Identification of Biomarkers for Predicting Nasopharyngeal Carcinoma Response to Radiotherapy by Proteomics

Xue-Ping Feng, Hong Yi, Mao-Yu Li, Xin-Hui Li, Bin Yi, Peng-Fei Zhang, Cui Li, Fang Peng, Can-E Tang, Jian-Ling Li, Zhu-Chu Chen, and Zhi-Qiang Xiao

Abstract

Radiotherapy is the primary treatment for nasopharyngeal cancer (NPC), but radioresistance remains a serious obstacle to successful treatment in many cases. To identify the proteins involved in this resistance and to evaluate their potential for predicting NPC response to radiotherapy, we first established a radioresistant subclone cell line (CNE2-IR) derived from NPC cell line CNE2 by treating the cells with five rounds of sublethal ionizing radiation. Proteomics was then performed to compare the protein profiles of CNE2-IR and CNE2, and a total of 34 differential proteins were identified. Among them, 14-3-3σ and Maspin were downregulated and GRP78 and Mn-SOD were upregulated in the radioresistant CNE2-IR compared with control CNE2, which was confirmed by Western blot. Immunohistochemistry was performed to detect the expression of the four validated proteins in the 39 radioresistant and 51 radiosensitive NPC tissues and their value for predicting NPC response to radiotherapy were evaluated by receiver operating characteristic analysis. The results showed that the downregulation of 14-3-3σ and Maspin and the upregulation of GRP78 and Mn-SOD were significantly correlated with NPC radioresistance and the combination of the four proteins achieved a sensitivity of 90% and a specificity of 88% in discriminating radioresistant from radioresistant NPC. Furthermore, the resistance to ionizing radiation can be partially reversed by the overexpression of 14-3-3σ in the CNE2-IR. The data suggest that 14-3-3σ, Maspin, GRP78, and Mn-SOD are potential biomarkers for predicting NPC response to radiotherapy and their dysregulation may be involved in the radioresistance of NPC.

Introduction

Nasopharyngeal carcinoma (NPC) is an endemic disease with incidence rates of 15 to 50 per 100,000 in southern China and Southeast Asia. In addition, it poses one of the most serious public health problems in these areas (1). Histologically, these usually belong to WHO types II and III. In the West, NPC occurs sporadically and, histologically, usually belongs to WHO type I. The disease tends to be more sensitive to ionizing radiation than other head and neck cancers. Additionally, types II and III are more sensitive to ionizing radiation than WHO type I. Thus, the primary treatment for NPC at that point is radiotherapy. More accurate tumor localization by computed tomography and better radiotherapy technique have contributed to the improvement in the local control of this disease (2). Even in relatively advanced disease, combined radiochemotherapy may increase survival (3). Nevertheless, radioresistance remains a serious obstacle to successful treatment in many cases. Some of the NPC patients present local recurrences and distant metastases after radiotherapy due to radioresistance and the majority of these patients surrender recurrence and metastasis within 1.5 year after treatment (4, 5). Hence, revealing the molecular mechanism of NPC radioresistance and identifying subgroup of radioresistant NPC patients are urgently needed for personalized therapy.

Although a fraction of genes such as elements of cell cycle control, apoptosis/antiapoptosis, and DNA repair are believed to play a key role in the ionizing radiation–induced cell damages, our understanding of radioresistance in cancer at a molecular level is limited. Microarrays have been used to assess genes involved in radioresistance in various types of cancer (6–11). For examples, analysis for gene expression profiles of radioresistant NPC cell lines using a cDNA array found that at least two genes, gp96 and GDF15, involved in the radioresistance of NPC cell lines (11). However, there was no overlap in the genes found to be involved in radioresistance in various studies. This may be because of distinct tissue specificity, but it is also possible that there is some fundamental mechanism underlying radioresistance that has not yet been elucidated. High throughputs proteomics has introduced a new approach to radioresistant research that aims at identifying differential expression proteins associated with the development of cancer radioresistance.
providing new opportunities to reveal the molecular mechanism underlying NPC radioresistance.

At present, histologic grading and clinical tumor-node-metastasis (TNM) staging are still the main guidelines as to whether the NPC patients receive higher radiation dose or combined more aggressive chemotherapy. NPC patients with the same histologic grading and clinical TNM staging usually received the same therapeutic regimens. As cancer sensitivity to radiotherapy not only is associated with its morphology and clinical TNM staging, it also is affected by its intrinsic molecular characteristics. Hence, the finding of biomarkers to estimate NPC response to radiotherapy would be of clinical importance for the identification of subgroups of patients that could benefit from personalized therapeutic strategies.

In this study, we established a radioresistant NPC subclone cell line CNE-2-IR to search for proteins responsible for NPC radioresistance by a proteomic approach. Thirty-four differential proteins were identified in the radioresistant CNE-2-IR and control CNE-2 cells and the expression levels of four differential proteins (14-3-3σ, Maspin, GRP78, and Mn-SOD) were confirmed. Because 14-3-3σ, Maspin, GRP78, and Mn-SOD proteins are involved in tumor radiosensitivity and/or chemosensitivity from other studies (12–15), we further investigated their predictive values for NPC radioresensitivity by immunohistochemical detection of their expressions in the radiosensitive and radioresistant NPC tissues. Furthermore, we examined the correlation of 14-3-3σ expression level with NPC radioresistance by over-expressing 14-3-3σ in CNE-2-IR cells. Our findings provide substantial evidence that 14-3-3σ, Maspin, GRP78, and Mn-SOD are potential biomarkers for predicting NPC response to radiotherapy.

Materials and Methods

Patients and tissues. One hundred ninety-six NPC patients who were treated by curative-intent radiotherapy (a total dose of 60–70 Gy) using a modified linear accelerator in the Xiangya Hospital of Central South University, China from August 2006 to July 2008 were reviewed. Among these patient, 90 NPC patients without distant metastasis (M0; WHO staging II and III) at the time of diagnosis, comprising 39 radioresistant and 51 radiosensitive patients, were recruited in this study. Radioresistant NPC patients were defined as ones with persistent disease (incomplete regression of tumor) at >6 weeks after completion of radiotherapy or ones with recurrent disease at the nasopharynx and/or neck nodes at >2 months after completion of radiotherapy (16). Radiosensitive NPC patients were defined as ones without the local residual lesions at >6 weeks or recurrence at >2 months after completion of radiotherapy (16). Distant metastasis was excluded by skeletal, thoracic, and upper abdominal imaging before radiotherapy. NPC tissue biopsies from these 90 patients were obtained at the time of diagnosis before any therapy with an informed consent and were used for immunohistochemical staining. Diagnoses were established by experienced pathologists based on the 1978 WHO classification (17). This study was approved by the ethics committee of Xiangya School of Medicine, Central South University, China. The clinicopathologic parameters of NPC tissues used in the present study are shown in Supplementary Table S1.

Establishment of radioresistant NPC CNE2 subclone cell line. Poorly differentiated NPC cell line CNE-2 was seeded at a density of 1 x 10^5 per T25 flask in DMEM (Invitrogen) with 10% FCS and 1% antibiotics, and cultured in an incubator at 37°C with humidified 5% CO2. After 24 hours of culture, various doses from 0 to 12 Gy were given to determine the sublethal dose for the cell line. The radiation was delivered at room temperature at 300 cGy/min with a linear accelerator (2100EX, Varian). To establish radioresistant subclone cell line, CNE-2 cells received the sublethal dose of irradiation (11 Gy). After treatment, the surviving cells were selected and cultured to produce the first generation of the subclone cells. Again, the subclone cells received a sublethal dose of irradiation and the surviving cells were cultured to produce the next generation of subclone cells. Up to five generations of subclone NPC cells were produced, and we defined the fifth-generation cells as the radioresistant subclone cell line and named for CNE-2-IR. CEN-2 cells, used as a control, were treated with the same procedure, except they were sham irradiated. Experiments were performed with the CNE-2-IR cells within 4 to 10 passages after the termination of irradiation.

Clonogenic survival assay. Radioresistance was measured by clonogenic survival assay following exposure to irradiation. Briefly, cells were plated in six-well culture plates and were exposed to a range of radiation doses (2–10 Gy). After irradiation, the cells were cultured for 12 days and the number of surviving colonies (defined as a colony with >50 cells) was counted. The survival fraction was calculated as the numbers of colonies divided by the numbers of cells seeded times plating efficiency. Plating efficiencies were calculated as colonies per 10 cells. Three independent experiments were done.

Cell growth analysis in response to irradiation. Cells were plated in a 24-well culture plates (2.5 x 10^4/well). After incubation for 24 hours, the cells were irradiated with 6 Gy. Cell growth was monitored by counting cell numbers at various time intervals. Three independent experiments were done in triplicate.

Flow cytometry analysis of cell cycle in response to irradiation. Cells were growing in the complete medium and were harvested at 24 hours after irradiation with 6 Gy. The cell pellets were fixed with ice-cold 70% ethanol in PBS at −20°C for 1 hour and then centrifuged at 1,500 rpm for 5 minutes. The pellets were incubated with 0.5% Triton X-100 (Sigma) and 0.05% RNase (Sigma) in 1 mL PBS at 37°C for 30 min and then centrifuged at 1,500 rpm for 5 min. The cell pellets were incubated with 40 μg/mL propidium iodide (Sigma) in 1 mL PBS at room temperature for 30 minutes. Samples were immediately analyzed by a FACScan flow cytometry (Becton Dickinson). The distribution of cell cycle was determined using CellQuest Pro and the ModFit software. Three independent experiments were done.

Two-dimensional electrophoresis. Cells were lysed in lysis buffer (7 mol/L urea, 2 mol/L thiourea, 100 mmol/L DTT, 4% CHAPS, 40 mmol/L Tris, 2% Pharmalyte, 1 mg/mL DNase I) at 37°C for 1 hour and then centrifuged at 15,000 rpm for
30 minutes at 4°C. The supernatant was transferred and the concentration of the total proteins was determined using the two-dimensional Quantification kit (Amersham Biosciences). Two-dimensional electrophoresis was performed to separate 600 μg of protein samples as previously described by us (18). After SDS-PAGE, the protein spots were visualized by staining with colloidal Coomassie (Bio-Rad Laboratories). Triplet gels were made for each cell line.

**Image analysis.** To minimize the contribution of experimental variations, three separate two-dimensional gels were prepared for each cell line. The stained two-dimensional gels were scanned with the MagicScan software on Imagescanner (Amersham Biosciences) and were analyzed using PDQuest system (Bio-Rad Laboratories). Spot intensities were quantified by calculating the spot volume after normalization of the image using the total spot volume normalization method multiplied by the total area of all the spots. An average 2-DE map for each cell line was constructed with the PDQuest image analysis software after spot matching and the expression level of a protein spot was determined by its average intensity volume of three gels. The average 2-DE maps were used to perform the different expression analysis. The differences of the matched protein spots between the two cell lines was calculated by the ratio of their average expression levels and proteins were classified as being differentially expressed between the two cell lines when average spot intensity was showed a difference 2-fold variation. Significant differences in protein expression levels were determined by Student’s \( t \) test with a set value of \( P < 0.05 \).

**Mass spectrometry analysis.** All the differential protein spots showing consistent difference between the two cell lines in triplicate experiments were excised from stained gels using punch, were in-gel trypsin digested, and analyzed with a Voyager System DE-STR 4307 matrix-assisted laser desorption/ionization–time-of-flight (MALDI-TOF) Mass Spectrometer (MS; ABI) to get a peptide mass fingerprint as previously described by us (18). The protein spots identified by MALDI-TOF MS were also subjected to analysis of electrospray ionization quadrupole–time of flight MS (Micromass, Waters). Briefly, the samples were loaded on to a precolumn (320 μm*50 mm, 5 μm C18 silica beads, Waters) at 30 μL/min flow rates for concentrations and fast desalting through a Waters CapLC autosampler and then eluted to the reversed-phase column (75 μm*150 mm, 5 μm, 100 Å, LC Packing) at a flow rate of 200 nL/min after flow splitting for separation. MS/MS spectra were performed in data-dependent mode in which up to four precursor ions above an intensity threshold of 7 counts per second were selected for MS/MS analysis from each “scan.”

**Database search.** In peptide mass fingerprint map database searching, Mascot Distiller was used to obtain the monoisotopic peak list from the raw mass spectrometry files. Peptide matching and protein searches against the Swiss-Prot database were performed using the Mascot search engine (http://www.matrixscience.com/) with a mass tolerance of ±50 ppm. Protein scores of >64 (threshold) indicate identity or extensive homology \( (P < 0.05) \) and were considered significant. In tandem mass spectrometry data database query, the MS/MS data (PKL format file) was imported into the Mascot search engine with a MS/MS tolerance of ± 0.3 Da to search the Swiss-Prot database. For the protein matching of two or more unique peptides, individual ion scores of >28 (threshold) indicate identity or extensive homology \( (P < 0.05) \) and were considered significant. For the protein matching only one unique peptide, individual ion scores of >35 (threshold) indicate identity or extensive homology \( (P < 0.01) \) and were considered significant.

**Western blot.** Western blot was performed to detect the expression of 14-3-3σ, Maspin, GRP78, and Mn-SOD in CNE-2-IR and CNE-2 cells as previously described by us (19). Briefly, 40 μg of lysates were separated by 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. Blots were blocked with 5% nonfat dry milk for 2 hours at room temperature and then incubated with 1:2,000 dilution of monoclonal mouse anti–14-3-3σ antibody (Santa Cruz Biotechnology), 1:800 dilution of monoclonal mouse anti-Maspin antibody (Neomarkers), 1:2,000 dilution of polyclonal goat anti-GRP78 antibody (Santa Cruz Biotechnology), or 1:3,000 dilution of polyclonal rabbit anti-Mn-SOD antibody (Santa Cruz Biotechnology) for 2 hours at room temperature, followed by incubation with 1:3,000 dilution of horseradish peroxidase–conjugated secondary antibody for 1 hour at room temperature. The signal was visualized with an enhanced chemiluminescence detection reagent. \( β \)-Actin was detected simultaneously using 1:5,000 dilution of monoclonal mouse anti–\( β \)-actin antibody (Sigma) as a loading control. Three independent experiments were done.

**Immunohistochemistry.** Immunohistochemical analysis of 14-3-3σ, Maspin, GRP78, and Mn-SOD in the 39 radiosensitive and 51 radiosensitive NPC tissue samples was performed on formalin-fixed and paraffin-embedded tissue sections as previously described by us (19). Briefly, 4 μm of tissue sections were treated with an antigen retrieval solution [10 mmol/L sodium citrate buffer (pH 6.0)]; incubated with anti-14-3-3σ antibody (1:400 dilution), anti-Maspin antibody (1:100 dilution), anti-GRP78 antibody (1:400 dilution), or anti-Mn-SOD antibody (1:600 dilution) overnight at 4°C; and then were incubated with 1:1,000 dilution of biotinylated secondary antibody followed by avidin-biotin peroxidase complex (DAKO). Finally, tissue sections were incubated with 3’3’-diaminobenzidine (Sigma) until a brown color developed and were counterstained with Harris’ modified hematoxylin. In negative controls, primary antibodies were omitted.

**Evaluation of staining.** Sections were blindly evaluated by two investigators in an effort to provide a consensus on staining patterns by light microscopy. A quantitative score was performed by adding the score of staining area and the score of staining intensity for each case to assess the expression levels of the proteins as previously described by us (19). At least 10 high-power fields were chosen randomly and >1,000 cells were counted for each section. First, a quantitative score was performed by estimating the percentage of immunopositive cells: 0, no staining of cells in any microscopic fields; 1+, <30% of tissue stained positive; 2+, between 30% and 60% stained positive; and 3+, >60%...
stained positive. Second, the intensity of staining was scored by evaluating the average staining intensity of the positive cells (0, no staining; 1+, mild staining; 2+, moderate staining; 3+, intense staining). Finally, a total score (ranging from 0–6) was obtained by adding the area score and the intensity score for each case. A combined staining score of ≤2 was considered to be negative staining (no expression); a score between 3 and 4 was considered to be moderate staining (expression); and a score between 5 and 6 was considered to be strong staining (high expression).

**Statistical analysis of immunohistochemical data.** Statistical analyses were performed with the statistical package SPSS 13.0. Difference of the protein expression in the radiosensitive and radioresistant NPC tissues was analyzed using Mann-Whitney U test. In addition, the four proteins were individually and, as a penal, assessed for its ability to discriminate between radiosensitive and radioresistant NPC patients by evaluating its receiver operating characteristic (ROC) curve based on the immunohistochemistry scores described by Hu and colleagues (20). Briefly, we built a logistic regression model and conducted ROC curve analyses to evaluate overall predictive power of individual and the combined four proteins. The optimal cut point was determined for each protein by identifying the value that yielded the maximum corresponding sensitivity and specificity. ROC curves were then plotted based on the set of optimal sensitivity and specificity values. The area under the curve and other attributes were computed through the numerical integration of the ROC curves. Sensitivity, specificity, positive predictive value, and negative predictive value of the four proteins were calculated individually and as a penal. A two-sided P < 0.05 was considered significant.

**Overexpression of 14-3-3σ in radioresistant CNE2-IR cells.** pcDNA3-14-3-3σ plasmid and empty vector pcDNA3 (21) were transfected into the radioresistant CNE-2-IR cells with the Lipofectamine 2000 Reagent (Invitrogen), respectively. After 14 days of selection in RPMI 1640 containing 10% FCS and 400 mg/mL G418 (Invitrogen), the expression of 14-3-3σ was determined by Western blot. Clonogenic survival assay was done to determine the resistant level in the transfected cells at 24 hours after exposed a range of radiation doses (2–10 Gy).

### Results

**Establishment and validation of radioresistant NPC subclone cell line.** The sublethal radiation dose was determined by dose titration for the NPC CNE-2 cell line. Nearly all NPC CNE-2 cells were killed after 5 days of irradiation at a dose of 12 Gy and no cells were recovered at doses higher than 12 Gy. A dose of 11 Gy was therefore considered sublethal and used for the selection of CNE-2-radioresistant subclones. The first generation of subclone cells was generated from the culture of the surviving fraction of the parental cells irradiated with the sublethal dose. Similarly, the second generation of subclone cells was generated from the culture of the surviving fraction of the first-generation cells irradiated with the sublethal dose. Finally, the fifth generation of CNE-radioresistant subclones was established and designated as CNE-2-IR.

To verify the radioresistant phenotypes of CNE2-IR, CNE-2-IR and control CNE-2 cells were irradiated with a range of radiation doses (2–10 Gy) and were examined by clonogenic survival assay. As shown in the Fig. 1A, the surviving colonies of the CNE-2-IR cells were significantly more and bigger than control CNE-2 cells. As shown in the Fig. 1B, CNE-2-IR showed decreased radiosensitivity compared with the level of control CNE-2 and dose-modifying factors were 2.04 and 1.56, respectively, at 10% and 1% of isosurvival levels for CNE-2-IR. Furthermore, CNE-2-IR and control CNE-2 were subjected to 6 Gy radiation to examine the effect on cell growth. As shown in the Fig. 1C, cell growth delay after 6 Gy of irradiation was less for CNE-2-IR than for control CNE-2. For ~5-fold increase in cell number, the delay was ~32 hours for CNE-2-IR compared with ~61 hours for control CNE-2.

The difference in response to radiation between the two cell lines was further studied by cell cycle analysis using flow cytometry. As showed in Supplementary Table S2, no difference was induced by ionizing radiation in G0–G1 phases at 24 hours after 6 Gy of irradiation, whereas compared with control CNE-2 cells, more CNE-2-IR cells were found detained in S phase with less cells in G2-M phase. These results suggest that the regulation of cell cycle induced by ionizing radiation stress is altered in the radioresistant CNE-2-IR, which is also consistent with the typical radioresistant phenotype. The above results indicate that CNE-2-radioresistant subclone cell line (CNE-2-IR) was established.

**Screening for radioresistance-associated proteins by proteomic analysis.** Comparative proteomic study of CNE-2-IR and control CNE-2 was performed to identify the protein associated with CNE-2-IR radioresistance. Two representative 2-DE maps from CNE-2-IR and control CNE-2 were shown in Fig. 2A. After comparing the average 2-DE maps, 41 differential protein spots (≥2-fold) in the two cell lines were detected and subjected to the analysis of both MALDI-TOF MS and ESI-Q-TOF MS, 34 differential proteins of which were successfully identified. The MS/MS results of a spot 22 are shown in Fig. 2B and C. The amino acid sequence of a doubly charged peptide from spot 22 with m/z 951.9672 was identified as LAEQARYEDMAAFMK, which is a part of 14-3-3σ sequence, and the query result indicated that the protein spot 22 is 14-3-3σ. The detailed information of all the identified proteins is summarized in the Supplementary Table S3 and Table 1.

**Validation of the identified proteins.** To confirm the expression levels of the differential proteins identified by proteomics, the expression of four identified proteins (14-3-3σ, Maspin, Mn-SOD, and GRP78) in the CNE-2-IR and control CNE-2 cells was detected by Western blot. As shown in Fig. 3A, the expression of Mn-SOD and GRP78 was significantly higher, whereas 14-3-3σ and Maspin was significantly lower in the CNE-2-IR than that in the control CNE-2 cells, which is consistent with the results of proteomic analysis.

**Expression of 14-3-3σ, Maspin, Mn-SOD, and GRP78 proteins in radioresistant and radiosensitive NPC tissues.** The results of both proteomics and Western blot showed
that the expression levels of 14-3-3σ, Maspin, Mn-SOD, and GRP78 were associated with the radiosensitivity of NPC cells. To examine the relevance of this finding to clinical NPC radiosensitivity, we detected the four-protein expression in the radioresistant and radiosensitive NPC tissues. As shown in Fig. 3B and Table 2, compared with the radiosensitive NPC, 14-3-3σ and Maspin were significantly downregulated, whereas Mn-SOD and GRP78 were significantly upregulated in the radioresistant NPC. The results indicate the association of the four-protein expression abnormality with the clinical NPC radiosensitivity.

**Value of 14-3-3σ, Maspin, Mn-SOD, and GRP78 as biomarkers for predicting NPC response to radiotherapy.** The ability of 14-3-3σ, Maspin, Mn-SOD, and GRP78 in distinguishing the radiosensitive and radioresistant NPC tissues was analyzed by determining the ROC curves of the four proteins individually and as a panel. The area under curve values of the four proteins are listed in Supplementary Table S4 together with their individual and collective values of merit. When individual protein serves as a biomarker for predicting NPC radiosensitivity, their sensitivity and specificity are 64% to 87% and 66% to 88% in discriminating radiosensitive from radioresistant NPC samples, respectively (Supplementary Table S4; Fig. 4A). As a panel, the four proteins achieved a sensitivity of 90% and a specificity of 88% in discriminating radiosensitive from radioresistant NPC samples (Supplementary Table S4; Fig. 4A).

**14-3-3σ Overexpression decreased the radioresistance of CNE-2-IR.** To address the question whether the expression levels of the four proteins may affect the radiosensitivity of CNE-2-IR, we selected 14-3-3σ, one of the four proteins, to be further studied because our previous study showed that the downregulation of 14-3-3σ was associated with the poor survival of patients with NPC as an independent prognostic indicator (19). We generated a stably transfected CNE-2-IR cell line overexpressing 14-3-3σ and measured the radioresistant levels of 14-3-3σ-overexpressing and vector-transfected CNE-2-IR cells after irradiation with a range of radiation doses (2–10 Gy) using a clonogenic survival assay. As shown in Fig. 4B, transfection of pcDNA3-14-3-3σ plasmid into CNE-2-IR cells increased 14-3-3σ expression compared with the vector-transfected cells. As shown in Fig. 4C, the 14-3-3σ-overexpressing CNE-2-IR showed increased radiosensitivity compared with the vector-transfected cells. As shown in Fig. 4C, the 14-3-3σ-overexpressing CNE-2-IR showed increased radiosensitivity compared with the vector-transfected CNE-2-IR and dose-modifying factors were 0.717 and 0.744, respectively, at 10% and 1% of isosurvival levels for 14-3-3σ-overexpressing CNE-2-IR. The result indicates that 14-3-3σ downregulation confers a significant protection against ionizing radiation, and increased the resistance of NPC cells to irradiation.

**Discussion**

Radioresistance continues to be a major problem in the treatment of NPC (4, 5). The molecular mechanisms underlying NPC radioresistance are still unclear, and till now, there have not been effective biomarkers for predicting NPC radiosensitivity. Identification of NPC radioresistance-associated proteins will be helpful for finding biomarkers...
to estimate NPC response to radiotherapy and deciphering the molecular mechanisms of NPC radioresistance.

In this study, we established radioresistant NPC subclone cell line CNE-2-IR by sublethal doses of radiation and used proteomic approach to screen for differential proteins in the CNE-2-IR cells. Consequently, 34 differential proteins were identified and four differential proteins (14-3-3σ, Maspin, GRP78, and Mn-SOD) were confirmed by Western blot, suggesting that the proteins identified by proteomic approach are actually differential expression proteins. Immunohistochemistry was performed to detect the expressions of 14-3-3σ, Maspin, GRP78, and Mn-SOD in the radiosensitive and radioresistant NPC tissues, and the correlation of their expression levels with NPC radioresistance was evaluated. The results showed that the downregulation of 14-3-3σ and Maspin and upregulation of Mn-SOD and GRP78 were significantly correlated with NPC radioresistance. In addition, a panel of the four proteins achieved a sensitivity of 90% and a specificity of 88% in discriminating radiosensitive from radioresistant NPC. Furthermore, transfection of 14-3-3σ-expressing plasmid to upregulate the expression of 14-3-3σ, one of the four differential proteins, rendered radioresistant CNE-2-IR cells more sensitive to radiation, which strongly indicated that the downregulation of 14-3-3σ play an important role in the development of NPC radioresistance. The above results suggest that 14-3-3σ, Maspin, GRP78, and Mn-SOD are potential biomarkers for predicting NPC response to radiotherapy.
Table 1. Differential expression proteins between CNE-2-IR and control CNE-2 identified by mass spectrometry

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<tr>
<th>No.</th>
<th>Swiss-Prot AC</th>
<th>Protein name</th>
<th>MW (Da)</th>
<th>pI</th>
<th>Coverage</th>
<th>Unique peptides</th>
<th>Scores</th>
<th>Peptide sequence identified</th>
<th>Expression ratio (CNE2-IR/Control CNE2)</th>
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<tr>
<td>1</td>
<td>Q9Y4L1</td>
<td>Oxygen-regulated protein 150K precursor</td>
<td>111,266</td>
<td>5.16</td>
<td>2%</td>
<td>2</td>
<td>78</td>
<td>LPATEKPVLLSK YFQHLLGK</td>
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<td>2</td>
<td>P11142</td>
<td>Heat shock cognate 71-kDa protein</td>
<td>88,805</td>
<td>5.44</td>
<td>3%</td>
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<td>109</td>
<td>SQIHDIVLGGSTR MVNHIAEFK</td>
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<td>3</td>
<td>P68361</td>
<td>Tubulin α-1B chain</td>
<td>50,804</td>
<td>4.94</td>
<td>17%</td>
<td>5</td>
<td>178</td>
<td>AVFVDLEPTIDEVR</td>
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<td>4</td>
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<td>50,503</td>
<td>4.95</td>
<td>6%</td>
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<td>5</td>
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<td>γ-IFN–inducible protein Ifi-16</td>
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<td>4.9</td>
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<td>2</td>
<td>82</td>
<td>LISEMHSFIQIK GLEVINDYHFR</td>
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<td>6</td>
<td>P28331</td>
<td>NADH-ubiquinone oxidoreductase 75-kDa subunit, mitochondrial (precursor)</td>
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<td>7</td>
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<td>6.81</td>
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<td>MYLGYEYTAIR</td>
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<td>8</td>
<td>P12532</td>
<td>Creatine kinase, ubiquitous mitochondrial (precursor)</td>
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<td>P04264</td>
<td>Keratin, type II cytoskeletal 1</td>
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<td>4</td>
<td>174</td>
<td>SLDDLSIAEK WELLOQVDSTR SLLNQFASIDK</td>
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<td>10</td>
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<td>78-kDa glucose-regulated protein</td>
<td>72,187</td>
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<th>MW (Da)</th>
<th>pl</th>
<th>Coverage</th>
<th>Unique peptides</th>
<th>Scores</th>
<th>Peptide sequence identified</th>
<th>Expression ratio (CNE2-IR/Control CNE2)</th>
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<tr>
<td>13</td>
<td>Q04695</td>
<td>Keratin, type I cytoskeletal 17</td>
<td>40,524</td>
<td>4.9</td>
<td>18%</td>
<td>5</td>
<td>321</td>
<td>TMQALEIELOQLSMK</td>
<td>↓0.15</td>
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<td>14</td>
<td>P23381</td>
<td>Tryptophanyl-tRNA synthetase</td>
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<td>Template-activating factor-I</td>
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<td>55</td>
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<td>↑3.25</td>
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<tr>
<td>22</td>
<td>P31947</td>
<td>Peroxiredoxin-4</td>
<td>30,749</td>
<td>5.86</td>
<td>4%</td>
<td>1</td>
<td>55</td>
<td>KLETAVNLAWTQNSNTR</td>
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<tr>
<td>23</td>
<td>P21976</td>
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<td>30,868</td>
<td>8.62</td>
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<td>3</td>
<td>176</td>
<td>SENLEFTSSGSAANTETTK</td>
<td>↑2.27</td>
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<th>No.</th>
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<th>Coverage</th>
<th>Unique peptides</th>
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<td>128</td>
<td>LATGSNEITIPFTESR LFDQAFGLPR</td>
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<tr>
<td>28</td>
<td>Q06830</td>
<td>Peroxisome-1</td>
<td>22,324</td>
<td>8.27</td>
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<td>4</td>
<td>300</td>
<td>GLFIIIDKGILR TIAQDYGVKLAKDEGISFR QITVNDLPVGR LNCQVIGASVDHFCFHLAWVNTPK</td>
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<td>Protein DJ-1</td>
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<td>54%</td>
<td>7</td>
<td>451</td>
<td>EGPDYVLPGLNLGAQNLSESAMK GAEEMETVPDVMR GLIAICAGPTALLAHEIGFGSK VTVAGLKDVPVQCSR DVICPDASLEDAK DVICPDASLEDAK KGLIAACAGPTALLAHEIGFGSK</td>
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<td>P03755</td>
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<td>5</td>
<td>191</td>
<td>AEDGSVIDYLDODAR GVDEVTIVNLNR GLGTDEDSLIEICSR SALSGHLETVILGLLK</td>
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<td>Voltage-dependent anion-selective channel protein 3</td>
<td>32,060</td>
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<td>LTLDIFVPNTRGK</td>
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<td>32</td>
<td>P31943</td>
<td>Heterogeneous nuclear ribonucleoprotein H</td>
<td>49,198</td>
<td>5.89</td>
<td>4%</td>
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<td>135</td>
<td>SNNVEMDWWLKV VHIIEGPDGR</td>
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<td>33</td>
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<td>155</td>
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<td>34</td>
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<td>1</td>
<td>49</td>
<td>EAVCFIPSEGHTLQEQVLVEGGR</td>
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Abbreviations: AC, accession code; MW, molecular weight; pl, isoelectric point.
14-3-3σ, a potential tumor suppressor protein, can negatively regulate cell cycle progression by inducing G2-M phase arrest (22, 23). It has been shown that 14-3-3σ is transactivated by p53 in response to DNA damage and, in turn, interacts with p53 and positively regulates p53 activity (24). It is known that p53 is involved in the complex response to ionizing radiation, such as inducing irreversible growth arrest and apoptosis, and sensitizes cells to radiochemotherapy (25). As a negative regulator of cell cycle and mediator of p53 function in response to DNA, 14-3-3σ may also play a

### Table 2. The difference of 14-3-3σ, Maspin, Mn-SOD, and GRP78 expression in the radioresistant and radiosensitive NPC tissues

<table>
<thead>
<tr>
<th></th>
<th>Score</th>
<th></th>
<th></th>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Low (0–2)</td>
<td>Moderate (3–4)</td>
<td>High (5–6)</td>
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<td>18</td>
<td>9</td>
<td>12</td>
<td>0.0001</td>
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<tr>
<td></td>
<td>51</td>
<td>9</td>
<td>17</td>
<td>25</td>
<td></td>
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<tr>
<td>Maspin</td>
<td>39</td>
<td>17</td>
<td>18</td>
<td>4</td>
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<td></td>
<td>51</td>
<td>19</td>
<td>22</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>GRP78</td>
<td>39</td>
<td>7</td>
<td>14</td>
<td>18</td>
<td>0.0000</td>
</tr>
<tr>
<td></td>
<td>51</td>
<td>20</td>
<td>16</td>
<td>15</td>
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<tr>
<td>Mn-SOD</td>
<td>39</td>
<td>6</td>
<td>14</td>
<td>19</td>
<td>0.0000</td>
</tr>
<tr>
<td></td>
<td>51</td>
<td>18</td>
<td>17</td>
<td>16</td>
<td></td>
</tr>
</tbody>
</table>

NOTE: P < 0.01 by Mann-Whitney U test, radioresistant versus radiosensitive NPC tissues.
critical role in sensitizing tumor cells to radiochemotherapy. Indeed, decreased expression of 14-3-3σ has been observed in drug-resistant human breast cancer cells (12). Our previous study showed that 14-3-3σ was decreased in NPC and that its downregulation was associated with the poor survival of NPC patients as an independent prognostic factor which indicates that 14-3-3σ downregulation may cause radioresistance in NPC (19). It is also a reason for selecting 14-3-3σ to examine the effects of its expression in CNE-2-IR cells. Our results showed that 14-3-3σ was downregulated in the radioresistant NPC cells and tissues; its downregulation was associated with NPC radioresistance; and upregulating 14-3-3σ expression lead to the partial radiosensitization of CNE-2-IR cells. The data strongly suggest that 14-3-3σ might be a biomarker for predicting NPC response to radiotherapy. However, we found that the overexpression of 14-3-3σ did not completely reverse the radioresistant phenotype of CNE-2-IR, indicating that there are other factors contributing to radioresistance other than 14-3-3σ in NPC.

Maspin is a serpin with tumor suppressive properties; can inhibit tumor growth, angiogenesis, invasion, and metastasis; and promote apoptosis (26). Maspin is also an important apoptosis-sensitizing factor, making tumor sensitive to drug-induced apoptosis (27, 28). It has been shown that Maspin expression was directly correlated with radiochemotherapy response in head and neck squamous cell carcinoma (13, 29) and the use of Maspin as a therapeutic target against cancer through the reexpression of Maspin by pharmacologic intervention have been explored (30). In this study, Maspin was downregulated in the radioresistant NPC cells and tissues and its downregulation was associated with NPC radioresistance, which together with reporters suggests that the decrease of Maspin expression in NPC increased its radiation resistance and Maspin might be a biomarker for predicting NPC response to radiotherapy.

The glucose-regulated protein GRP78, an endoplasmic reticulum stress–induced molecular chaperone, plays a critical role in the endoplasmic reticulum stress and in maintaining cellular homeostasis as an important stress response survival protein (31, 32). GRP78 is induced in cancers due to endoplasmic reticulum stress, including glucose deprivation, acidosis, and severe hypoxia (33), and is also induced by
chemotherapeutic drugs and radiation, conferring tumor resistance to chemotherapeutic agents and radiation (14, 34). Knockdown of GRP78 sensitizes tumor cells to drug treatment in multiple types of cancer (35). Given the importance of GRP78 in cancer cell survival, it represents a prime target for anticancer treatment. Furthermore, GRP78 can serve as a biomarker for predicting treatment response in breast cancer patients (14). In the present study, GRP78 is upregulated in the radioresistant NPC cells and tissues, and its upregulation was associated with NPC radiosensitivity, which together with reporters suggests that overexpression of GRP78 increased NPC radioresistance and GRP78 might be a biomarker for predicting NPC response to radiotherapy.

Mn-SOD, one of the mitochondrial antioxidant enzymes, plays a central role in protecting cells against reactive oxygen species injury (36). Exposure of cells to ionizing radiation leads to the formation of reactive oxygen species that can damage DNA, RNA, protein, and lipid components, and is associated with radiation-induced injury (36). Mn-SOD can scavenge reactive oxygen species produced by ionizing radiation and protect cancer cells from radiation-induced oxidative damage (37). Both in vitro and in vivo studies showed that the expression level of Mn-SOD negatively correlates with tumor radiosensitivity (15, 38–40). In the present study, Mn-SOD was upregulated in the radioresistant NPC cells and tissues, and its upregulation was associated with NPC radioresistance, which together with reporters suggests that the overexpression of Mn-SOD increased NPC radiation resistance and Mn-SOD might be a biomarker for predicting NPC response to radiotherapy.

In summary, we used proteomic approach identifying 34 differential proteins in the radioresistant NPC cell line CNE2-IR. We further showed that the expression abnormality of 14-3-3σ, Maspin, GRP78, and Mn-SOD might contribute to NPC radiosensitivity and be potential biomarkers for predicting NPC response to radiotherapy. The findings reported here could have clinical value in distinguishing radiosensitive from radioresistant NPC and in identifying subgroups of NPC patients that could benefit from personalized therapeutic strategies.

 Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

Identification of Biomarkers for Predicting Nasopharyngeal Carcinoma Response to Radiotherapy by Proteomics

Xue-Ping Feng, Hong Yi, Mao-Yu Li, et al.

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