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Proteomic Analysis of Bladder Cancer Cells Reveals Potential Candidates of Biomarkers in Bladder Tumorigenesis

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Abstract. *Background: Bladder cancer is the most prevalent type of cancer of the urinary tract in Taiwan. In order to identify the molecular basis of bladder carcinogenesis, we analyzed the proteomic profiling of transitional cell carcinoma (TCC) cell lines to search for novel biomarkers for human bladder cancer. Materials and Methods: Two human TCC cell lines (TSGH8301, Grade II and BFTC905, Grade III) were selected for proteomic analysis. The candidate genes were identified by tandem mass spectrometry. Results: Eight differentially-expressed spots were revealed by high resolution 2-D electrophoresis. Five genes were identified by spectrometry as showing higher expression in the TSGH8301 cells, i.e. heat- shock protein 27, maspin, prohibitin and glutathione S-transferase P1-1 and Chaperonin-containing t-complex polypeptide 1 β subunit. In contrast, S100A4 and annexin V exhibited higher levels in the BFTC905 cells. The differential expression patterns of the identified genes were confirmed by immunoblotting and further analyzed using a variety of TCC cell lines. Conculsion: Our studies implicate the potential role of these de-regulated proteins in bladder cancer and warrant further investigation in clinical samples.*

Bladder cancer is the most prevalent type of cancer of the urinary tract. In Taiwan, most of the cases of bladder cancer are of transitional cell carcinoma (TCC) histology and epidemiological studies indicate that the incidence of TCC is unusually high on the southwest coast of Taiwan where blackfoot disease is endemic (1). The majority of TCC (>70%) are diagnosed as superficial lesions and are easily cured by transurethral dissection and chemotherapy (2, 3). However, these tumors have a high frequency of recurrence after transurethral dissection and some of them will progress to invasive and potentially fatal metastatic tumors (4). Cytoscopic examination and histological confirmation of bladder biopsy are currently the most reliable methods for therapeutic interventions and clinical outcome. However, it is impossible to predict recurrence by these invasive procedures. Therefore, it is necessary to identify biomarkers that are useful in prognosis, progression and clinical medicine.

Like many types of cancer, most research in the field of bladder cancer has centered on tumor suppressor genes and oncogenes. Previous studies have indicated that inactivation of the p53 protein by point mutation is associated with tumor stage and grade, progression and patient survival (5-8). Reduced expression of the retinoblastoma gene, *Rb*, is related to tumor grading and progression (9, 10). A metastasis suppressor gene, *nm23-H1*, is reported to be inversely correlated with size and staging (11).

In addition to tumor suppressor genes, it has also been demonstrated that alteration of proto-oncogenes is related to the progression of bladder cancer, including *ras*, EGFR and the Erb family (12-15). Overexpression of EGFR is associated with muscular invasion, whereas expression of ErbB3 and ErbB4 is implicated in tumor invasion. Furthermore, co-expression of EGFR with ErbB2 and/or ErbB3 is an indicator of tumor recurrence and patient survival (15).

Collectively, these observations indicate that carcinogenesis of the bladder involves multiple genetic alterations. However, the frequency of these alterations is associated with the heterogeneity of bladder cancer and its diverse geographical and etiological background. For instance, mutation of *ras* ranges from 10% in the UK and Japan (13, 14) to 45% in the USA (12). In addition, the gene alteration may not occur in the majority of tumor samples. This is

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illustrated by observations that alteration of p53 occurs in less than 10% of superficial TCC and that *Rb* is inactivated in 40% of samples (10). Given the fact that genetic change varies in distinct regions, an important step towards gaining an insight into carcinogenesis of the bladder in Taiwan is to profile protein expression and to look for markers that are deregulated. Initially, we established the proteome map and have found differential protein expression between TSGH8301 and BFTC905 cells, which were established from local superficial (Stage A, Grade II) and invasive TCC (Stage D1, Grade III), respectively (16, 17). Further analysis by mass spectrometry indicates altered expression of tumor suppressor and metastasis-related genes as bladder cancer progresses to a higher grade.

Materials and Methods

Cell lines. Both TSGH8301 and BFTC905 cells were derived from patients with TCC in Taiwan. The TSGH8301 cells were established from superficial bladder cancer (Stage A, Grade II) (16), whereas the BFTC905 cells were from invasive TCC (Stage D1, Grade III) (17). The UB09 (Grade II), UB37 (Grade II) and UB47 (Grade III) cell lines have recently been established from Taiwanese TCC patients in our laboratory. RT4 and J82 were purchased from ATCC. The RT4 cells were maintained in McCoy's 5A (Sigma-Aldrich Co.), while the remaining cell lines were cultured at 37˚C in DMEM (Life Technologies Inc., Grand Island, NY, USA) supplemented with 10% fetal bovine serum.

Sample preparation. Cancer cells were harvested after trypsinization, washed and lysed in lysis buffer (7M urea, 2M thiourea, 4% CHAPS, 1 mM EDTA, 1 mM PMSF, 100 U/ml aprotinin, 100 mM dithiothreitol). The samples were then centrifuged at 14,000 rpm for 20 min. The supernatant was collected and centrifuged again at 85,000 rpm in an Optima ultracentrifuge (Beckman, Fullerton, CA, USA) for 2 h. After centrifugation, the supernatant was cleaned with the 2-D clean kit (Amersham-Pharmacia Biotech Inc., Piscataway, NJ, USA) and the protein pellet was dissolved in rehydration buffer and stored at –80˚C. The protein concentration was determined by DC protein assay kit (BioRad, Hercules, CA, USA).

Isoelectric focusing (IEF). The IPG gel strips (11cm, pH 3-10 or pH 4-7) were rehydrated for 16 h with 200 μl rehydration buffer (7M urea, 2M thiourea, 4% CHAPS, 2% dithiothreitol, 0.5% IPG buffer and a trace amount of bromophenol blue) containing 60 μg of protein. The proteins were then focused at 200V, 1000V, 5000V and 8000V with a total of 32,000 voltage-hours.

SDS-PAGE. After isoelectric focusing, the gel strips were equilibrated in equilibration buffer (6M urea, 30% glycerol, 2% SDS) containing 2% dithiothreitol for 15 min and then in equilibration buffer containing 5% iodoacetamide for a further 15 min. The gel was loaded onto the top of an acrylamide gel (12.5%) and sealed with 0.5% agarose. The proteins were separated at 20 mA per gel until bromophenol blue reached the bottom of the gel.

Silver staining and image analysis. The 2-D plus one silver staining kit (Amersham-Pharmacia) was utilized to detect proteins, employing a modified protocol as described elsewhere (18). To look for proteins showing disparity in expression, the proteome maps of bladder cancer cells were first analyzed by ImageMaster 2-D Elite (Amersham Pharmacia) and by PDQuest (BioRad) software. A total of eleven pairs of well-focused gels from TSGH8301 and BFTC905 cells were compared. Differentially-expressed spots detected by computer analysis were further examined by visualization. The intensity of a spot was calculated and normalized as a percentage of the total number of all spots of a gel and analyzed by Student's *t*-test. In all cases, a *p* value less than 0.05 was considered to be significantly different.

In-gel digestion and liquid chromatography-tandem mass spectrometry (LC-MS/MS). The in-gel digestion and mass spectrometric analysis were carried out as described previously, with some modifications (19). Electrospray ionization tandem mass spectrometry was performed using a ThermoFinnigan LCQ Deca ion trap mass spectrometer interfaced with an Agilent 1100 HPLC system. The digested peptides were separated in an Agilent ZORBAX 300SB-C18 column (150 x 0.3 mm, 3 μm particle diameter, 300 A pore size) using a mobile phase of solution A (0.1% formic acid in water) and solution B (0.085% formic acid in acetonitrile). The peptides were eluted at a flow rate of 5 μl/min with an acetonitrile gradient consisting of 5-16% solution B in 5 min, 16-20% solution B in 40 min, and 20- 65% solution B in 40 min. The peptides eluted from the HPLC were introduced on-line to the ESI source and the spectra were acquired as successive sets of three scan modes (MS, Zoom and MS/MS scans), as described previously (19). The acquired collision-induced dissociation spectra were interpreted with TurboSequest software (ThermoFinnigan, San Jose, CA, USA) that matches predicted tandem mass spectra against a non-redundant protein database.

Western blotting. Total cell lysates (50 μg of protein) were separated by 10% acrylamide gel electrophoresis and transferred onto the PVDF membrane. The membrane was blotted with primary antibody overnight, followed by incubation with HRP-conjugated secondary antibody, and visualized using chemiluminescence (Amersham Pharmacia). The antibodies utilized were obtained from the following sources: maspin (BD Biosciences, San Diego, CA, USA); prohibitin (Lab Vision, Fremont, CA, USA); glutathione S-transferease P1-1 (Stressgen Bioreagents, Victoria, British Columbia, Canada); actin (Chemicon International, Temecula, CA, USA); and heat-shock protein 27 (Upstate Biotechnology, Lake Placid, NY, USA).

Results

Molecular profiling of TCC cell lines. In this study, we started with 2D electrophoresis at pH 3-10 as the first dimension and SDS-PAGE (12.5%) as the second dimension, to establish the proteome maps. Most of the proteins detected were in the area ranging from pH 4 to 8 (data not shown). To further amplify this region, we exploited a gel strip of pH 4-7 as the first dimension. As shown in Figure 1, proteins extracted from Grade II TSGH8301 and Grade III BFTC905 cells were well resolved and around 700 spots were observed by image analysis.

The differential expression patterns of the proteins between these two cell lines were confirmed by image and statistical

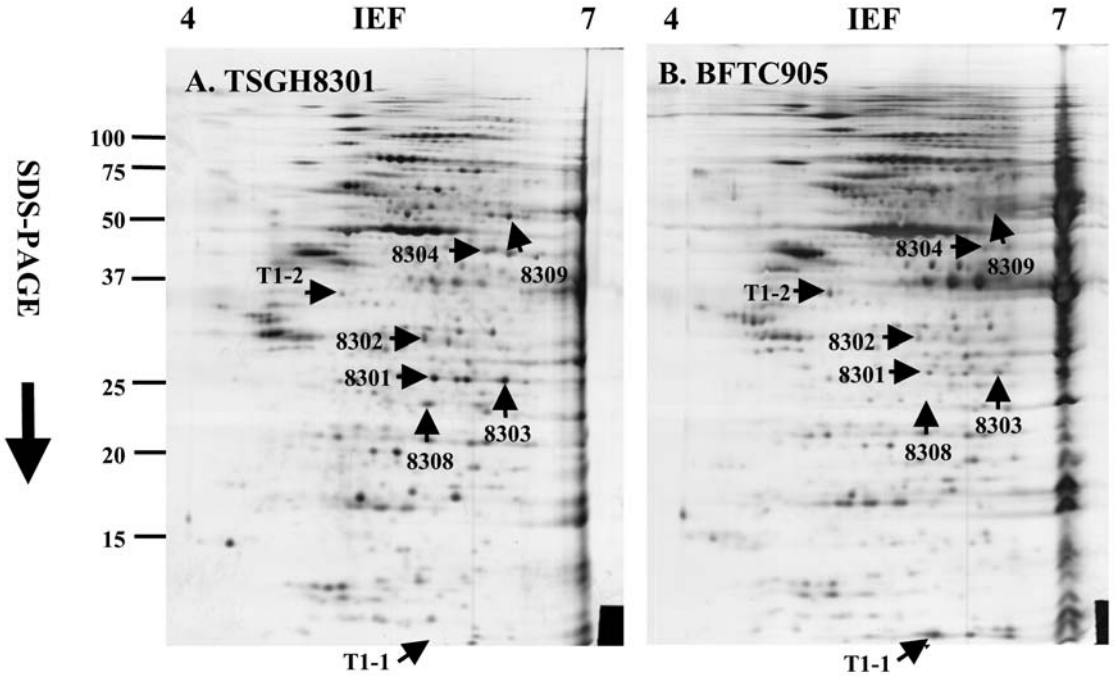

Figure 1. *Proteome maps of TSGH8301 and BFTC905 cells. Proteins were separated by 2-D PAGE, using 11 cm pH 4-7 gel strip and 12.5% SDS-PAGE. The paired gels are representative of eleven independent experiments. The expression profiles of the proteins were analyzed by PDQuest (BioRad) software. Note that the differentially-expressed spots are indicated by arrows.*

Table I. *Identification of differentially-expressed proteins by tandem mass spectrometry in TCC cell lines.*

Spot No.	Identity	Protein coverage	pI/Molecular mass (kDa)		Potential functions
			Experimental	Theoretical	
Up-regulation in TSGH8301 cells (Grade II TCC)					
8301	HSP27	48%	5.7/24.8	5.98/22.7	Heat-shock response to stress Differentiation
8302	Prohibitin	23%	5.61/27.6	5.57/29.8	Tumor suppressor Cell cycle regulation
8303	HSP27	31%	6.4/24.8	5.98/22.7	Heat-shock response to stress Differentiation
8304	Maspin	8%	6.0/42	5.72/42	Tumor suppressor Angiogenesis inhibitor
8308	Glutathione transferase P1-1 chain A	54%	5.77/24.4	5.74/23.2	Oxidative reduction Cancer resistance
8309	Chaperonin-containing t-complex polypeptide 1 β subunit	14%	6.7/60	6.01/57.5	Molecular chaperone
Up-regulation in BFTC905 cells (Grade III TCC)					
T1-1	S100A4	20%	5.74/12	5.85/11.7	Metastasis
T1-2	Annexin V	62%	5.08/32.8	4.94/36	Ca^{2+} -dependent phospholipid-binding protein

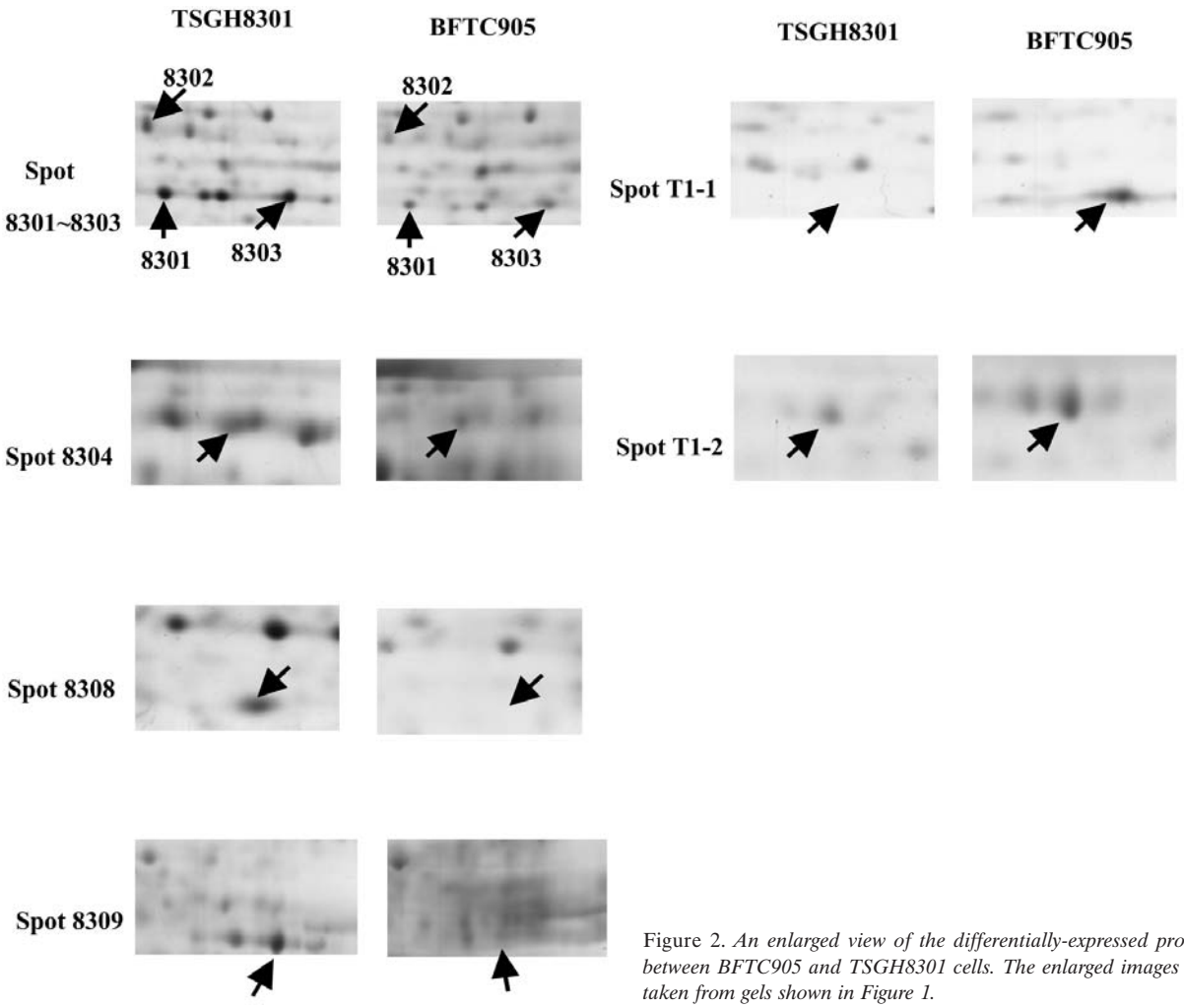

Figure 2. *An enlarged view of the differentially-expressed proteins between BFTC905 and TSGH8301 cells. The enlarged images were taken from gels shown in Figure 1.*

analysis. A total of eight differentially-expressed spots were observed after comparison of eleven pairs of gels. Of these spots, as indicated in Figure 1, six were down-regulated (spots 8301-8304, 8308, 8309) and two were up-regulated (T1-1 and T1-2) in BFTC905 (Grade III) cells as compared to TSGH8301 (Grade II) cells. The selected areas containing differentially-expressed spots were enlarged in Figure 2.

Identification of differentially-expressed proteins. After establishing the proteome map, the spots were excised, digested with trypsin and analyzed by tandem mass spectrometry. The results of the mass spectrometric analysis are summarized in Table I. Of the six overexpressed spots in TSGH8301 (Grade II) cells, two were identified as heat-shock protein 27 (HSP27) (spots 8301 and 8303). It is interesting to note that these two spots migrated at the same molecular mass, but focused at a different isoelectric point (experimental pI 5.7 and 6.4, Figure 1), indicating the same protein with different post-translational modification. The other two spots (spots 8302 and 8304), showing higher expression levels in TSGH8301 cells, were identified as prohibitin and maspin by mass spectrometry, respectively. Spot 8308 was identified as glutathione transferase (GST) P1-1 chain A and spot 8309 as the β subunit of the Chaperonin-containing t-complex polypeptide 1 (CCT-β). The up-regulated spots in BFTC905 cells were T1-1 and T1-2. Spot T1-1 was determined as S100A4 protein, which belongs to the S100 family of calcium-binding proteins and plays an important role in the metastasis of bladder cancer (20). Spot T1-2 was identified as annexin V, a Ca^{2+}-dependent phospholipid-binding protein (21). The experimental molecular mass and isoelectric points of the above proteins were comparable to the theoretical values deduced from a protein database, with the exception of HSP27 which showed

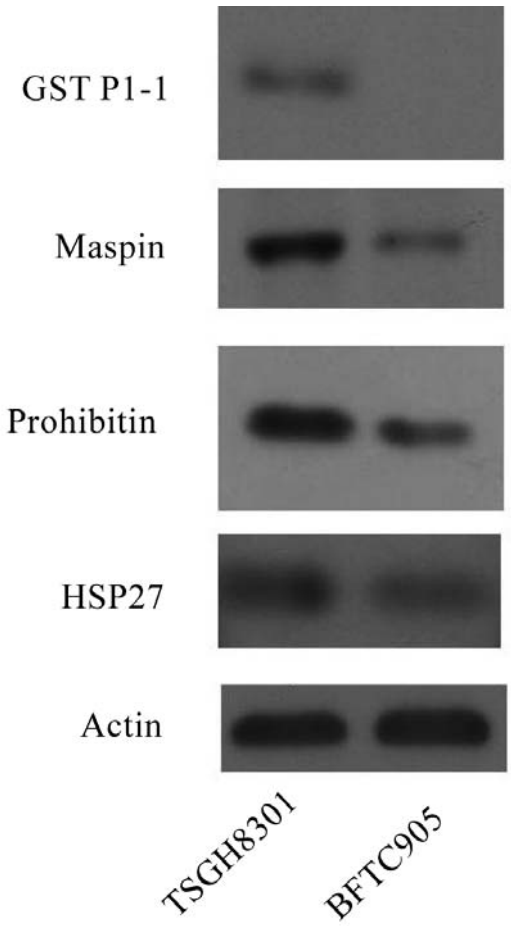

Figure 3. *Expression of glutathione S-transferase P1-1, maspin, prohibitin and HSP27 in TSGH8301 and BFTC905 cells by Western blotting. These blots are representatives of three independent experiments. GST P1-1: glutathione S-transferase P1-1; HSP27: heat-shock protein 27.*

two pI values (Table I). Taken together, in higher grade bladder cancer (BFTC905) cells, metastasis-associated genes were overexpressed (such as S100A4). In contrast, in the lower grade bladder cancer cells (TSGH8301), stress-related or tumor suppressor genes were overexpressed.

Confirmation by immunoblotting. The accuracy of 2-D PAGE results is highly dependent on the reproducibility of the separation and analytic tools. To confirm the 2-D PAGE results, Western blotting was used to assess the expression of GST P1-1, maspin, prohibitin and HSP27 at translational level in TSGH8301 and BFTC905 cells. Consistent with the proteomics results, above four genes (GST P1-1, maspin, prohibitin and HSP27) were highly expressed in TSGH8301 cells as compared to the expression in BFT905 cells (Figure 3). Overall, our proteomic results were consistent with the observation from immunoblotting.

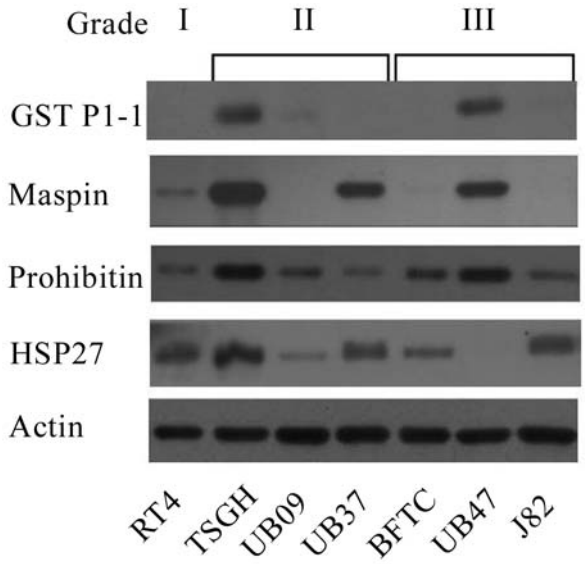

Figure 4. *Expression of glutathione S-transferase P1-1, maspin prohibitin and HSP27 in TCC cell lines. Protein expression in seven bladder cancer cell lines was evaluated by Western blot analysis. The grading of each cell line is indicated on the top of the blots. The results are representative of three experiments. TSGH: TSGH8301 cells; BFTC: BFTC905 cells.*

The expression patterns of de-regulated proteins in the TCC cell line panel. To further reveal the potential significance of de-regulated proteins identified by proteomic analysis, we assessed the expression patterns of maspin, GST P1-1, HSP27 and prohibitin by Western blotting in a panel of seven TCC cell lines representing various pathologic Grades. As shown in Figure 4, a bipolar expression pattern was found for maspin, which was abundantly expressed in TSGH8301, UB37 and UB47 cells, but undetectable in UB09, BFTC905 and J82 cells. Similarly, GST P1-1 was easily detected only in two TCC cell lines (TSGH8301 and UB47). On the other hand, prohibitin was universally expressed in all the TCC cell lines tested and its occurrence was highest in TSGH8301 and UB47 cells. Except for UB47 cells, HSP27 was observed in all TCC cell lines with a heterogeneous expression pattern. Taken together, these results show the differential expression patterns of the identified genes in various bladder cancer cell lines. Their potential roles in bladder cancer require further exploration.

Discussion

Using a gel-based proteomic approach, we identified eight differentially-expressed proteins between Grade II TSGH8301 and Grade III BFTC905 cells. Most of the down-regulated proteins observed in the present study were consistent with the prior reports (22-25). For instance, Celis

et al. found an up-regulation of GST P1-1 in low-grade, superficial bladder cancer, and a down-regulation of maspin in invasive TCCs (23). Prohibitin was shown to be up-regulated in two cases of primary invasive tumors (24). The overexpression of S100A4 in BFTC905 cells (representative of invasive/ metastatic TCC) is consistent with the findings that S100A4 is positively-associated with tumor metastasis or reduced patient survival (20). The higher expression of HSP27 in TSGH8301 cells (representative of low-grade, superficial tumor) also corroborates with an inverse relationship between HSP27 and the stage classification of primary tumors (25).

However, there is a disparity between our data and an earlier study showing a down-regulation of annexin V in invasive TCCs (23). We determined higher expression of annexin V in Grade III BFTC905 cells. This discrepancy may be explained, in part, by the paradigm of gene expression affected by *in vitro* culture (22). Alternatively, it might reflect bias derived from the cell line selection utilized for the experiments. As shown in Figure 4, the expression of de-regulated proteins was heterogeneous in the TCC cell lines of similar grades. These experimental results suggest that the protein expression might be different against a different genetic background. De-regulated proteins, that were found using cell-based comparison, should be further examined in the clinical tumor samples. As a whole, our results by Western blotting substantiate the stability and accuracy of the proteomics system. However, the inconsistency between gene expression levels and tumor cell grading warrants deeper exploration. Nonetheless, this study provides the potential biomarker candidates for in bladder tumorigenesis.

Regarding significance in the clinical setting, some of the de-regulated proteins identified by the present study might be of interest for further investigation. For example, the role of prohibitin is not known in bladder cancer. Prohibitin is an evolutionarily-conserved protein located in the inner membrane of mitochondria (26), and is believed to block DNA synthesis and to negatively regulate cell proliferation. It has also been shown to be up-regulated in two cases of primary invasive bladder tumors (24). Overexpression of prohibitin has been reported in endometrial adeno-carcinoma, hepatocellular carcinoma and breast cancer (27-29). Our results indicate that prohibitin was expressed at lower levels in one Grade I (RT4) and two Grade II cell lines (UB09 and UB37) (Figure 4). Comparatively, two (BFTC905 and UB47) of the three Grade III cell lines expressed higher levels of prohibitin, suggesting that prohibitin expression tends to increase in late-stage bladder cancer cells. These findings imply that prohibitin may play a positive role in the pathogenesis of epithelial cancer and more studies are needed to elucidate the role of prohibitin in bladder cancer.

Except for the differential biological properties of TSGH8301 and BFTC905 cells (*e.g.* histological grading and tumor stage), the BFTC905 and UB cell lines were all derived from bladder cancer patients with long-term exposure to high arsenic well water. In addition, prohibitin is ubiquitously expressed in these cell lines (Figure 4). Arsenic has been linked to bladder cancer development in the southern part of Taiwan (30). In addition, prohibitin is ubiquitously expressed in these cell lines (Figure 4). Whether prohibitin may also be involved in arsenic-related bladder carcinogenesis deserves further investigation (31, 32). The finding that HSP27 is down-regulated in BFTC905 cells differs from the results reported by Liu *et al.* (32). Currently, it is not known whether this is due to cell line selection or a common mechanism connected with arsenic exposure. Establishment of the proteome map from primary tumors of arsenic-related TCC is required to elucidate the molecular mechanism of arsenic-related carcinogenesis.

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