that occur upon cisplatin treatment. We could identify several proteins to be altered in a panel of HNSCC cell lines upon cisplatin treatment for 24 h. Further, one of the upregulated proteins was validated by Western blot analysis not only in cells exposed to cisplatin but also in cisplatin resistant cells.

## Materials and methods

### Cell culture conditions

Four HNSCC cell lines, A253, FaDu, Det562, and CAL27 were used in this study. All these cell lines were from ATCC. A253 is derived from submaxillary salivary gland of an epidermoid carcinoma patient. A253 cells were cultured in MaCoy's medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin mixture. FaDu, a squamous cell carcinoma cell line derived from a patient with hypopharyngeal tumor was grown and maintained in Roswell Park Memorial Institute (RPMI) medium containing 10% fetal bovine serum and 1% penicillin/streptomycin mixture. Det562 is a pharyngeal carcinoma cell line and CAL27 is a squamous cell carcinoma cell line derived from tongue. These two cell lines were cultured in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin mixture. All these cells were grown at 37°C in a humidified 5% CO<sub>2</sub> incubator.

### Sensitivity of HNSCC cells to cisplatin

Cisplatin used in the study was purchased from Selleckchem Chemicals (catalog #: S1166) and cisplatin stock was prepared as per the manufacture details. HNSCC cells were seeded at a density of  $3 \times 10^3$  cells per well in a 96-well plate. Post-24 h, cells were treated with varying concentration of cisplatin ranging from 1–8  $\mu$ M for 72 h. Viability of cells was further determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The absorbance was measured at 570 and 650 nm using a Microplate Spectrophotometer (Multiskan GO, Thermo Scientific, Bremen, Germany).

### Sample preparation for mass spectrometry analysis

HNSCC cells were grown to 70–80% confluency. Once they reached the required confluency, cells were treated with

cisplatin at their respective IC<sub>50</sub> for 24 h. Untreated cells served as control. Post-treatment, cells from both the conditions were washed with 1X PBS thrice and harvested in 2% SDS buffer containing protease and phosphatase inhibitors. The samples were then sonicated, centrifuged and protein concentration was determined using bicinchoninic acid (BCA) assay. Equal amount of protein lysate from untreated and cisplatin treated condition was subjected to reduction, alkylation and trypsin digestion as described previously.<sup>11</sup> The peptides were then subjected to label-free quantification using mass spectrometry.

### LC-MS/MS analysis

The dried peptides were reconstituted 0.1% formic acid before injecting into the mass spectrometer. Data was acquired on the Orbitrap Fusion Tribrid mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) coupled with Easy n-LC 1000 liquid chromatography system (Thermo Fisher Scientific). Data for Det562 cell line pair was acquired on Q Exactive HF-X Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific) coupled with Dionex Ultimate 3000 HPLC system. Data were acquired in triplicates. Samples were loaded on to a two-column setup, the peptides were first loaded onto the trap column at a flow rate of 6  $\mu$ l/min, followed by separation in the analytical column with a gradient of 8–38% solvent B (96% Acetonitrile in 0.1% Formic acid for Easy nLC 1000 and 80% Acetonitrile in 0.1% Formic acid for Dionex Ultimate 3000) over 165 minutes at a flow rate of 280 nl/min with a total run time of 180 minutes. The peptides were analyzed on an orbitrap in DDA mode with a cycle time of 3 seconds. Resolution at 200 m/z was 120,000 for full scan and 30,000 for MS2. HCD was used for fragmentation of the selected parent ions at 34% NCE (Normalized Collision Energy). Dynamic exclusion was set to 30 seconds. AGC target was set to 4e5 for full scan and 1e5 for MS2 with maximum injection time of 50 ms and 54 ms, respectively.

### Database searches for peptide and protein identification

Data from LC-MS/MS analysis was searched using Proteome Discoverer (version 2.2, Thermo Scientific, Bremen, Germany) suite against NCBI RefSeq database (release 89 with known contaminants). We used SequestHT and MS Amanda as search engines. The search parameters included acetylation on N-terminal of protein; oxidation on methionine as dynamic modification, and carboxyamidomethylation on cysteine as static modification. The enzyme used to generate peptides was trypsin with a maximum of two missed cleavages. Precursor mass tolerance of 10 ppm and fragment mass tolerance of  $\pm 0.05$  Da was applied. FDR of 1% was applied at the peptide spectrum match (PSM), peptide and protein level. Minora feature detector node was used with feature mapper for label free quantification of peptides and proteins.

### Availability of data

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE<sup>12</sup> partner repository with the dataset identifier PXD045610.

### Bioinformatics analysis

The pathway enrichment analysis of the differentially regulated proteins was performed using Reactome Pathway database using the ClueGo (version 2.5.8) plug-in in Cytoscape (version 3.9.1) software.<sup>13,14</sup> The protein-protein interaction network was constructed using the software Pathvisio.<sup>15</sup>

### Western blot analysis

CAL27 and Det562 cells were treated with cisplatin at IC<sub>50</sub> for 24 h and harvested in RIPA buffer containing protease and phosphatase inhibitors. Similarly, untreated and cisplatin resistant CAL27 and Det562 cells were cultured to 70% confluence and harvested in RIPA lysis buffer. Western blot analysis was carried out as described previously<sup>11</sup> and was repeated thrice. Antibody to TUBB3 was purchased from Abcam (Catalog #: ab231083).  $\beta$ -

actin antibody used as loading control was purchased from Sigma (Catalog #: A5316).

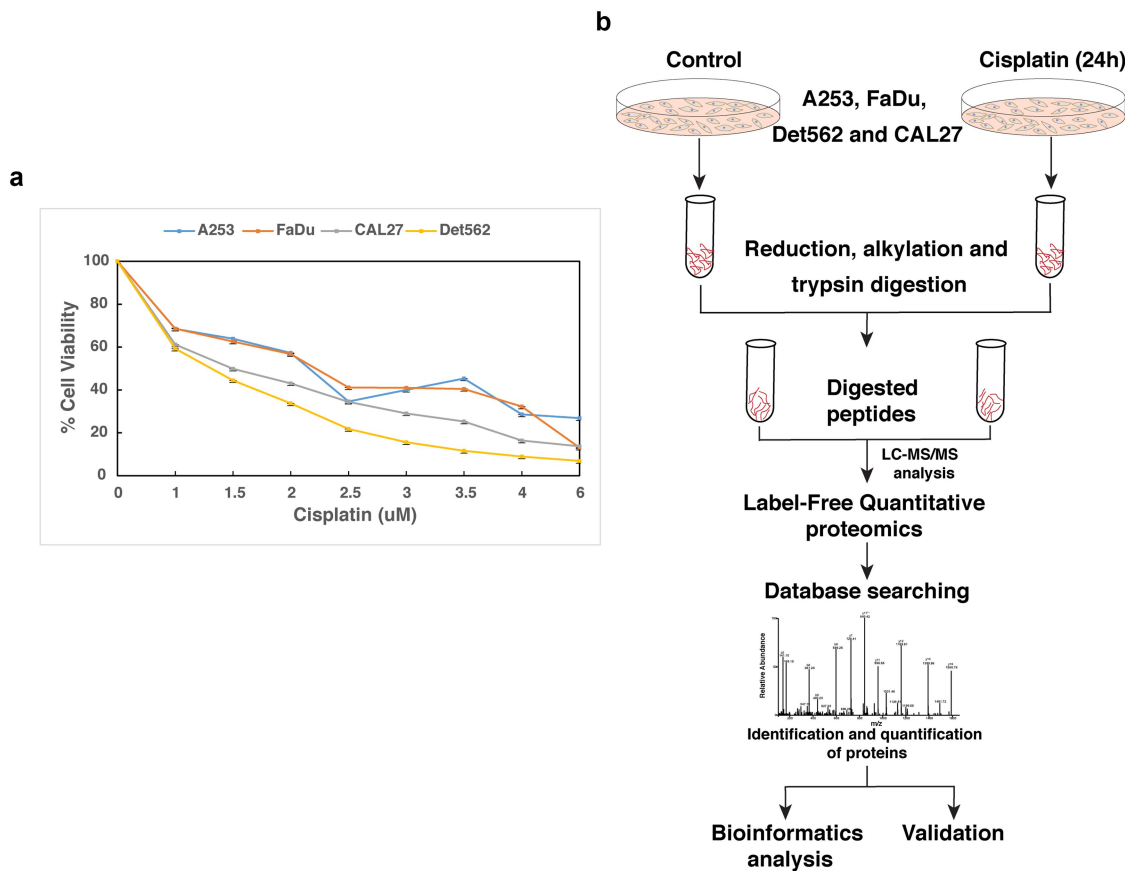
### Cellular proliferation assay

Cisplatin resistant and control CAL27 and Det562 cells were seeded at a density of  $3 \times 10^3$  cells per well in triplicate in 96-well plates. Cellular proliferation was monitored for a period of 6 days. The proliferation rate of cells was determined by MTT assay as described above at different time intervals, day 2, day 4, and day 6.

## Results

### Determination of half-maximum inhibitory concentration (IC<sub>50</sub>) of HNSCC cell lines

HNSCC cell lines, A253, CAL27, Det562, and FaDu were treated with varying concentrations of cisplatin and their IC<sub>50</sub> was determined by MTT assay. As shown in Figure 1a, the IC<sub>50</sub> for A253, FaDu, Det562, and CAL27 was found to be 1.5  $\mu$ M, 2  $\mu$ M, 1.5  $\mu$ M, and 2  $\mu$ M, respectively. Further, to understand the early molecular changes induced in response to cisplatin treat-



**Figure 1.** (a) Response of HNSCC cell lines, A253, CAL27, Det562 and FaDu to cisplatin: HNSCC cells were treated with varying concentration of cisplatin for 72 h and IC<sub>50</sub> was determined by MTT assay. The IC<sub>50</sub> of A253, FaDu, Det562 and CAL27 was found to be 1.5  $\mu$ M, 2  $\mu$ M, 1.5  $\mu$ M and 2  $\mu$ M, respectively (b) Workflow employed to identify proteins altered in response to cisplatin. HNSCC cells were treated with cisplatin at their IC<sub>50</sub> for 24 h. Untreated cells served as control. Equal amount of protein from both the conditions was subjected to trypsin digestion followed by mass spectrometry analysis.

ment, mass spectrometry-based label-free quantitative (LFQ) proteomic approach was employed.

### Mass spectrometry-based analysis of head and neck cancer cells treated with cisplatin

Studies have shown that a 24 h time point would be an appropriate time point to profile the perturbed proteome in response to a pharmacological agent and to elucidate the mechanisms of action of several drugs.<sup>4,6</sup> Cisplatin is shown to induce migration and invasion of cancer cells and expression of several proteins implicated in cisplatin resistance following 24 h treatment.<sup>16,17</sup> A comparative phosphoproteomic analysis of ovarian cancer cells treated with cisplatin for 6 h and 24 h, showed an enhancement of signaling events at 24 h treatment compared to 6 h treatment duration.<sup>5</sup> Thus, in this study, we choose an 24 h timepoint to understand the early proteomic changes that occur upon cisplatin treatment. HNSCC cell lines were grown to 70% confluency and treated with cisplatin at IC<sub>50</sub> for 24 h. Following 24 h treatment, cells from both the conditions were harvested and processed for mass spectrometry analysis as shown in Figure 1b. LC-MS/MS analysis led to the identification of 5,060, 4,816, 4,537, and 4,142 proteins in A253, FaDu, Det562, and CAL27, respectively. Among these, 230 proteins were upregulated and 122 proteins were found to be downregulated in cisplatin treated cells

compared to untreated cells (with high confidence and 2-fold cutoff). Complete list of proteins identified in the study is provided in Supplementary Table S1. Partial list of differentially regulated proteins is provided in Table 1.

### Effects of cisplatin on DNA

DNA is the primary target of cisplatin and binding of cisplatin results in the formation of inter- and intra-strand crosslinks. To overcome these damages, cells activate DNA damage repair pathways such as nucleotide excision repair pathway, trans lesion polymerase, and homologous recombination.<sup>18</sup> In this study, approximately 60 proteins associated with DNA damage response and repair exhibited altered expression levels following cisplatin treatment. A list of some of these proteins is provided in Table 2. The protein-protein interaction (PPI) network for these proteins was manually constructed based on evidence from published literature using the tool, Pathvisio. Figure 2 illustrates the resulting protein-protein interaction network. Among 64 proteins implicated in DNA damage response and repair, around 50 proteins were found to interact in the PPI network.

### Pathway analysis of differentially expressed proteins

A pathway-based enrichment analysis of differentially regulated proteins was carried using Reactome pathway

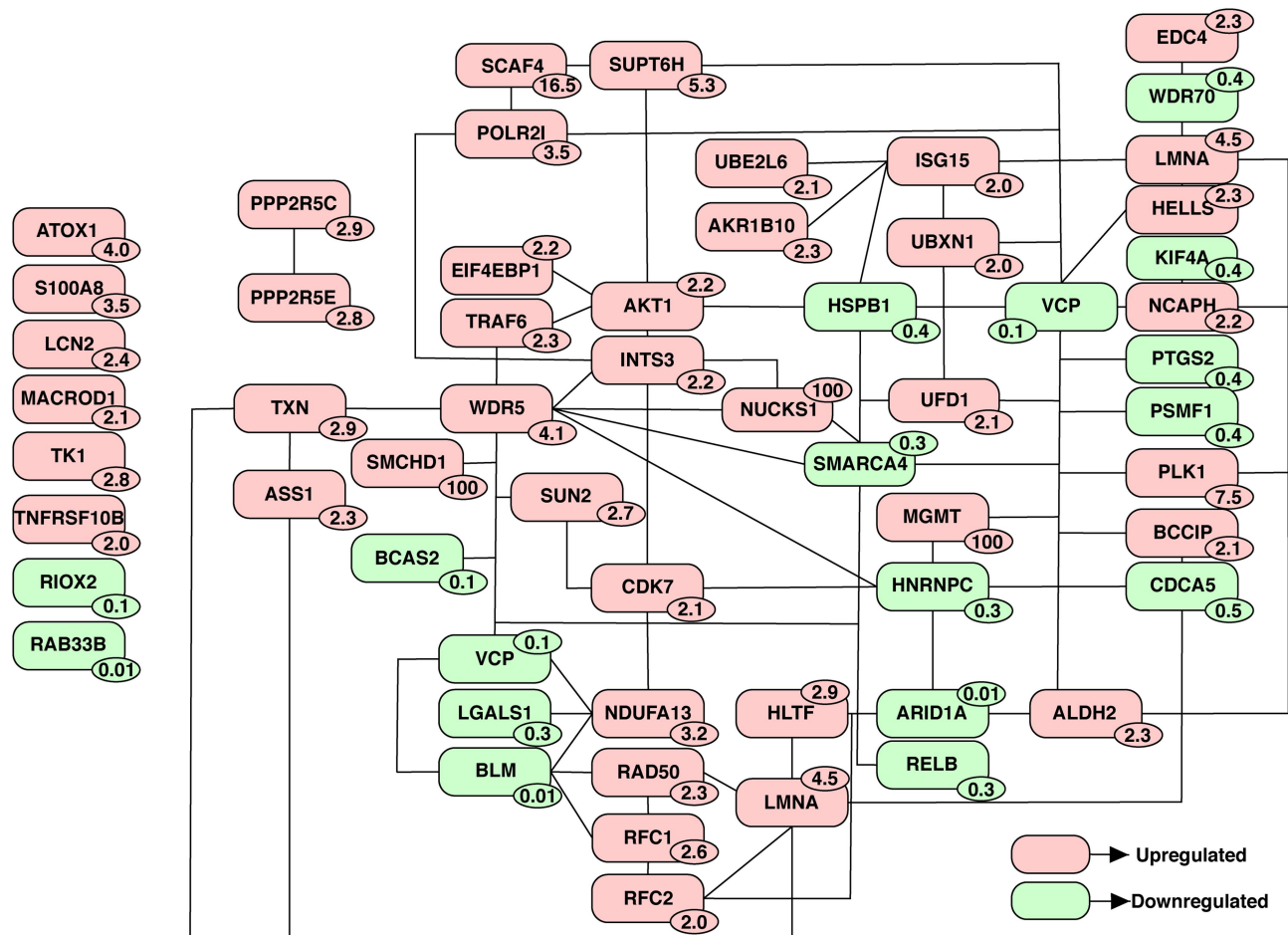
**Table 1.** Partial list of proteins that are differentially regulated in cisplatin treated HNSCC cell lines compared to untreated cells.

Gene Symbol	Description	Cisplatin treated (24 h)/untreated (Fold change)	Associated biological process
CREBBP	CREB-binding protein	16.9	Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism
PLK1	serine/threonine-protein kinase PLK1	7.5	Cell communication; Signal transduction
POLR2I	DNA-directed RNA polymerase II subunit RPB9	3.5	Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism
EIF4EBP1	eukaryotic translation initiation factor 4E-binding protein 1	2.2	Chromatin remodeling
AKT1	RAC-alpha serine/threonine-protein kinase	2.2	Cell communication ; Signal transduction
HMGA2	high mobility group AT-hook 2	0.3	Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism
TSSC4	tumor suppressing subtransferable candidate 4	0.4	spliceosomal snRNP assembly
KIF4A	kinesin family member 4A	0.4	Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism
SMARCA4	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4	0.3	Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism
THBS1	thrombospondin 1	0.2	Cell growth and/or maintenance

**Table 2.** Partial list of proteins involved in DNA damage response and repair and are differentially expressed upon cisplatin treatment.

Gene Symbol	Description	Cisplatin treated (24 h)/untreated (Fold change)	Associated Biological Process
MGMT	methylated-DNA-protein-cysteine methyltransferase	100	DNA Methylation
ATOX1	copper transport protein ATOX1	4	Copper ion transport
AKR1B10	aldo-keto reductase family 1 member B10	2.3	Cellular detoxification of aldehyde
NDUFA13	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 13	3.2	Apoptotic signaling pathway
RAD50	DNA repair protein RAD50	2.3	DNA repair
CDK7	cyclin-dependent kinase 7	2.1	Cell cycle
HSPB1	heat shock protein family B (small) member 1	0.4	Signal transduction
SMARCA4	transcription activator BRG1	0.3	Chromatin remodeling
HNRNPC	heterogeneous nuclear ribonucleoprotein C	0.3	Chromatin remodeling
LGALS1	galectin 1	0.3	Apoptotic process
RELB	transcription factor RelB	0.3	Signal transduction
PTGS2	prostaglandin-endoperoxide synthase 2	0.4	Enzyme binding





**Figure 2.** Protein-protein interaction (PPI) network of proteins implicated in DNA damage response and repair. Proteins associated with DNA damage and repair were shortlisted from list of proteins altered upon cisplatin treatment. An PPI network was created based on literature evidence using the tool, Pathvisio. The number on each protein indicates the fold change observed upon cisplatin treatment compared to untreated cells.

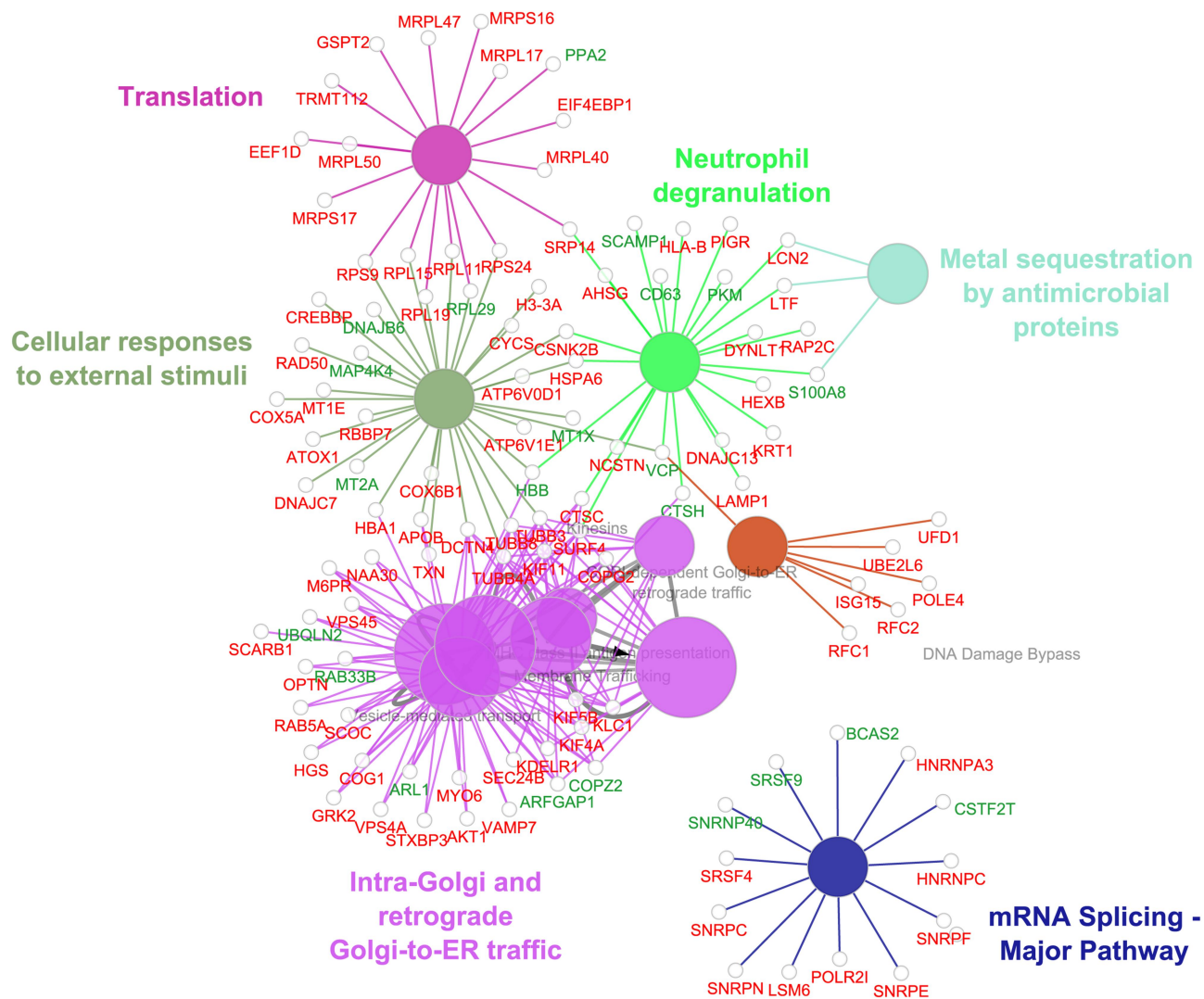
database in Cytoscape software. In addition to proteins implicated in DNA damage bypass pathway, proteins involved in translation and mRNA splicing were found to be enriched (Figure 3). Some of the proteins implicated in DNA damage bypass pathway include Ubiquitin conjugating enzyme E2 L6 (UBE2L6), ISG15 ubiquitin like modifier (ISG15) and DNA polymerase epsilon 4, accessory subunit (POLE4), replication factor C subunit 1 (RFC1), and replication factor C subunit 2 (RFC2) were found to be upregulated in this study. Several proteins reported to play a role in mRNA translation were enriched in response to cisplatin treatment. The expression of several ribosomal proteins, ribosomal protein S9 (RPS9) ribosomal protein S24 (RPS24) ribosomal protein L15 (RPL15), ribosomal protein L11 (RPL11), ribosomal protein L19 (RPL19), and ribosomal protein L29 (RPL29) involved in translation were also found to be altered in cisplatin treated HNSCC cell lines.

Further, proteins associated with mRNA splicing were found to be altered in response to cisplatin treatment. Heterogeneous nuclear ribonucleoprotein C (HNRNPC), heterogeneous nuclear ribonucleoprotein A3 (HNRNPA3), BCAS2 pre-mRNA processing factor (BCAS2), small nuclear ribonucleoprotein polypeptides, SNRPC, SNRPN, SNRPF, and SNRPE and serine and arginine rich splicing factors SRSF9 and

SRSF4 were enriched. Heterogeneous nuclear ribonucleoproteins, HNRNPC and HNRNPA3 have been previously reported to be overexpressed in cisplatin resistant cell lines.<sup>19,20</sup> It has been shown that HNRNPC overexpression promotes chemoresistance in gastric cancer cell lines.<sup>19</sup> Splicing factor, serine and arginine rich splicing factor 4 (SRSF4) has been shown to play a role in cisplatin induced pre-mRNA splicing and apoptosis of gastric cancer cells.<sup>21</sup>

### Validation of tubulin beta 3 class III (TUBB3) expression in cisplatin treated HNSCC cells

To determine the role of proteins that were altered upon cisplatin treatment (24 h) in platinum resistance, we compared data from this study with proteins previously linked to platinum resistance. A study by Huang D., *et al.*, has reported over 900 proteins to be associated with platinum resistance.<sup>22</sup> Of the 343 proteins that were differentially regulated upon cisplatin treatment in this study, we observed an overlap of over 45 proteins with those known to be associated with platinum resistance (Figure 4a). This serves as a proof-of-concept that these proteins undergo alterations as early as 24h and could play a role in developing platinum resistance. Tubulin beta 3 class III (TUBB3) is one of the proteins that was found to be



**Figure 3.** Pathway enrichment analysis of proteins altered in response to cisplatin treatment was carried out using ClueGo plugin in Cytoscape. This analysis revealed enrichment of proteins implicated translation, mRNA splicing and DNA damage bypass pathways among others to be enriched upon cisplatin treatment. Proteins highlighted in red indicates upregulation and downregulated proteins are in green.

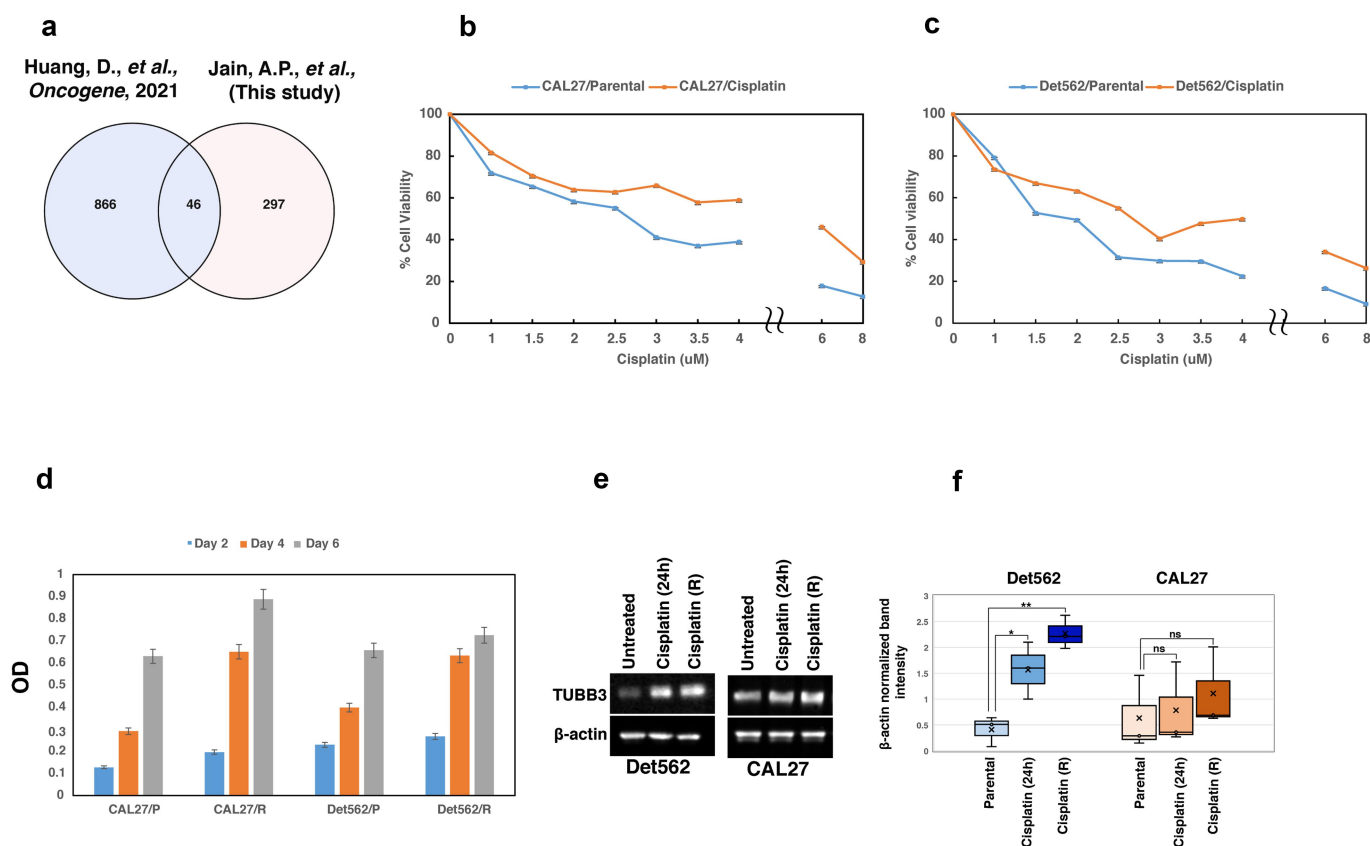
common and is also reported in literature to be overexpressed upon acquisition of platinum resistance.<sup>23–25</sup> Mass spectrometry analysis revealed upregulation of TUBB3 in response to cisplatin treatment (24 h) compared to control cell lines. We further validated the expression of TUBB3 in CAL27 and Det562 cells treated with cisplatin for 24 h and in cells that have acquired resistance to cisplatin on chronic treatment.

Cisplatin resistant, CAL27 and Det562 cell lines were developed as described previously.<sup>26</sup> Briefly, CAL27 and Det562 cells were treated with cisplatin at their  $IC_{50}$  for 72 h. Following this, the cells were maintained in cisplatin-free media until the surviving cells recovered. This process was repeated for 3 months. The  $IC_{50}$  of parental and cisplatin treated cells was then determined by MTT assay. As shown in Figure 4b–c, chronic treatment of CAL27 and Det562 cells with cisplatin led to an increase in  $IC_{50}$  compared to the parental cells indicating the acquisition of cisplatin resistance phenotype. Further, we observed that cisplatin resistant, CAL27 and Det562 cells exhibited higher cellular proliferation rate compared to untreated or parental cells (Figure 4d). Western blot

analysis confirmed the overexpression of TUBB3 in cells treated with cisplatin for 24 h, as well as in cisplatin resistant CAL27 and Det562 cell lines compared to untreated cells (Figure 4e–f). These results indicate that subsets of proteins implicated in protection against cisplatin are activated as early as 24 h and these could be molecules that enable cells to evade cisplatin induced damage.

## Discussion

Studies have shown that determining response of cells to short-term drug treatment will provide in-sights into the i) mechanisms of action of drugs and ii) adaptive mechanisms employed by cells to counteract the anti-proliferative effects of drugs.<sup>4,6</sup> In the past, mass spectrometry-based proteomic approaches have been successfully employed to elucidate the mechanisms of action of various drugs.<sup>4,6,7</sup> However, the precise mechanisms of action of cisplatin are not completely understood, despite its widespread use in cancer treatment. The current study demonstrates the successful application of label-free quantitative proteomic approach to examine the alterations



**Figure 4.** (a) Venn diagram showing the list of proteins that were common between this study and proteins associated with platinum resistance. (b-c) Development of cisplatin resistant cell lines. CAL27 and Det562 cells were treated with cisplatin at their  $IC_{50}$  for 3 months. The  $IC_{50}$  of both the cells were found to increase with chronic cisplatin treatment indicating acquisition of resistant phenotype. (d) CAL27 and Det562 cisplatin resistant cells exhibited higher cellular proliferation rate compared to untreated or parental cell lines; ((e-f) Expression of TUBB3 in untreated and cisplatin treated CAL27 and Det562. TUBB3 was found to be overexpressed in cells exposed to cisplatin compared to untreated cells. Cisplatin resistant cells (R) also showed a higher expression of TUBB3 compared to cells treated with cisplatin for 24 h. The expression of TUBB3 was found to be statistically significant in Det562 cells.  $\beta$ -actin was used as loading control.

in the cellular proteome of multiple HNSCC cell lines induced by short-term cisplatin treatment. Using more than one cell type in a study will lead to ii) high proteome coverage, ii) an in-depth knowledge on the signaling mechanisms induced by cisplatin, and iii) enhanced statistical power.

Exposure of HNSCC cell lines to cisplatin for 24 h resulted in the alterations of various cellular processes including DNA damage response pathway, translation and mRNA splicing. The response of cells to a genotoxic insult can result either in apoptosis of cells due to damaged DNA or repair of DNA damage that enables cell survival. A literature-based analysis of data from this study revealed several proteins implicated in DNA damage response and repair to be altered. Proteins such as RAD50 double strand break repair protein (RAD50), Aldo-keto reductase family 1 member B10 (AKR1B10), aldehyde dehydrogenase 2 family member (ALDH2), and S100 calcium binding protein A8 (S100A8) implicated in DNA damage response pathway were found to be altered within 24 h of treatment. Further, we observed proteins implicated in translation and mRNA splicing that are associated with cisplatin resistance to be enriched. These results indicate that the cells activate protective mechanisms to cisplatin as early as 24 h of exposure and these proteins may contribute to the development of cisplatin resistance.

In addition to proteins implicated in regulating diverse cellular functions, a subset of differentially expressed proteins from our study was found to be associated with platinum resistance. LC-MS/MS analysis revealed several microtubule proteins such as TUBB3, TUBB4A, and TUBB8 to be altered upon cisplatin treatment in HNSCC cell lines. Microtubules are the cytoskeletal proteins implicated in various processes including cell motility, intracellular transport, and mitosis.<sup>27</sup> TUBB3 is reported to be expressed in neuronal cells, melanocytes, and follicular lymphoid cells.<sup>28</sup> In cancers such as HNSCC, lung, ovarian, and bladder cancer, TUBB3 is shown to be overexpressed.<sup>29–32</sup> Patients with high expression of TUBB3 exhibit poor survival rate and resistance to docetaxel, paclitaxel- and cisplatin-based chemotherapies.<sup>24,33–37</sup> Head and neck cancer patients positive for TUBB3 expression and undergoing induction chemotherapy with 5-Fluorouracil (5-FU) and cisplatin and 5-FU, cisplatin and docetaxel exhibited lower response rate. In addition, they also showed a higher disease progression and reduction in overall survival rate compared to TUBB3-negative patients.<sup>32</sup> These studies indicate the significance of TUBB3 as a predictive and prognostic marker not only in HNSCC patients but also in other cancers. In the current study, we observed upregulation of TUBB3 upon cisplatin treatment. Western blot analysis further confirmed the overexpression of TUBB3 upon short-

term and chronic exposure to cisplatin in CAL27 and Det562 cell lines. The exact mechanisms of role of TUBB3 in inducing cisplatin resistance is not known. However, a study by Gan, P.P., *et al.*, has shown that silencing of TUBB3 sensitizes non-small cell lung carcinoma (NSCLC) cells to DNA damaging agents including cisplatin and induces apoptosis in response to cisplatin treatment indicating its role in drug sensitivity.<sup>38</sup> Though the current study provides a clue that TUBB3 can serve as a reliable predictive marker of cisplatin sensitivity in HNSCC patients, additional *in vitro* and *in vivo* studies are warranted to ascertain its clinical utility. Targeted mass spectrometry-based validation of TUBB3 in large cohort of samples from HNSCC patients undergoing neoadjuvant therapy and from chemotherapy responders and non-responders will enhance the knowledge on utility of TUBB3 as a predictive marker. In addition to TUBB3, the present study, however, does not rule out the role of other molecules in inducing cisplatin resistance.

## Conclusions

In summary, this study reveals the molecular level alterations that occur in response to short-term treatment to cisplatin in head and neck cancer. We demonstrate that certain molecules that play a role in the development of cisplatin resistance are activated as early as 24 h. These could be the molecules that enable cells to adapt to cisplatin and escape cell death resulting in the acquisition of resistance phenotype. However, more *in vivo* and *in vitro* studies are warranted to understand the role of these proteins in inducing cisplatin resistance and their role as potential predictive markers of cisplatin therapy.

## Disclosure statement

No potential conflict of interest was reported by the author(s).

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## Authors' contributions

A.P.J and V.N conceived and designed the study. A.P.J. and V.N. were involved in cell culture and performed all assays. V.G. F.A.B and G. D. performed proteomics experiments, fractionation and carried out mass spectrometric analysis of samples. S.M. carried validation experiments. A.P.J., G.D., and V.N. prepared the manuscript, figures and tables. All the authors have read and approved the final manuscript.

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