

# Sperm protein 17 is a novel marker for predicting cisplatin response in esophageal squamous cancer cell lines

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Expression of sperm protein 17 (Sp17) mRNA has been reported in various malignancies. In an earlier study, we reported the upregulation of Sp17 transcripts in primary esophageal squamous cell carcinomas (ESCCs) using differential display and detected Sp17 transcripts in 86% of ESCCs by RT-PCR, whereas no transcripts were detected in the paired normal esophageal tissues. Herein we hypothesized that Sp17 might be used as a marker for detecting the response of anticancer therapies in ESCCs. Our results indicated that Sp17 protein levels in esophageal squamous cancer cell lines decreased in response to treatment with (i) the HSP90 activity inhibitor geldanamycin, (ii) the tyrosine kinase inhibitor erlotinib and (iii) cisplatin (chemotherapeutic agent commonly used in management of ESCC). In contrast, the Sp17 levels did not decrease in response to radiation therapy and treatment with the chemotherapeutic agent, gemcitabine. Further investigations showed that cisplatin induced decrease in Sp17 levels was due to transcriptional inhibition and cisplatin-resistant cell lines did not show this decrease in Sp17 levels in response to cisplatin treatment. In addition, we also carried our mass spectrophotometric analysis to identify the binding partners of Sp17 to characterize its possible involvement in esophageal tumorigenesis and chemoresistance.

Cancer of the esophagus has been reported as the 9th most common malignancy in the world, with esophageal squamous cell carcinoma (ESCC) occurring most frequently in developing countries.<sup>1,2</sup> This malignancy is usually detected at an advanced stage, requiring multimodality treatment. Despite improvements in its detection, surgical resection and neoadjuvant therapy, the overall survival of esophageal cancer patients remains lower than that of patients with many other solid tumors.<sup>3,4</sup> The prognosis of patients who do not respond to neoadjuvant therapy appears to be poorer than that of patients who had surgery alone.<sup>5,6</sup> Also, ineffective therapy or excessive therapy to the resistant tumors may exacerbate the severity of disease. The treatment modalities such as chemotherapy, which mainly includes cisplatin and 5-fluorouracil alone, or in combination with radiation, have

significantly increased the survival rate of ESCC patients.<sup>7</sup> However, recently reported trials using cisplatin-based adjuvant chemotherapy in resected esophageal cancers, with one exception, have failed to result in improved survival compared to surgery alone.<sup>8-10</sup> These data suggest the need for predictive markers to allow tailored chemotherapy alone or in combination with radiation, to increase the number of complete pathological responses following neoadjuvant approaches. Recently, molecular markers have been identified using innovative, molecular-driven technologies to find predictive and prognostic markers of response to neoadjuvant and adjuvant therapies in esophageal cancer.

Sperm protein 17 (Sp17) was originally identified as a novel cancer-testis antigen in various malignancies, including multiple myeloma.<sup>11,12</sup> Recent emerging studies on Sp17 indicated that its expression is not restricted to testis only but is expressed in other tissues also, albeit at low levels.<sup>13</sup> Sp17 is expressed frequently at the transcript and protein levels in epithelial ovarian cancers and thus represents a novel ovarian tumor antigen.<sup>14</sup> Overexpression of Sp17 protein has been observed in gastrointestinal, breast carcinoma, melanoma and esthesioneuroblastoma.<sup>15,16</sup> In an earlier study, we reported upregulation of Sp17 transcripts in esophageal tumor tissues using differential display reverse transcription (RT)-polymerase chain reaction (PCR).<sup>17</sup> We further detected Sp17 transcripts in 86% of ESCCs by RT-PCR, but no transcripts were detected in the paired normal esophageal tissues. Importantly, we showed that the circulating levels of anti-Sp17 antibodies

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were significantly elevated in ESCC patients as compared to the normal subjects and increasing Sp17 antibody titers were observed to be associated with progressive disease.<sup>18</sup> Recently, Sp17 was found to be involved in the chemoresistance of ovarian cancer to paclitaxel.<sup>19</sup> However, the status of Sp17 protein levels in chemosensitive and resistant upper-aerodigestive tract cancer cell lines has not been determined. The pathways in which Sp17 might play an important role remain to be investigated.

Our study was undertaken to address two questions in esophageal squamous cancer cell lines: (i) Do Sp17 levels change in response to chemotherapy and other anticancer treatments in ESCC? (ii) Does Sp17 hold promise as a molecular marker of response to these treatments in ESCC? We also carried out the mass spectrometric analysis to investigate the binding partners of Sp17 in ESCC cell lines in an attempt to investigate its roles in cellular pathways in esophageal carcinogenesis.

## Material and methods

### Cell culture

The human esophageal squamous cell carcinoma cell line TE13 and cisplatin-resistant head and neck squamous cell carcinoma cell lines UMSCC-5Pt and UMSCC10BPt were a gift from Prof. T. Nishihira (Department of Gastroenterology, Iwaki Kyoritsu General Hospital, Iwaki, Japan) and Dr. Thomas E. Carey (University of Michigan, Ann Arbor, MI), respectively. All these cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum. All experiments were conducted in serum-containing media. Cells ( $6 \times 10^5$ ) were plated in 100-mm culture dishes 2 days prior to treatment. For all *in vitro* experiments, cells were released from flasks using 0.01% trypsin and 0.2 mmol/l EDTA in PBS, and cultures were between 30% and 50% confluence at the time of harvest.

### Reagents

Drugs and antibodies: Poly(ADP-ribose) polymerase (PARP) and actin antibodies were purchased from Cell Signaling Technology (Beverly, MA). Sp17 antibody was acquired from Delta Biolabs (Santa Cruz, CA) and phosphorylated Histone H<sub>2</sub>AX ( $\gamma$ -H<sub>2</sub>AX) was from Upstate Group LLC (Charlottesville, VA). Erlotinib was kindly provided by Genentech (San Francisco, CA). Cisplatin and gemcitabine were acquired from Bedford Laboratories (Bedford, OH) and Eli Lilly, respectively (Indianapolis, IN). Geldanamycin was purchased from Sigma (St. Louis, MO). Irradiation of cells was performed using an ortho-voltage machine in the radiation core facility of the University of Michigan Medical School, MI.

### Drugs or radiation treatments

For drug treatments, the most commonly used concentrations of various drugs in esophageal cancer were used.<sup>20–25</sup> It was also ensured that these concentrations are reported to be clinically achievable in esophageal cancer patients. Cell lines

were treated with geldanamycin (1  $\mu$ M), erlotinib (3  $\mu$ M), cisplatin (10  $\mu$ M) and gemcitabine (300 nM) for 2 hr, and then the old media was replaced with drug-free fresh media. Plates were harvested at the various time points mentioned in results. Drugs were diluted in the same media which was used for growth of the cells and untreated controls were used to achieve the basal levels which were compared to the treatments at various time points. For radiation, we used 4 Gy, which is also a clinically achievable dose of ionizing radiation.

Cisplatin-resistant head and neck cancer cell lines UMSCC-5Pt and UMSCC10BPt which expressed Sp17 were treated with cisplatin to determine the effect of drug treatment in these resistant cell lines and compare with cisplatin treatment response in the sensitive cell line TE-13.

### Western blotting

After drug treatments and radiation, at different time points the cells were harvested and protein samples were prepared for analysis of proteins by western blotting. Briefly, cells were scraped using PBS containing sodium orthovanadate and protease inhibitor mixture (Roche Diagnostic, Indianapolis, IN). Detergent-soluble proteins were extracted by incubating cells at 4°C for 15 min in extraction buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% Triton X-100, 0.1% SDS, 1 mM EDTA, 100 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM phenylmethylsulfonyl fluoride and 1  $\mu$ g/ml aprotinin]. After particulate materials were removed by centrifugation at 13,000 rpm for 15 min at 4°C, protein concentrations in the supernatant were determined using Bradford reagent (Bio-Rad Laboratories, Hercules, CA). Aliquots containing 40  $\mu$ g of protein were diluted with an equal volume of loading buffer [63 mM Tris-HCl, 2% (w/v) SDS, 10% (v/v) glycerol and 0.005% (w/v) bromophenol blue], heated to 95°C for 5 min and applied to SDS-polyacrylamide gels. Separated proteins were electrophoretically transferred to polyvinylidene fluoride membrane. Membranes were incubated for 1 hr at room temperature in blocking buffer consisting of 3% BSA and 1% normal goat serum in Tris-buffered saline [137 mM NaCl, 20 mM Tris-HCl (pH 7.6) and 0.1% (v/v) Tween 20] and were subsequently incubated overnight at 4°C with 1  $\mu$ g/ml primary antibody in blocking buffer, washed and again incubated for 1 hr with horseradish peroxidase-conjugated secondary antibody (Southern Biotechnology, Birmingham, AL). After three additional washes in Tris-buffered saline, bound antibody was detected by enhanced chemiluminescence plus reagent (Amersham Biosciences, Piscataway, NJ). Some of the membranes were stripped using Restore stripping buffer Pierce (Rockford, IL) and reprobed with another antibody. For quantification of relative protein levels, immunoblot films were scanned and analyzed using ImageJ 1.32j software (NIH, Bethesda, MD). Unless otherwise indicated, the relative protein levels shown represent a comparison to untreated control.

### Immunocytochemistry

To confirm the changes in Sp17 levels observed by immunoblotting, immunocytochemical analysis was performed in TE13 and UMSCC5Pt and 10BPt. Cells were treated with cisplatin and proceeded for immunocytochemistry at 24 and 48 hr of treatments and the results were compared to untreated controls. Immunohistochemical staining was performed by an immunoperoxidase method using labeled streptavidin–biotin complex (LSAB2 System; DAKO, Carpinteria, CA). Briefly, TE 13 cells were plated on a glass slide by centrifugation using Cytospin 4 (Thermoshendon, Pittsburg, PA), air-dried for 1 hr at room temperature and fixed with cold acetone for 10 min. After a brief washing in PBS, slides were blocked with 5% normal goat serum for 1 hr and then incubated with rabbit polyclonal anti-human Sp 17 antibody (dilution, 1:100). After overnight incubation, the slides were washed (3× PBS) and then incubated with secondary antibody at room temperature for 1 hr. 3,3-diaminobenzidine (DAB) was used as a chromogen and Mayer's hematoxylin nuclear stain was used as a counterstain. The stained slides were mounted with mounting medium Sigma-Aldrich (St. Louis, MO) and analyzed under an epifluorescence microscope (Labophot-2; Nikon, Melville, NY). Pictures were captured using a Photometrics Coolsnap CF color camera (Nikon) and MetaMorph version 4.6.5 software (Universal Imaging, Downingtown, PA).

### Real-time reverse transcription–polymerase chain reaction

To investigate whether the changes in Sp17 levels occur at RNA levels or protein levels, we performed quantitative real-time polymerase chain reaction (qRT-PCR) analysis. TE13, UMSCC5Pt and UMSCC10BPt cell lines were harvested at various time points after cisplatin treatment, and total RNA was extracted using an RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instruction. qRT-PCR was performed using the Smart Cycler System (Cepheid, Sunnyvale, CA) with Platinum SYBR Green kit (Invitrogen, Carlsband, CA). Primer sequences used for qRT-PCR for *Sp17*, which generated a 102-bp PCR product, are as follows: forward 5'-AAA CAAATAGTCTTCAAAAATGAGGAAA-3' and reverse 5'-AAG AAGGTTGATGGATTTGGA-3'. Optimal annealing temperature was determined and the melt curve was carefully monitored to ensure PCR results.  $2^{\Delta\Delta C_T}$  method,<sup>26</sup> which reflected fold change in expression of the gene between treated cells and untreated cells, was employed using GAPDH as the normalized control.

### Immunoprecipitation and mass spectrometry

To determine the binding partners of Sp17, the cellular proteins interacting with Sp17 were immunoprecipitated using anti-Sp17 antibodies and the immunoprecipitates were subjected to liquid chromatography and mass spectrometry analysis. TE 13 cells were lysed in a minimum volume of immunoprecipitation lysis buffer [Tris-HCl buffer (50 mmol/l, pH

8), containing 150 mmol/l NaCl and 1% NP40] and pre-cleared by incubation with 50  $\mu$ l of Trublott anti-mouse/anti-rabbit Ig immunoprecipitation beads for 1 hr at 4°C. The pellet was discarded and the supernatant was subjected to immunoprecipitation. Cell lysates (500  $\mu$ g protein) were incubated with 5  $\mu$ g of specific antibody for 1 hr at 4°C. Twenty microliters of Trublott anti-mouse/anti-rabbit Ig immunoprecipitation beads was added and the pellet was further incubated on a rotating device overnight at 4°C. The pellet was then washed 4 times in ice-cold lysis buffer. The supernatant was discarded and the pellet was resuspended in 50  $\mu$ l of the sample buffer. The samples were fractionated by SDS-PAGE. Several bands were excised and subjected to liquid chromatography/MS/MS analysis in the core Mass Spectrometry Facility at the University of Michigan Medical School.

### MS-MS analysis

To analyze MS-MS results, the data were collected using a linear ion-trap instrument equipped with nanospray probe (Thermo Fisher, Waltham, MA). HPLC conditions were 5–95% buffer B (95% acetonitrile containing 1% acetic acid) for 40 min. Mass spectrometer was operated in a dual play mode wherein the instrument switched between full MS mode and MS/MS mode to collect collision-induced dissociation (CID) data on the 5 most abundant ions. CID data were analyzed using Bioworks 3.2 (Finnigan, Wayne, MI) by searching against a human international protein index (IPI) protein database.

### Ingenuity pathway analysis

Ingenuity pathway analysis (IPA) (Ingenuity® Systems, Redwood City, CA; www.ingenuity.com) was used to identify the protein networks in which Sp17 and the proteins coimmunoprecipitated with Sp17 antibody identified in our study are involved. Our aim was to determine the biological functions that are most significant to the genes in the network. Proteomics data were uploaded into IPA Knowledge Base as a tab-delimited text file of IPI accession numbers. IPA used abbreviation names of these proteins to navigate the literature database. For the detailed information on IPA, visit www.ingenuity.com. Eligible molecules serve as “seeds” for generating these networks. Networks are scored based on the number of network eligible molecules contained in these networks. The score =  $-\log(p\text{-value})$ . The higher the score, the lower is the probability of finding the observed number of network eligible molecules in a given network by random chance (Ingenuity® Systems; www.ingenuity.com).

### Statistical analysis

The results are presented as mean  $\pm$  SE of at least 3 experiments. Student's *t*-test was used to assess the statistical significance of differences. A significance level threshold of  $p < 0.05$  was used in our study.

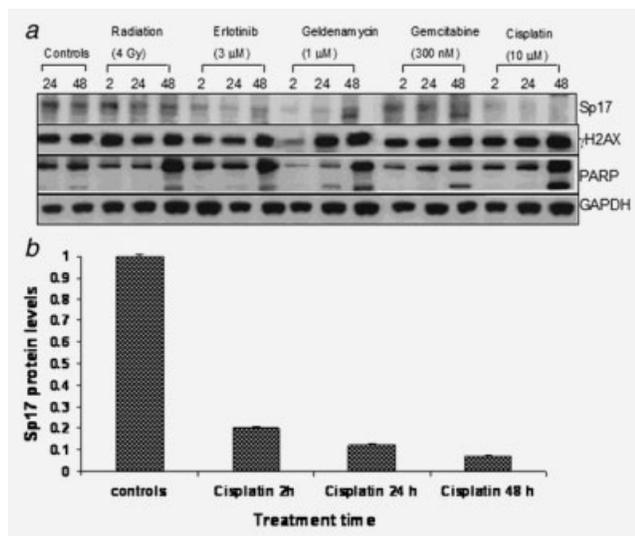


Figure 1. (a) Effects of radiation (4 Gy), erlotinib (3  $\mu$ M), geldanamycin (1  $\mu$ M), gemcitabine (300 nM) and cisplatin (10  $\mu$ M) on Sp17, PARP,  $\gamma$ H<sub>2</sub>AX and GAPDH levels in TE13 cell line. Conditions of drug treatments and immunoblotting are described in the Material and methods section. (b) Bar diagram showing the changes in Sp17 protein levels in response to cisplatin in the 3 independent immunoblotting experiments.

## Results

### Cisplatin treatment resulted in rapid and prolonged degradation of Sp17

We sought to determine the changes in Sp17 levels in response to various anticancer treatments, namely erlotinib, geldanamycin, gemcitabine, cisplatin and radiation. The esophageal cancer cells, TE13, were treated with erlotinib (3  $\mu$ M), geldanamycin (1  $\mu$ M), gemcitabine (300 nM) and cisplatin (10  $\mu$ M), as per details given in the Methods section or with radiation (4 Gy) and the protein levels of Sp17, PARP,  $\gamma$ H<sub>2</sub>AX and GAPDH were determined. Of these treatments, geldanamycin and cisplatin resulted in rapid degradation of Sp17 protein (within 2 hr); however, the levels increased further in case of geldanamycin (24 and 48 hr), whereas cisplatin caused prolonged decrement in Sp17 levels (48 hr) (Fig. 1a). More than 90% of Sp17 protein degradation was observed at 48 hr of cisplatin treatment than controls (Fig. 1b;  $p < 0.001$ ). Erlotinib also caused reduction in Sp17 levels (2 hr); however, at 48 hr the levels were close to control levels. Maximum amount of PARP cleavage was observed in cisplatin-treated cells, showing high sensitivity of these cells to cisplatin. Radiation treatment did not cause an increase in  $\gamma$ H<sub>2</sub>AX levels, whereas the levels increased in response to chemotherapeutic drugs such as gemcitabine and cisplatin (Fig. 1). An increase in the levels of  $\gamma$ H<sub>2</sub>AX in response to treatment with anticancer drugs is an indicator of unrepaired DNA damage. Increased PARP cleavage and  $\gamma$ H<sub>2</sub>AX levels in response to chemotherapy suggests that TE13 is a drug-responsive cell line.

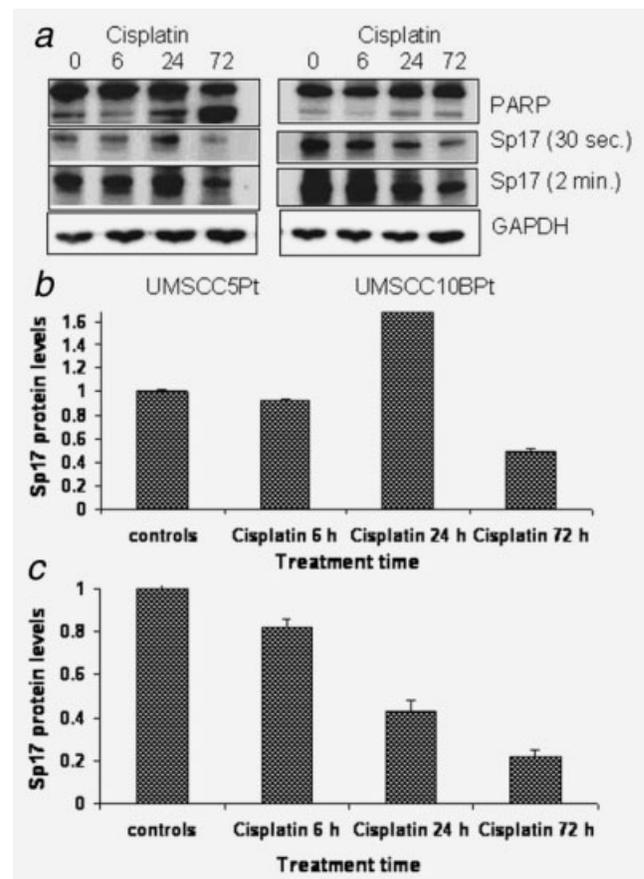
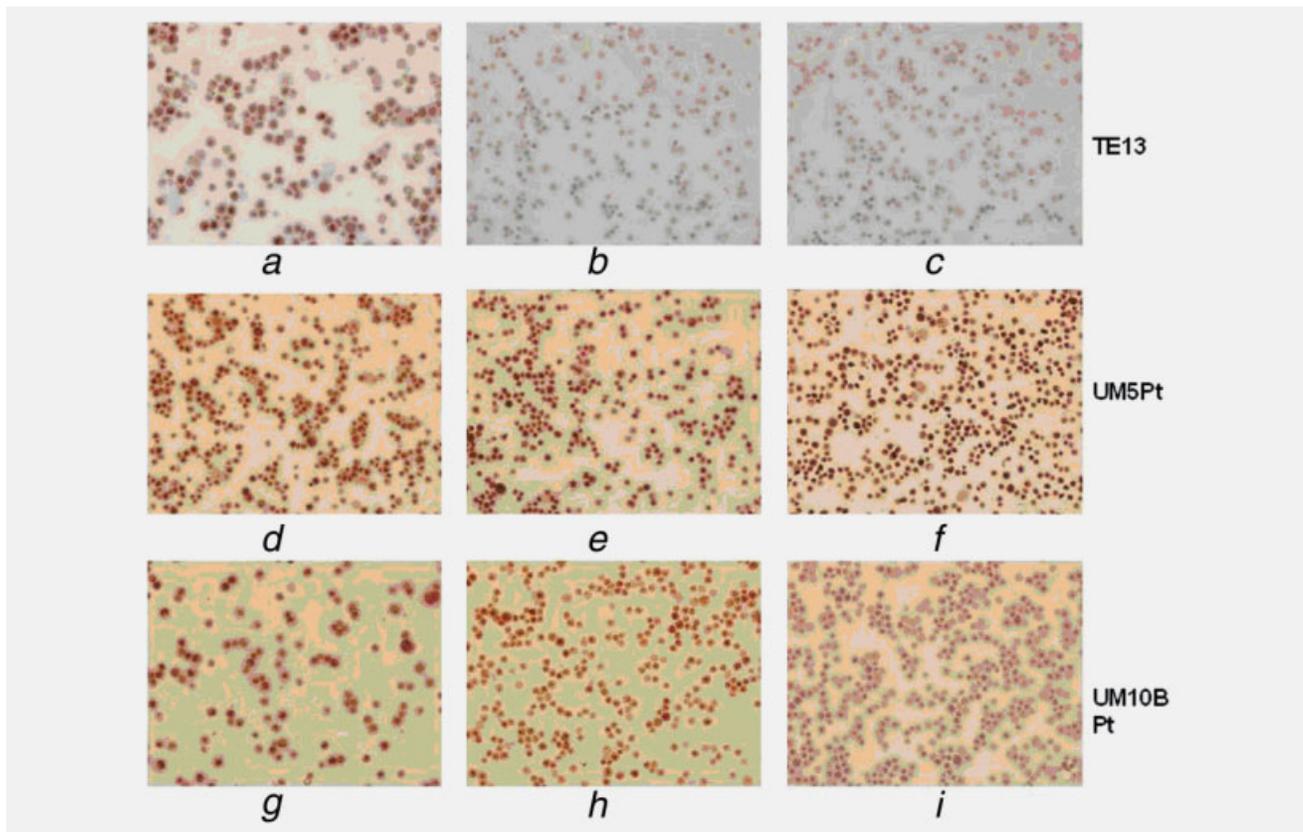


Figure 2. (a) Effects of cisplatin on Sp17, PARP and GAPDH levels in relatively cisplatin-resistant UMSCC5Pt and UMSCC10BPt cell lines. (b, c) Bar diagram showing the changes in Sp17 protein levels in response to cisplatin in UMSCC5Pt and UMSCC10BPt. Results are the indicative of the 3 independent immunoblotting experiments.

### In cisplatin-resistant head and neck squamous cell lines, Sp17 levels did not decrease significantly

Being convinced with the decrease in levels of Sp17 in response to cisplatin in a sensitive cell line, we next investigated if there is a change in Sp17 levels in response to cisplatin in cisplatin-resistant head and neck squamous cell lines, UMSCC5Pt and UMSCC10BPt cells, which expressed Sp17 (Fig. 2a). Cisplatin treatment in these cisplatin-resistant cell lines did not cause a significant decrease in Sp17 levels. Although, the 2 cell lines behaved differently with respect to Sp17 protein degradation in UMSCC5Pt, at 72 hr, Sp17 levels were 49% of the controls ( $p > 0.05$ ), whereas in UMSCC10BPt the remaining Sp17 protein levels were only 22% ( $p < 0.05$ ) (Figs. 2b and 2c, respectively). The decrease in Sp17 levels was not significantly different in UMSCC5Pt and UMSCC10BPt cells at 24 hr, whereas 90% of Sp17 protein levels were reduced than control levels in the cisplatin-sensitive TE13 cell line. Also, there was only a modest increase in cleaved PARP levels in UMSCC5Pt and almost



**Figure 3.** (a–i) Results of immunocytochemistry experiments indicating changes in Sp17 protein levels in response to cisplatin in cisplatin-sensitive TE13 and relatively cisplatin-resistant UM5Pt and UM10BPt cell lines. [Color figure can be viewed in the online issue, which is available at [www.interscience.wiley.com](http://www.interscience.wiley.com).]

undetectable PARP cleavage in UM5Pt, suggesting that these 2 cell lines are comparatively resistant to cisplatin-induced apoptosis than TE13.

#### Immunocytochemistry data confirmed lowered expression of Sp17 after cisplatin treatment in cisplatin-sensitive cells

Using immunocytochemistry, we further investigated Sp17 levels in response to cisplatin treatment in the cisplatin-sensitive TE13 and cisplatin-resistant UM5Pt and UM10BPt cells. After cisplatin treatment, equal numbers of cells were plated for immunocytochemical analysis. Sp17 expression was found to be significantly decreased in response to cisplatin compared to the untreated controls in cisplatin-sensitive TE13 cells, whereas the expression did not decrease in the cisplatin-resistant UM5Pt and UM10BPt cells (Fig. 3).

#### Quantitative real-time PCR analysis confirmed reduction in Sp17 transcripts on cisplatin treatment in cisplatin-sensitive cells

To investigate further whether the reduction of Sp17 levels after cisplatin treatment occurs at the protein or RNA levels, we carried out qRT-PCR analysis of the Sp17 transcripts. Cis-

platin-sensitive, TE13 and resistant UM5Pt and UM10BPt cells were plated at a cell density of  $2.5 \times 10^3$  cells/well in a 6-well plate and treated with 10  $\mu$ M cisplatin for 2, 24, 48 and 72 hr. qRT-PCR analysis revealed a significant reduction in expression of Sp17 after 24-hr treatment as compared to the untreated control cells (Fig. 4a). A difference of 3 to 4 threshold cycles ( $C_T$ ) was observed between control and cisplatin-treated TE13 cells. Decreased Sp17 expression was observed in 48 and 72 hr treated cells (upto >80%;  $p < 0.001$ ; Fig. 4b). There was no significant difference in expression of Sp17 levels in both UM5Pt and UM10BPt cells after cisplatin treatment (from 2 to 72 hr) as compared to untreated cells (Figs. 4a and 4b). These data suggest that cisplatin-induced reduction in Sp17 expression occurs at the transcript level.

#### Novel binding partners of Sp17 protein

The biological functions of Sp17 in human cancers remain largely unknown. The above results and our earlier reports<sup>17,18</sup> suggest that it might be involved in tumor growth and development as well as drug resistance. We speculated that Sp17 might be important in signaling pathways and it acts in co-ordination with many factors. Therefore, we carried out immunoprecipitation analysis using Sp17 antibody, to

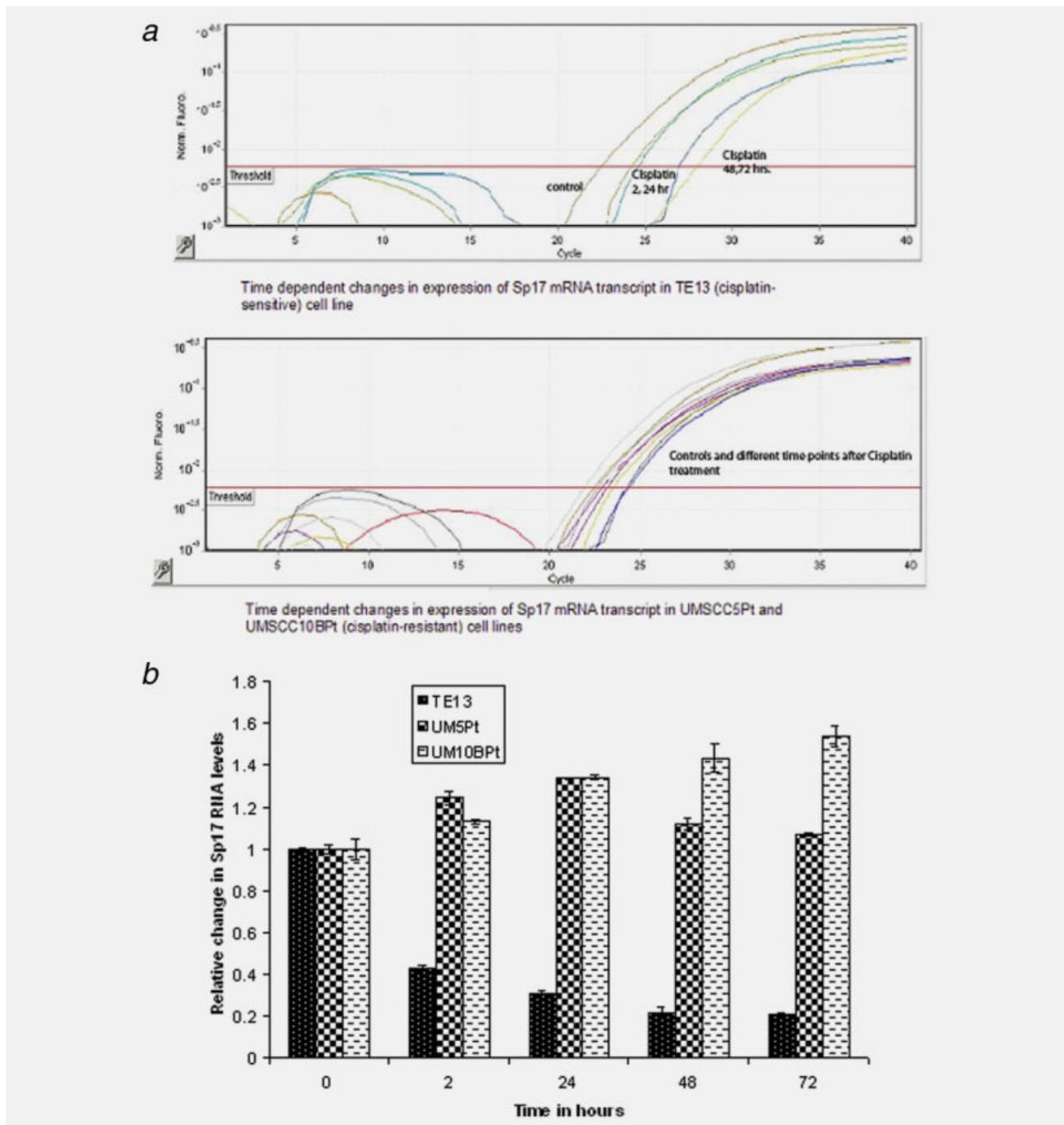


Figure 4. (a) Kinetics of Sp17 mRNA in response to cisplatin at various time points in TE13, UM5CC5Pt and UM5CC10Pt cell lines. Sp17 mRNA at various time points 2, 24, 48 and 72 hr after cisplatin treatment were compared to controls. The melting curves depicting Sp17 mRNA start to separate as early as at 2 hr after cisplatin treatment in case of TE13 showing the decrease in levels, whereas the curves did not separate in case of UM5CC5Pt and UM5CC10Bt showing no change in Sp17 mRNA levels in these cell lines. (b) Bar diagram showing quantitation of Sp17 mRNA in the 3 cell lines. Results are the indicative of the 3 independent real-time PCR experiments. (c) Network analysis using ingenuity pathways analysis software. Network analysis classified proteins into 2 networks on the basis of functions published in the literature; the merged network is shown. Solid lines show direct interactions/regulations, whereas dashed lines show indirect interactions/regulations of proteins at ends of the lines. [Color figure can be viewed in the online issue, which is available at [www.interscience.wiley.com](http://www.interscience.wiley.com).]

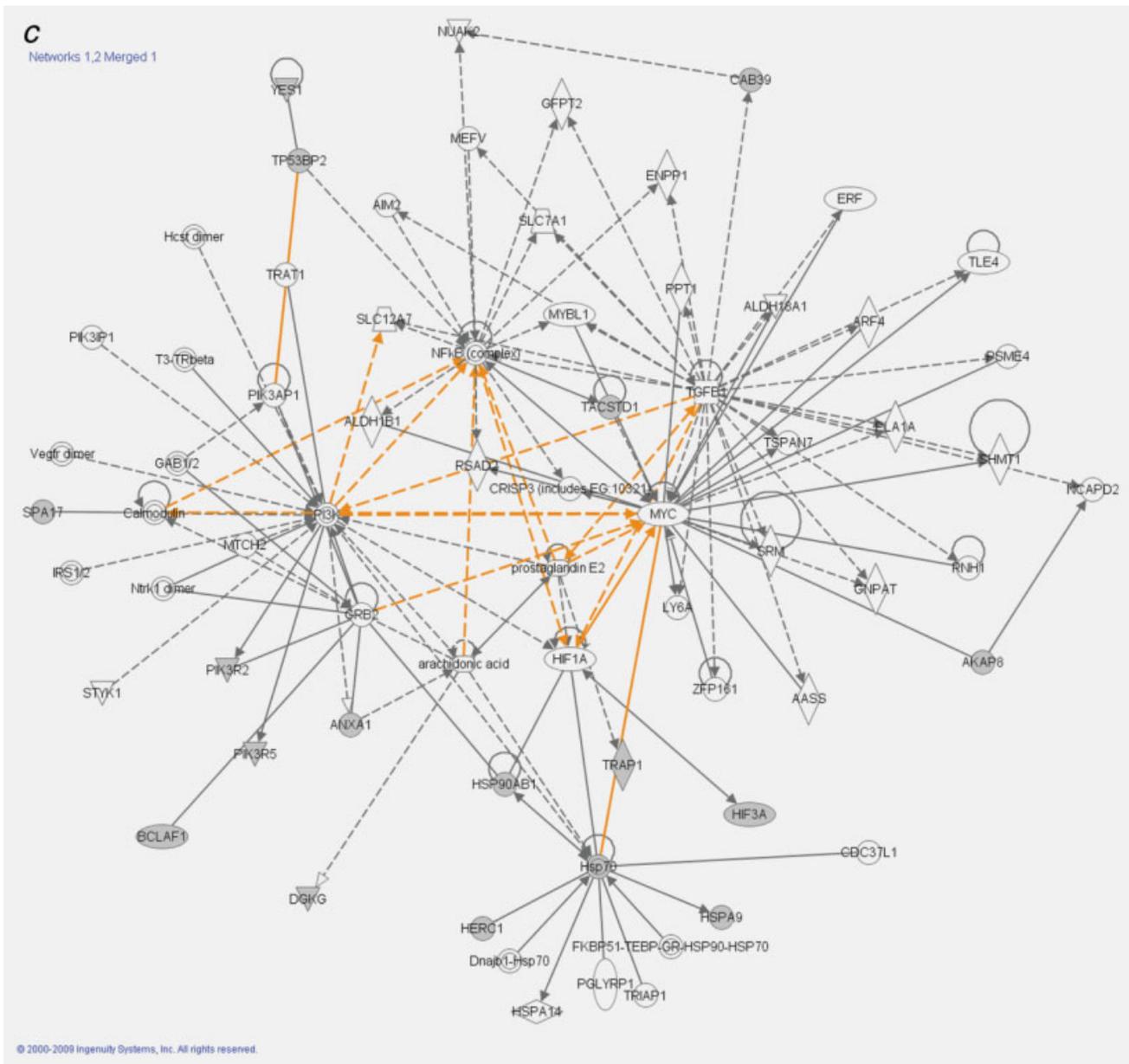


Figure 4. (Continued)

identify the binding partner(s) of Sp17. Mass spectrometric analysis of Sp17 immunoprecipitates resulted in identification of several proteins that might act as the binding partners of Sp17 and have known roles in cell survival, cell signaling, tumor growth and metastasis. These proteins are listed in Table 1. The structural organization of Sp17 confirms that some of these proteins might be binding to Sp17.

Protein network analysis using IPA suggested that 16 network eligible proteins were components of 2 major networks with scores 26 and 11 comprising 11 and 5 network proteins identified in our study, respectively (Supporting Information Table). Figure 4c depicts the merged 2 novel networks to shed light on the biological significance of the proteins identified in a cellular context. The complexity of Figure 4c dem-

onstrates the numerous interactions between Sp17 and its putative interaction partners that are components of inflammation, stress response, cell signaling, growth and proliferation—cellular processes known to be implicated in esophageal cancer development. These networks suggest direct interaction of Sp17 with calmodulin which in turn shows connections with PI3-Kinase, NFkB, TGFβ1, myc, HIF1A, HSP90AB1 and HSP 70.

**Discussion**

Molecular markers that can predict the response of various therapeutic interventions in esophageal cancer patients are urgently needed. We sought to investigate the changes in levels of a cancer-testis antigen, Sp17, in response to radiation

**Table 1.** List of proteins that were found to be interacting with Sp17 (Sp17-binding partners) in TE13 cell line

Significant hits	Abbreviation	Molecular weight (kD)	Functions
Tumor-associated calcium signal transducer 1 precursor	TACSTD1	35	Cell–cell adhesion
ERBB3 isoform 1 of receptor tyrosine–protein kinase erbB-3 precursor	HER3 precursor	~180	Receptor tyrosine kinase signaling
Isoform 1 of Bcl-2-associated transcription factor 1	BCLAF1	106	Bcl-2 family member repressor
Hypoxia inducible factor 3, alpha subunit	HIF3A	72	Hypoxia sensing, angiogenesis
Annexin A1	ANXA1	38	Membrane fusion, exocytosis
Membrane-associated ring finger	MARCH7	78	Ring-finger domain ubiquitin ligase
Tumor protein p53-binding protein, 2	TP53BP2	125	Inhibition of apoptosis
Heat shock 70-kDa protein 9	HSPA9	73	HSP70 family, molecular chaperones
TNF receptor-associated protein/heat shock protein 75 kDa, mitochondrial precursor	TRAP1	80	Molecular chaperones
Heat shock protein 90 kDa alpha (cytosolic), class B member 1	HSP90AB1	86	HSP90 family, molecular chaperones
Phosphoinositide 3-kinase regulatory subunit 6	PIK3R5	97	PI3K signaling
Phosphoinositide 3-kinase regulatory subunit 2	PIK3R2	85	PI3K signaling
Diacylglycerol kinase, gamma	DGKG	89	PI3K signaling
Vav 2 oncogene	VAV 2	60	Receptor tyrosine kinase signaling
Proto-oncogene tyrosine–protein kinase Yes	YES1	60	Receptor tyrosine kinase signaling
Guanine nucleotide exchange factor p532	HERC1	72	Ras signaling; GTP-binding proteins
Ras-related protein-19	RAB19	49	Ras signaling; GTP-binding proteins
A kinase anchor protein 8	AKAP8	95	Protein kinase A signaling
Calcium-binding protein 39	CAB39	39	Calcium signaling

Data are representative of 2 independent MS-MS experiments.

and chemotherapeutic drugs including erlotinib, geldanamycin, gemcitabine and cisplatin in esophageal squamous carcinoma cell lines. Our salient findings demonstrated that Sp17 protein levels decreased in response to treatment with (i) the HSP90 activity inhibitor geldanamycin, (ii) the tyrosine kinase inhibitor erlotinib and (iii) cisplatin (the chemotherapeutic agent commonly used in management of ESCC). In contrast, the Sp17 levels did not decrease in response to radiation therapy or treatment with the chemotherapeutic agent gemcitabine. Further investigations showed that the cisplatin induced decrease in Sp17 levels was due to transcriptional inhibition, and cisplatin-resistant cell lines did not show this decrease in Sp17 levels in response to cisplatin treatment.

Notably, in the TE13 cell line, of the aforementioned 5 treatments, cisplatin and geldanamycin caused rapid degradation of Sp17 protein at 6 hr, which correlated with PARP cleavage at 48 hr. The geldanamycin-induced Sp17 degradation suggests that Sp17 might be a substrate/client protein for HSP90 activity, as geldanamycin and other ansamycin derivatives are known to degrade HSP90 substrate proteins.<sup>27</sup> Among the HSP90 substrate proteins, Erb2, Src, Akt and ChK1 are important for cell cycle regulation and the list is growing.<sup>28,29</sup> Whether Sp17 plays a role in DNA damage and repair is yet to be determined; however, our MS-MS data

showed its interaction with both HSP70 and HSP90, suggesting a possible link.

Importantly, only cisplatin caused almost 90% degradation of Sp17 protein in TE13 cells at 48 hr. As increased PARP cleavage and  $\gamma$ H2AX levels were also indicative of apoptosis and unrepaired DNA damage in response to cisplatin at 48 hr, the degradation of Sp17 might be proposed to be a marker for cisplatin response. Hence, the next step was to look at Sp17 levels in cisplatin-resistant cell lines. In one of our earlier reports, we found upregulation of Sp17 mRNA in oral cancer patients as compared to healthy control subjects.<sup>30</sup> Therefore, we used head and neck squamous cell carcinoma cell lines, UM5CC5Pt and UM5CC10BPt, as our model for cisplatin resistance. Cisplatin treatment in these resistant cell lines did not cause a significant decrease in Sp17 levels or affected PARP cleavage, suggesting that Sp17 levels correlated with cisplatin response and apoptosis, whereas treatment with other chemotherapeutic agents such as erlotinib and geldanamycin as well as radiation did not have such effects on Sp17 levels, implicating Sp17 in cisplatin resistance. Importantly, because its downregulation correlated with cisplatin sensitivity and resultant apoptosis, we propose Sp17 to be a putative marker for predicting cisplatin response prior or early in the course of treatment. It is noteworthy that the

risk factors for esophageal cancer development might vary across different ethnic groups, and therefore, it is possible that the response to a particular type of treatment may be different because of distinct signaling pathways. Notably, a recent report demonstrating the involvement of Sp17 in chemoresistance in human epithelial ovarian cancer is in agreement with our findings.<sup>19</sup> However, these authors did not determine the phenotype in sensitive cisplatin cell lines and the influence on Sp17 protein levels was also not reported. Our results also indicated that the reduction in Sp17 protein levels is due to the downregulation of Sp17 transcripts in response to cisplatin. At present, we do not know what is/are the transcriptional repressor(s) of Sp17 in response to cisplatin; nevertheless, several reports observed the differential gene expression profiles of cisplatin-sensitive and resistant tumors.<sup>31–33</sup>

The biological role of Sp17 in the development of esophageal cancer is yet unknown. Immunoprecipitation and mass spectrometry data combined with IPA showed the interaction of Sp17 with the calcium-binding protein calmodulin and cAMP-dependent protein kinase, which are expected from its structural organization. The N-terminal domain of Sp17 is almost totally conserved among all species studied and has a high homology to the cAMP-dependent protein kinase A regulatory subunit II, which is essential for protein dimerization and interaction with protein kinase A-anchoring proteins.<sup>34</sup> Sp17 protein has a central sulphated carbohydrate-binding domain and its C-terminal contains an “IQ” motif which is a Ca<sup>2+</sup>/calmodulin-binding domain.<sup>35</sup> Expectedly, the binding partners of Sp17 include A kinase anchor protein 8 and calcium-binding protein 39. In addition, the central portion of Sp17 contains a region that binds carbohydrates and is implicated in cell–cell adhesion,<sup>36</sup> which might help in cell adhesion and angiogenesis during tumorigenesis.

The goal of this pathway analysis approach was to establish a hypothesis to unravel new protein connections between Sp17 and components of cellular pathways. In this context, IPA of the identified novel binding partners of Sp17 revealed

their involvement in cell proliferation, cell–cell adhesion, angiogenesis, molecular chaperones, PI3K signaling and receptor tyrosine kinase signaling. It also revealed some major hubs in these networks where multiple connections arrive or irradiate to the rest of the network—Sp17 direct interaction with calmodulin which is connected to other major hubs that include PI3-kinase, NFκB, TGFβ1, myc, HIF1A, HSP90AB1 and HSP70, suggesting that Sp17 may be found to be indirectly implicated in cell proliferation, stress response and cell signaling. Functional testing is however warranted for the validation of Sp17 interactions with the individual proteins deduced from this approach. In this context it is noteworthy that 2 of the proteins identified in these networks, namely A-kinase anchoring protein (AKAP) and ropporin or rhophilin-associated protein 1, have been reported to be associated with Sp17. Lea *et al.*,<sup>37</sup> demonstrated the association of Sp17 with A-kinase anchoring protein 3 (AKAP3) in flagella. In another report, a yeast 2-hybrid system using Sp17 identified ropporin as a novel cancer-testis antigen in hematologic malignancies.<sup>38</sup> Further, both ropporin and AKAP-associated sperm protein have been shown to bind AKAP3.<sup>39</sup> Ropporin binds rhophilin, a GTPase Rho-binding protein, involved in the Rho signal transduction pathway.<sup>40</sup> In this way, AKAP3 acts as a scaffold protein for 2 independent signal transduction pathways. These studies support our network-based approach for unraveling novel interaction partners and functions of Sp17 in esophageal tumorigenesis.

Collectively, our data strongly suggest Sp17 to be a novel marker for predicting cisplatin response in ESCC. Further studies in this direction should focus on its involvement in various signaling pathways involved in DNA damage and repair.

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