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Silencing of high-mobility group box 2 (HMGB2) modulates cisplatin and 5-fluorouracil sensitivity in head and neck squamous cell carcinoma

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Abstract

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6 Conflict of interest

The authors have declared no conflict of interest.

Dysregulation of protein expression is associated with most diseases including cancer. Mass spectrometry-based proteomic analysis is widely employed as a tool to study protein dysregulation in cancers. Proteins which are differentially expressed in head and neck squamous cell carcinoma (HNSCC) cell lines compared to the normal oral cell line could serve as biomarkers for patient stratification. To understand the proteomic complexity in HNSCC, we carried out iTRAQ-based mass spectrometry analysis on a panel of HNSCC cell lines in addition to a normal oral keratinocyte cell line. LC-MS/MS analysis of total proteome of the HNSCC cell lines led to the identification of 3,263 proteins, of which 185 proteins were overexpressed and 190 proteins were downregulated more than 2-fold in at least two of the three HNSCC cell lines studied. Amongst the overexpressed proteins, 23 proteins were related to DNA replication and repair. These included high mobility group box 2 (HMGB2) protein, which was overexpressed in all three HNSCC lines studied. Overexpression of HMGB2 has been reported in various cancers, yet its role in HNSCC remains unclear. Immunohistochemical labeling of HMGB2 in a panel of HNSCC tumors using tissue microarrays revealed overexpression in 77% (54 of 70) of tumors. The HMGB proteins are known to bind to DNA structure resulting from cisplatin-DNA adducts and affect the chemosensitivity of cells. We observed that siRNA-mediated silencing of HMGB2 increased the sensitivity of the HNSCC cell lines to cisplatin and 5-FU. We hypothesize that targeting HMGB2 could enhance the efficacy of existing chemotherapeutic regimens for treatment of HNSCC.

Keywords

antimetabolite; biomarker; drug resistance; *in vitro* labeling

1 Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer and is a major cause of cancer mortality and morbidity [1]. Despite several advancements in our understanding of HNSCC, the 5 year survival rate remains unchanged at 50% for the last three decades. This highlights the need for biomarkers that can be used for early diagnosis of HNSCC or can serve as prognostic markers. Mass spectrometry based proteomic approaches are useful in the discovery of dysregulated proteins which can likely serve as markers for early detection, disease prognosis or patient stratification [2].

Considerable efforts have been made to characterize the protein alterations in HNSCC. However, most of these studies were based on two-dimensional gel electrophoresis (2DE) and matrix-assisted laser desorption/ionization (MALDI) based techniques [3]. High resolution mass spectrometry enables in-depth proteomic profiling and hence can pave way for the discovery of new biomarkers/targets; leading to more effective treatment regimens that have the potential to reduce morbidity and mortality. Our lab has previously carried out quantitative proteomic analysis comparing the secretome from HNSCC cell lines with normal oral keratinocyte cell line [4]. In another recent study, 90 putative biomarkers were reported using label free quantitative proteomics approach integrated with gene expression analysis [5]. To identify proteins which are differentially expressed in HNSCC cell lines compared to the normal cell line, we carried out an iTRAQ-based quantitative proteomic analysis of HNSCC cell lines compared to a normal oral keratinocyte cell line. iTRAQ is an

in vitro chemical labeling strategy that enables simultaneous identification and quantitation of proteome changes from up to 8 different conditions in a single experiment. It is a robust technique compared to other proteomic strategies such as label free approach which requires a longer analysis time and SILAC, a metabolic labeling strategy that allows quantitation of only 5 conditions in a single experiment. Additionally, as iTRAQ labeling involves isobaric tags it causes an increase in the precursor ion intensity resulting in improved MS/MS fragmentation and hence more confident peptide and protein identifications. Liquid chromatography and high resolution tandem mass spectrometry (LC-MS/MS) analysis led to the identification of 375 differentially expressed proteins in at least two of the three HNSCC cell lines compared to normal keratinocytes. Amongst the differentially expressed proteins, high mobility group box B (HMGB2) expression was found to be more than two fold higher in all three HNSCC cell lines studied. Literature evidence indicates both HMGB1 and HMGB2 as prognostic markers for bladder carcinoma [6] and squamous cell carcinoma of skin [7]. Elevated HMGB2 expression has previously been linked to poor prognosis in hepatocellular carcinoma [8] and ovarian cancer [9]. Studies have indicated that high mobility group proteins increase the sensitivity to cisplatin in lung cancer cell lines [10]. Chemotherapy is a major component of HNSCC treatment and is used in conjunction with surgery and radiotherapy [11]. Cisplatin and 5-fluorouracil (5-FU) are administered along with docetaxel in the management of locally advanced HNSCCs [12]. Resistance to cisplatin is a common phenomenon in HNSCC with the response rate to treatment being 25% [13–14]. Resistance to 5-FU during the course of treatment is also a common occurrence and a leading cause of failure of therapy [12].

Several mechanisms of cisplatin resistance have been described [15–16] and it is evident that the cellular processing of platinum provides molecular mechanisms involved in cisplatin response. Cisplatin binds to the N7 of the purine bases to form DNA adducts [17]. The adducts are identified by many damage recognizing proteins which include HMGB1 and HMGB2 (also known as HMG1 and HMG2, respectively) [18] leading to activation ATR (ATM-and Rad3 related protein) kinases, ultimately resulting in the activation of p53, cell cycle arrest and apoptosis [19]. Nucleotide excision repair is one of the pathways known to remove cisplatin lesions from DNA [20]. HMGB proteins have been shown to bind to cisplatin-modified DNA and impairing nucleotide excision repair [18]. Despite these studies, resistance to cisplatin in HNSCC persists as a major problem. We addressed the role of HMGB2 in chemo responsiveness of HNSCC cells to cisplatin and 5-FU.

5-FU is an antimetabolite used widely in the treatment of HNSCC in combination with cisplatin. Antimetabolite agents are compounds which share a structural similarity with precursors of purines and pyrimidines and thereby interfere with their normal synthesis. 5-FU shares a structural similarity with uracil, a major component of RNA. Inside the cells, 5-FU is converted into various compounds including fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP) and fluorouridine triphosphate (FUTP). Thymidylate synthase is the enzyme responsible for *de novo* synthesis of deoxythymidylate and it is a crucial rate-limiting enzyme of DNA synthesis [21]. FdUTP forms a slow reversible covalent complex with thymidylate synthase leading to depletion of thymidine nucleotides and impairment of DNA synthesis. FUTP also mis-incorporates into RNA

affecting the normal functioning of RNA as well [12]. Although studies have been undertaken to understand the mechanisms of resistance to chemotherapeutic agents, effective strategies against 5-FU resistance in HNSCC have not yet been elucidated. Hence, identification of additional targets for personalized therapy is much needed, which will help overcome the resistance to chemotherapeutic agents

2 Materials and methods

2.1 Cell lines and culture conditions

OKF6/TERT1 is a normal oral keratinocyte cell line derived from floor of the mouth. It was a kind gift from Dr. James Rheinwald (Brigham and Women's Hospital, Boston, MA) and was cultured and maintained in KSFM (keratinocyte-serum free media; Life Technologies, Grand Island, NY) supplemented with bovine pituitary extract (25 mg/ml), calcium chloride (0.4 mM), epidermal growth factor (0.2 ng/ml) and 1% penicillin/streptomycin. HNSCC cell lines JHU-O22, JHU-O11 are derived from larynx; JHU-O29, JHU-O28 and FaDu are derived from base of tongue, oral cavity and pharynx respectively. These were cultured and maintained in RPMI medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. All cell lines were maintained in a humidified incubator with 5% CO₂ at 37°C.

2.2 iTRAQ labeling and SCX fractionation

Cells were lysed in 0.5% SDS and protein estimation was carried out using the BCA method. Equal amount of protein (200 µg) from each cell line was used for iTRAQ labeling, which was carried out essentially as described previously [22]. Briefly, reduction was carried out using tris (2-carboxyethyl) phosphine (TCEP) at 60°C for 1 h followed by alkylation using methyl methanethiosulfonate (MMTS) for 20 min at room temperature. Digestion was carried out using sequencing grade trypsin (Promega, San Luis Obispo, CA) at an enzyme to substrate ratio of 1:20 w/w for 16 h at 37°C. Tryptic digests were labeled with 8-plex iTRAQ reagents (AB SCIEX, Washington, D.C.). For technical replicate analysis, OKF6/TERT1 peptide digest were labeled with 113 and 114. Similarly, JHU-O11 was labeled with 115 and 116, JHU-O22 with 117 and 118 and JHU-O29 were labeled with 119 and 121 labels. The digests were incubated with labels for 2 h at room temperature. Labeled samples were pooled and vacuum dried.

Samples were reconstituted using Solvent A (10 mM KH₂PO₄, 25% acetonitrile, pH 2.8) and the pH was adjusted to pH 2.8 using ortho-phosphoric acid. The samples were fractionated on PolySULFOETHYL A column (200 × 4.6 mm, 5 µm, 200 Å, PolyLC Inc., Columbia, MD) using an Agilent 1260 series HPLC system equipped with binary pump, manual injector, variant wavelength detector and a fraction collector. Peptides were fractionated by a linear gradient of 0–60 % solvent B (10 mM KH₂PO₄, 350 mM KCl, 25% acetonitrile, pH 2.8) over 50 min at a constant flow rate of 350 µl/min. The collected fractions were pooled into 20 fractions, reconstituted in 0.1% formic acid, desalted using C₁₈ StageTips (3M Empore, St. Paul, MN) [23]. Samples were stored at –80°C until further analysis.

2.3 LC-MS/MS analysis

LC-MS/MS analysis was carried out using an LTQ-Orbitrap Velos mass spectrometer (Thermo Scientific, Bremen, Germany) interfaced with Easy-nLC II system (Thermo Scientific, Bremen, Germany). Nanospray source was fitted with an emitter tip 10 μm (New Objective, Woburn, MA) and a voltage of 2 kV was applied. Samples were loaded and washed for 5 min on an enrichment column (75 $\mu\text{m} \times 2 \text{ cm}$, Magic C₁₈Aq, 5 μm , 100Å using Solvent A (0.1% formic acid) using Solvent A (0.1% formic acid) at a flow rate of 3 $\mu\text{l}/\text{min}$. Samples were resolved on an analytical column (75 $\mu\text{m} \times 12 \text{ cm}$, Magic C₁₈ Aq, 3 μm , 100Å,) at a flow rate of 350 nl/min using a linear gradient of 7–30% of Solvent B (0.1% formic acid in 95% acetonitrile) over 75 minutes. MS and MS/MS scans were acquired using the Orbitrap mass analyzer at a mass resolution of 60,000 at 400 m/z and 15,000 at 400 m/z, respectively. Full MS scans were acquired in m/z range of 350–1800 m/z. In each duty cycle, 20 most abundant ions with charge states ≥ 2 were sequentially isolated to a target value of 50,000 ions and fragmented in the higher-energy collision dissociation cell using 41% normalized collision energy. Dynamic exclusion was enabled with precursors fragmented once and then excluded for next 45 seconds within 10 ppm error window. Isolation width was set to 1.90 m/z. The automatic gain control for full MS scan was set to 1×10^6 ions and for MS/MS scan was set to 5×10^4 ions with a maximum injection time of 100 ms and 300 ms respectively. The lock mass option was enabled for accurate mass measurements using polydimethylcyclsiloxane (m/z, 445.1200025) ion.

2.4 Data analysis

Proteome Discoverer (Version 1.4) software suite (Thermo Scientific, Bremen, Germany) was used to carry out protein identification and quantitation. Raw mass spectrometry data was searched against NCBI Human RefSeq protein database (Release 50 containing 33,833 sequences) supplemented with common contaminants using Mascot (version 2.2.0) and Sequest search algorithms. The search parameters included trypsin as the protease with maximum of 1 missed cleavage allowed; oxidation of methionine was set as a dynamic modification while static modifications included methylthio at cysteine and iTRAQ modification at N-terminus of the peptide and lysine. Precursor mass tolerance was set to 20 ppm and fragment mass tolerance was set to 0.1 Da. The false discovery rate (FDR) was calculated by carrying out decoy database searches and peptides scoring better than 1% FDR score cut-off were considered for further analysis [24]. iTRAQ quantitation for each identified PSM was carried out by taking the average of reporter ion intensities from technical replicates and peptides identified uniquely for each protein were considered for protein level quantitation. PSMs with more than 30% variability between any technical replicates were excluded from quantitation.

Proteins were classified based on the biological process, molecular function and subcellular localization using Human Protein Reference Database (HPRD: <http://www.hprd.org>), which is a Gene Ontology (GO) compliant database [25–26]. Network analysis using Ingenuity database was done for the 185 proteins which were overexpressed ≥ 2 fold in at least 2 of the HNSCC cell lines.

2.6 Data availability

The mass spectrometry data is publicly available and accessible to other researchers. The complete mass spectrometry data generated from this study has been deposited to the ProteomeXchange (<http://www.proteomexchange.org>) via PRIDE partner repository [27] with the dataset identifier PXD000737. The immunohistochemistry data was submitted to Human Proteinpedia [28] and can be visualized with the link http://www.humanproteinpedia.org/Experimental_details?exp_id=TE-531556 for adjacent non-neoplastic tissue and http://www.humanproteinpedia.org/Experimental_details?can_id=105422 for tumor tissues.

2.7 Immunohistochemistry

HNSCC tissue arrays (containing 70 cases of HNSCC) were obtained from Department of Otolaryngology, Johns Hopkins University. The TMAs were processed and immunohistochemical staining was carried out essentially as described previously [29]. Briefly, the formalin fixed paraffin embedded tissue sections were deparaffinised prior to antigen retrieval by addition of antigen retrieval buffer (Tris-EDTA buffer) for 20 min. A blocking solution (methanol and H₂O₂ mixed at 4:1 ratio) was used to quench the endogenous peroxidases and then washed with wash buffer (phosphate buffered saline with 0.05% Tween), polyclonal anti-HMGB2 antibody (Cat #: HPA003506, Sigma-Aldrich, St. Louis, MO) was used as a primary antibody at a dilution of 1:50. The sections were incubated overnight with primary antibody at 4°C. Then the sections were rinsed with wash buffer followed by incubation with rabbit secondary antibody conjugated horseradish peroxidase (Merck-Bangalore Genei, India) The staining was developed for 5 min using DAB chromogen (Dako, Glostrup, Denmark), followed by counterstaining with hematoxylin (Nice Chemicals, Kochi, India). The tissue sections were then examined under the microscope by pathologist and the immunohistochemical labeling was assessed to score the intensity of staining. The staining was scored on a scale of 0–2, where 0 represented absent staining, +1 represented faint staining and +2 represented intense staining. Cases that were +2 were considered as positively stained cases. Chi-square test was performed to determine statistical significance in difference of expression between HNSCC tissue and non-tumor tissue.

2.8 Western blot analysis

Whole cell extracts were prepared using RIPA buffer (10 mM Tris pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton-X-100, 0.1% SDS) containing protease inhibitors (Roche, Indianapolis, IN). Rabbit polyclonal antibody was obtained from Abcam (Cat# ab11973, Cambridge, UK). Western blot analysis was performed as previously described [30] using 30 µg protein lysate.

2.9 Cisplatin/5-FU sensitivity assays

HNSCC cells were seeded in 96-well plate (2×10^4 cells/well) and treated with cisplatin (0, 1, 2, 4, 6, 8, 10, 25, µg/ml) or 5-FU (0, 0.1, 0.5, 1, 2, 4, 8, 16 µg/ml) for 72 h. Cell viability was determined using MTT assays as previously described [31]. Absorbance was measured at 570 and 650 nm on a Microplate Spectrophotometer (Multiskan GO, Thermo Scientific,

Bremen, Germany). ON-TARGETplus SMARTpool scrambled siRNA and *HMGB2* siRNA were purchased from Dharmacon (Lafayette, CO). JHU-O28, JHU-O22, JHU-O29, and FaDu cells were seeded in 96-well plate (1×10^4 cells/well) and transfected with either scrambled siRNA or 10nM and 20nM *HMGB2* siRNA using RNAiMAX reagent (Invitrogen, Grand Island, NY) according to the manufacturer's instructions. 24 h post transfection, each cell line was treated with cisplatin or 5-FU for 72 h and MTT assays were performed to assess cell viability. All experiments were carried out in triplicate.

3 Results and Discussion

3.1 Identification of differentially expressed proteins in HNSCC cell lines

To characterize proteomic alterations in HNSCC, we employed an iTRAQ-based quantitative proteomics approach. The LC-MS/MS-based quantitative proteomics of a normal oral cell line, OKF6/TERT1, and a panel of three HNSCC cell lines, JHU-O11, JHU-O22 and JHU-O29, led to the identification of 3,263 proteins. The proteomic workflow used for this study is outlined in Fig. 1. Differentially expressed proteins were quantified based on relative intensities of the iTRAQ labels for unique peptides. The distribution of proteins which were differentially expressed in HNSCC cell lines compared to the normal cell line is depicted in Supporting Information Fig. 1. Amongst the differentially expressed proteins, 185 proteins were overexpressed (> 2 fold) and 190 proteins were downregulated (< 2 fold) in at least two out of the three HNSCC cell lines used in the current study compared to the normal oral cell line. Of these 375 proteins, 38 proteins were overexpressed and 75 proteins were downregulated in all the HNSCC cell lines used in this study. A partial list of proteins overexpressed in the HNSCC cell lines is shown in Table 1. The complete list of identified proteins and peptides is provided in Supporting Information Table 1 and 2, respectively.

Among the differentially regulated proteins, proteins that localized to membrane or cell surface included Ezrin (EZR), Midkine (MDK) and Calpastatin (CAST). These molecules have been reported in literature to be overexpressed in multiple cancers including HNSCC [32]. Ezrin has been associated with tumor growth and it has been proposed as a marker for poor prognosis in various tumors [33–37]. Midkine is a 13-kDa small, secreted growth factor that binds to heparin, which was initially discovered in embryonic carcinoma cells. Several studies have indicated the role of MDK in carcinogenesis related processes such as angiogenesis, transformation, proliferation, migration and mitogenesis [38–40]. MDK is overexpressed in HNSCC and is a known biomarker in HNSCC [41–43].

We also identified several novel and known nuclear proteins to be overexpressed in the HNSCC cell lines including, SET nuclear oncogene (SET), high mobility group box B (HMGB2) and insulin-like growth factor 2 mRNA binding protein 3 (IGF2BP3). Overexpression of SET and its association with tumorigenesis has been demonstrated in multiple cancers [9, 44–45]. Studies have shown association of members of the IGF2BP protein family in the development and progression of various cancers [46]. IGF2BP proteins are also known to be associated with cancer metastasis and expression of classical oncogenes such as MYC and KRAS [47]. Expression of IGF2BP3 in HNSCC and its role in

tumor progression has been established [48–49]. We report here for the first time the overexpression of HMGB2 in association with HNSCC.

We performed bioinformatics analysis of overexpressed proteins and categorized them based on the cellular localization and biological function (Supporting Information Fig. 2). The classifications were based on annotations in the Human Protein Reference Database [25–26]. Our analysis revealed that 27% of the overexpressed proteins localized to the nucleus and 25% localized to the cytoplasm. Amongst the other predominant protein groups, 15% and 12% of the proteins localized to mitochondria and plasma membrane, respectively. To gain insights into the altered pathways in HNSCC, network analysis was done for the 185 proteins overexpressed >2-fold in at least two of the HNSCC cell lines. Network analysis using Ingenuity database revealed that 23 proteins were associated with cellular processes involving DNA replication and repair. A list of these proteins is provided in Supporting Information Table 3. These proteins included high mobility group AT-hook 1 (HMGA1), high mobility group box 1 (HMGB1), high mobility group box 2 (HMGB2), flap structure-specific endonuclease 1 (FEN1), APEX nuclease 1 (APEX1), poly (ADP-ribose) polymerase 2 (PARP2), replication protein A1 (RPA1) and proliferating cell nuclear antigen (PCNA) which are known to play an important role in the DNA repair [50]. Alteration in the DNA repair pathway(s) may either induce resistance or sensitivity to chemotherapies [51]. HMGB2, one of the novel proteins identified by us in this study was overexpressed in all the HNSCC cell lines JHU-O29 (4.7-fold), JHU-O22 (3.9-fold) and JHU-O11 (2.2-fold). MS/MS spectra of representative peptides from SET, EZR, HMGB2 and IGF2BP3 are shown in Fig. 2.

3.2 Immunohistochemical validation of HMGB2 overexpression in HNSCC tissues

Having observed that HMGB2 is overexpressed in a panel of HNSCC cell lines, we investigated the expression profile of HMGB2 in primary HNSCC tumors using tissue microarrays. Overexpression of HMGB2 has been reported in various malignant tumors such as squamous cell carcinoma of skin, human bladder cancer and hepatocellular carcinoma, but there are no reports related to its expression in HNSCC [6–8]. The staining was scored on a scale of 0–2, where 0 represented absent staining, +1 represented faint staining and +2 represented intense staining. Cases scored as +2 were considered as positively stained cases. 77% (54/70) of the tested HNSCC cases demonstrated positive IHC staining. We observed expression of HMGB2 to be localized predominantly in the nucleus. Squamous epithelium from non-cancerous cases did not reveal nuclear staining of HMGB2. Staining pattern of HMGB2 in representative sections of non-tumor oral squamous epithelium and HNSCC cases (original magnification $\times 160$) are indicated in Fig. 3. A Chi-square test confirmed that the overexpression of HMGB2 in tumor tissues was statistically significant (p -value=3.75056e-14).

3.3 siRNA-mediated silencing of HMGB2 enhances sensitivity to cisplatin and 5-FU in HNSCC cells

High-mobility group box (HMGB) proteins are non-histone proteins that are highly conserved in eukaryotic organisms. HMGB proteins have DNA binding and bending capabilities which facilitate the binding of transcription factors to their cognate sites. [52]

Apart from its role in nuclear signaling, HMGB1 also plays a role during inflammation, cell differentiation and migration [53–55]. Though HMGB1 has been extensively characterized for its diverse role in cancer, relatively less is known about the function of HMGB2. Similar to HMGB1, HMGB2 has also been reported to be released into extracellular space under various inflammatory conditions and binds to receptors such as receptor for advanced glycation endproducts [56–57]. HMGB2 expression has been linked to response against chemotherapeutic agents such as cisplatin, oxaloplatin and cytarabine. [58–60]. In glioblastoma, HMGB2 overexpression conferred resistance to temozolomide treatment and siRNA-mediated silencing of HMGB2 improved sensitivity to the drug. [61]. Silencing of HMGB2 has been associated with increased sensitivity to ionizing radiation in colorectal cancer cells [62] In hepatocellular carcinoma, overexpression of HMGB2 has been associated with decrease in cisplatin- and etoposide-induced cell death, aggressive tumor growth and poor prognosis [8]

As mentioned previously, two of the common drugs used in the treatment of HNSCC are cisplatin and 5-FU [63]. Cisplatin forms adducts with DNA, which leads to intrastrand cross-links and DNA-protein crosslinks, resulting in inhibition of DNA replication [64]. The HMGB proteins are known to bind specifically to the cisplatin-DNA adducts conferring decreased sensitivity to cisplatin. Tumor resistance to chemotherapy is a major problem during therapy in HNSCC. The discovery of novel molecules/markers to predict and/or improve response to chemotherapy could be beneficial for the development of personalized treatment strategies.

We assessed the expression of HMGB2 in a panel of HNSCC cell lines and found detectable expression of the protein in all cell lines. Western blot analysis revealed a significant decrease in the endogenous expression of HMGB2 using 10nM and 20nM of HMGB2 siRNA in all the cell lines (Fig 4A)

Having observed that the HNSCC cell lines showed varied expression of HMGB2, we next studied the sensitivity of the cells to cisplatin and 5-FU. The cells were cultured for 72 h in media containing varying concentrations of cisplatin and 5-FU. Cell viability was assessed using MTT assays and the IC₅₀ values were determined. JHU-O22, JHU-O28 JHU-O29 and FaDu had an IC₅₀ 3.5µg/ml and 7µg/ml for cisplatin and 5-FU respectively, were considered chemo resistant and JHU-O11 which depicted an IC₅₀ of less 1µg/ml and 0.1µg/ml for cisplatin and 5-FU, respectively, was considered as chemo sensitive (Fig. 4 B,C).

To investigate the role of HMGB2 in the sensitivity of HNSCC cells to cisplatin and 5-FU, we assessed the sensitivity of JHU-O22; JHU-O28 JHU-O29 and FaDu cells to cisplatin and 5-FU using siRNA-mediated silencing of HMGB2. The cell lines which were deemed chemo resistant (JHU-O22, JHU-O28 JHU-O29 and FaDu) were transfected either with HMGB2 siRNA (10 nM or 20 nM) or with scrambled siRNA and treated with cisplatin or 5-FU. Silencing of HMGB2 in the HNSCC cells resulted in an increased sensitivity to both cisplatin and 5-FU (Fig. 5 A–D). Our data indicates that HMGB2 plays a crucial role in the responsiveness of HNSCC cell lines to two major chemotherapeutic agents. Further studies are warranted to understand the exact molecular mechanism associated with the role of

HMGB2 in conferring resistance in HNSCC. Also, studies using larger cohorts including follow up on patient prognosis are required before clinical applications.

4 Concluding remarks

Identification of differentially expressed proteins is crucial for biomarker discovery to facilitate early detection; disease prognosis; therapeutic intervention and prediction to drug response. Resistance to chemotherapeutic drugs represents a major problem for the long-term effectiveness in cancer regime. Dysregulation of various proteins drives drug resistance in cancer cells. Thus, novel markers to predict patient response and possible therapeutic interventions that prevent drug resistance are needed. In this study, using quantitative proteomics, we observed overexpression of HMGB2 in a panel of HNSCC cell lines with varied sensitivity to cisplatin and 5-FU. siRNA-mediated silencing of HMGB2 in HNSCC cell lines resulted in an increased sensitivity to cisplatin and 5-FU. Our current study does not rule out other mechanisms of resistance to cisplatin, such as decreased uptake or inactivation by nucleophilic compounds [65]. The exact molecular mechanism of increased sensitivity to cisplatin and/or 5-FU following suppression of HMGB2 is unclear from our current study. Regardless of the effect of HMGB2 in cisplatin or 5-FU induced DNA remodeling, our results clearly indicate for the first time a new therapeutic opportunity for patients who are non-responders to cisplatin or 5-FU. We hypothesize that HMGB2 may also serve as a predictive biomarker between responders and non-responders to chemotherapy in HNSCC, but studies in larger cohorts are warranted. Taken together, our results indicate that HMGB2 plays a role in HNSCC tumor resistance and provides new insights into the role of HMGB2 in chemo resistance in HNSCC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

cisplatin	cis-platinum (II) diamine-dichloride
5-FU	5-fluorouracil
iTRAQ	Isobaric Tags for Relative and Absolute Quantitation

HNSCC head and neck squamous cell carcinoma

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Figure 1. Workflow employed for iTRAQ-based quantitative proteomics of HNSCC cell lines 200 µg of protein from HNSCC cell lines was digested and iTRAQ labeled using 8plex iTRAQ reagents. SCX fractionation followed by LC-MS/MS analysis was carried out using LTQ-Orbitrap Velos mass spectrometer interfaced with Proxeon Easy nLC II system. The data was searched against Human RefSeq 50 using Proteome Discoverer software suite. Candidate proteins were validated using immunohistochemistry.

Figure 2. Representative MS/MS spectra of peptides from proteins overexpressed in HNSCC cell lines

(A) Ezrin (B) Insulin-like Growth Factor 2 mRNA-binding Protein 3 (C) SET nuclear oncogene (D) High Mobility Group Box 2.

Figure 3. Immunohistochemical validation of HMGB2 overexpression in HNSCC tissues
Representative sections from HNSCC tissue microarrays stained with anti-HMGB2 antibody
(A) Expression of high mobility group box 2 in HNSCC primary tissue (B) Expression of high mobility group box 2 in representative non-tumor oral squamous mucosa.

Figure 4. Cell viability of HNSCC lines in response to cisplatin and 5-FU
(A) HNSCC cell lines were transfected with scrambled siRNA or HMGB2 siRNA (10nM and 20nM) and Western blot analysis was performed using anti-HMGB2 antibody. β-actin was used as a loading control.
JHU-O11, JHU-O22, JHU-O28 JHU-O29 and FaDu were treated with indicated concentrations of cisplatin (B) and 5-FU (C) and MTT assays were performed.

Figure 5. Silencing of HMGB2 enhances sensitivity to cisplatin and 5-FU in HNSCC cells HNSCC cells (A) JHU-O22, (B) JHU-O28, (C) JHU-O29 and (D) FaDu were transfected with HMGB2-siRNA or scrambled siRNA and treated with cisplatin and 5-FU for 72 h and assayed for cellular survival using MTT assay. * denotes p-value <0.05 and ** denotes p – value <0.005

Table 1

A partial list of proteins overexpressed in HNSCC cell lines

Proteins overexpressed in HNSCC cell lines				
Gene symbol	Protein	Fold-change		
		JHU-O11	JHU-O22	JHU-O29
<i>BAG2</i>	BAG family molecular chaperone regulator 2	3.1	4.4	3.9
<i>BCAT2</i>	Branched chain amino-acid transaminase 2, mitochondrial	19.0	2.2	9.6
<i>CAST</i>	Calpastatin isoform b	4.0	5.6	14.5
<i>CLDN7</i>	Claudin-7 isoform 2 precursor	2.8	3.9	3.3
<i>STON2</i>	Stonin-2 isoform 1	6.9	5.7	5.1
<i>HMGA1</i>	High mobility group AT-hook 1	2.2	2.9	2.2
<i>HMGB2</i>	High mobility group box B	2.2	3.9	4.7
<i>PELP1</i>	Proline glutamic acid-and leucine-rich protein 1	11.0	2.6	16.3
<i>IGF2BP3</i>	Insulin-like growth factor 2 mRNA-binding protein 3	3.0	2.4	3.2
<i>EZR</i>	Ezrin	2.1	2.0	4.1
<i>MDK</i>	Midkine (neurite growth-promoting factor 2)	4.7	2.2	3.6
<i>SET</i>	SET nuclear oncogene	2.0	3.9	7.2