

Proteomic analysis of serum deprivation in tongue squamous cell carcinoma

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Abstract. The occurrence of tongue squamous cell carcinoma (TSCC) is closely correlated with serum components; however, the detailed mechanism remains to be fully elucidated. Proteomic analysis contributed to the discovery of potential biomarkers and provided an insight into TSCC at a molecular level. The present study investigated the effect of serum deprivation on the Tca-8113 TSCC cell line through protein profiling using two-dimensional gel electrophoresis and mass spectrometry, with the aim of improving TSCC diagnosis. The results showed that the Tca-8113 cells maintained proliferative capacity and resisted apoptosis following serum deprivation. A total of 43 proteins were upregulated and 45 were downregulated following serum deprivation for 24 h, compared with untreated controls (0 h). The upregulated caspase-7, heat shock protein 27 and Annexin A1, and the downregulated peroxiredoxin-6 and heat shock protein 70, were selected for verification using reverse transcription-polymerase chain reaction analysis following serum deprivation for 16 h. The results indicated that reactive oxygen species may be important in serum deprivation-induced oxidative stress.

Introduction

Tongue squamous cell carcinoma (TSCC) has a particularly high mortality rate among malignancies, predominantly due to the difficulty of early detection, and its incidence is increasing rapidly according to the American Cancer Society (1,2). Studies have shown that TSCC has a poorer prognosis and higher recurrence rate, compared with several other types of cancer due to metastasis being common (3). Clinical results suggest that a lack of available biomarkers

result in the majority of patients with TSCC not being diagnosed until an advanced stage. Therefore, understanding the molecular basis of tongue carcinogenesis may provide potential molecular markers for more rapid diagnosis of TSCC. Several biological molecules have been reported in the saliva and serum, and histological analysis has indicated that they are useful for screening and early detection of several types of cancer (4-6).

Serum is important for supporting cell viability and pH maintenance (7), whereas saliva is important in oral hygiene, food digestion and the repair of mucosal damage, as demonstrated by animal behavioural experiments and *in vivo* analysis of patients (8). Studies have suggested that saliva and serum are functionally similar and reflect the physiological state of the body (9-11), and that saliva is a non-invasive sample for potential clinical diagnosis. In the present study, serum deprivation experiments were performed on the Tca-8113 TSCC cell line to screen for potential biomarkers suitable for TSCC diagnosis. This was based on previous observations that serum deprivation synchronises proliferating cells at the G0 phase, induces apoptosis and stimulates the fibrotic response (12).

The results of the present study showed that the Tca-8113 cells resisted serum deprivation-induced apoptosis. Two-dimensional gel electrophoresis (2-DE) and mass spectrometry-based protein identification were used for the proteomic analysis of serum-deprived Tca-8113 cells. The results provided insight for potential diagnostic biomarkers or drug targets for the treatment of TSCC.

Materials and methods

Cell culture and MTT assay. The cells were routinely cultured, and MTT (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) assays were performed as previously described (13). Briefly, the TSCC Tca-811 cell line, provided by the Ninth People's Hospital of Shanghai (Shanghai, China) was maintained by routine culturing in RPMI-1640 medium with 10% fetal bovine serum (FBS) (both from Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) in a 37°C incubator with 5% CO₂. The cells were digested with 0.25% trypsin and seeded into 96-well plates (cat no. 3524; Corning Incorporated, Corning, NY, USA) at a density of 1x10⁴ cells/well and cultured for 18-24 h to 90% confluence.

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Table I. Reverse transcription-polymerase chain reaction primer sequences for the six genes.

Gene	Primer sequence	Length (bp)
Annexin A1	F: 5'-AAGACTTGGCTGATTCAGATGC-3' R: 5'-AACACTCTGCGAAGTTGTGGATA-3'	124
Peroxiredoxin 6	F: 5'-CGCATCCGTTTCCACGACTT-3' R: 5'-TGGCAAGCTCCCGATTCTAT-3'	271
Heat shock protein 27	F: 5'-AGTGGTTCGAGTGGTTAGGC-3' R: 5'-GGTTGACATCCAGGGACACG-3'	186
Heat shock protein 70	F: 5'-CTGCTGCGACAGTCCACTACC-3' R: 5'-TCGGCTCCGCTCTGAGATT-3'	177
Caspase-7	F: 5'-GAAGAGGCTCCTGGTTTGTG-3' R: 5'-CTGGCAACTCTGTTCATTACC-3'	106
GAPDH	F: 5'-GGGAAACTGTGGCGTGAT-3' R: 5'-GAGTGGGTGTCGCTGTTGA-3'	299

GAPDH, glyceraldehyde phosphate dehydrogenase; F, forward; R, reverse.

Serum deprivation was performed following washing twice with PBS. The cells were cultured for 12, 24, 36, 48 and 72 h following serum deprivation in the media at 37°C, with 10% serum as a control. Following cell culture, the culture medium were discarded, 200 μ l of 0.5 mg/ml MTT in PBS was added to wells, and the cells were cultured for 4 h at 37°C. Following removal of the supernatants, 200 μ l of DMSO was added and cells were shaken lightly for 15 min. The optical density value was measured using a microplate spectrophotometer (POWERWAVE 340; BioTek Instruments, Inc., Winooski, VT, USA) at 490 nm. All experiments were repeated four times and the mean \pm standard deviation of results were calculated.

Proteomic analysis. The proteomic experiments were performed as described previously (14). The steps were as follows: i) At least 1.0×10^8 Tca-8113 cells were harvested and lysed for sample preparation, and the bicinchoninic acid method was used for measurement of protein concentration; ii) each 80 μ g sample was subjected to 2-DE on an Ettan IPGphor II apparatus (GE Healthcare Life Sciences, Chalfont, UK) in triplicate. Silver-stained gels were scanned at 300 dpi resolution, and differential analysis was performed to assign spots. Student's t-test was used for filtering, and spots with a fold-change >1.2 were considered differentially expressed; iii) selected spots were subjected to MALDI-TOF mass spectrometry and tandem TOF/TOF mass spectrometry. The identified peptides were used for Basic Local Alignment Search Tool searches of the International Protein Index rat sequence database (<http://www.matrixscience.com>). The protein number, protein name, sequence coverage, score and Mr/pI were obtained and included in the analysis.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. The serum-deprivation treated Tca-8113 cells were prepared for one-step RT-qPCR SYBR Green assay as previous described (15). Briefly, RNA was extracted using the TRIzol LS reagent and the cDNA Synthesis kit (both from

Gibco; Thermo Fisher Scientific, Inc.) was used for cDNA synthesis. A total of 1 μ l reverse transcriptase product was used to amplify the six genes (Annexin A1, peroxiredoxin 6, heat shock protein 27, heat shock protein 70, caspase-7 and GAPDH). Primers (Table I) were designed based on the sequences of the **five differentially expressed proteins identified** in the proteomic analysis and GAPDH gene was used as reference. The primers were synthesized by Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China). The SYBR Green Real-Time PCR Master Mix (TOYOBO; Toyobo Life Science, Inc., Osaka, Japan) was used and the PCR cycling parameters were as follows: 94°C for 30 sec, followed by 35 cycles of 60°C for 30 sec and 72°C for 30 sec. A MyCycler thermal cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used for the PCR analysis. Each gene was quantified by $2^{-\Delta\Delta Cq}$ method (16) and the relative expression level was calculated by the ratio of each targeted gene to GAPDH gene.

Statistical analysis. Data are reported as the mean \pm standard deviation, and Student's t-tests were performed using the SPSS 16.0 software package (SPSS, Inc., Chicago, IL, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Serum deprivation inhibits cell proliferation. Serum deprivation inhibited the proliferation of cells, as exhibited in Fig. 1. When the Tca-8113 cells were subjected to serum deprivation for 12, 24, 36, 48 or 72 h, the proliferative activity was measured using an MTT assay. No significant differences were observed over time in the serum-deprived group ($P > 0.05$), however, proliferation continued to increase in the cells cultured in 10% FBS medium ($P < 0.05$).

Identification of differentially expressed proteins using proteomic analysis. As proliferation was inhibited in the Tca-8113 cells subjected to serum deprivation for 24 h, the

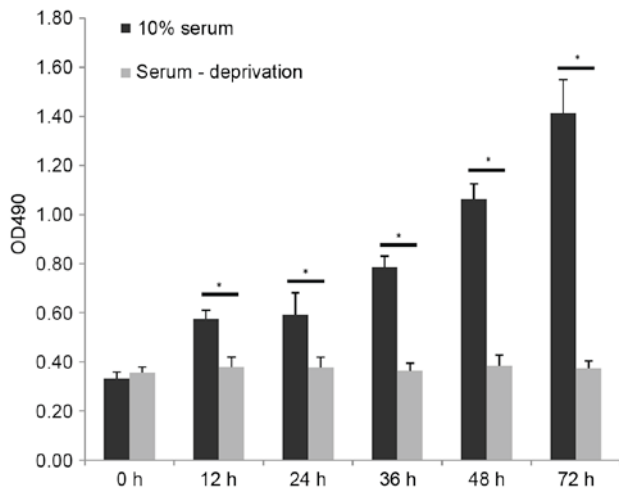


Figure 1. Proliferative activity of Tca-8113 cells subjected to serum deprivation. Experiments were repeated four times. * $P < 0.05$.

time-points of 0 h (control) and 24 h were selected for the comparison of triplicate samples using 2-DE and mass spectrometry. Representative 2-DE images are shown in Fig. 2. A total of 1,237 protein spots were detected on the 2-DE gel, with 43 significantly upregulated and 45 significantly downregulated at 24 h, compared with levels at 0 h ($P < 0.05$; average spot intensity difference > 1.2 -fold). Of these 88 potential differentially expressed proteins, 35 were identified using MALDI-TOF/MS, whereas the remaining 53 did not yield suitably complete polypeptide fragments or were too low in abundance for reliable identification. The results of the spot ID, average spot intensity, protein number, protein name, sequence coverage, score and Mr/pI are exhibited in Table II. The present study focussed on five differentially expressed proteins: Caspase-7 (spot 70), heat shock protein 27 (HSP 27; spot 390), Annexin A1 (spot 560), peroxiredoxin-6 (spot 405) and HSP 70 (spot 1194; Fig. 3). Notably, all are involved in mitochondrial oxidative stress, consistent with a previous conclusion that serum deprivation can induce apoptosis (7).

RT-PCR verification. The present study performed RT-PCR analysis to investigate the expression of caspase-7, HSP 27,

Annexin A1, peroxiredoxin-6 and HSP 70 in the Tca-8113 cells subjected to serum deprivation at two time-points (0 and 16 h). The stably expressed GAPDH mRNA was selected as an internal reference gene as the expression of cytoskeletal proteins was significantly disrupted. For example, the expression of β -actin was increased at the protein (Table II) and mRNA levels (data not shown), whereas the expression of tubulin was decreased (Table II). As shown in Fig. 4, the expression patterns of the five proteins were similar to the results obtained using 2-DE.

Discussion

Serum deprivation is widely used for cell cycle arrest and apoptosis, and various molecular mechanisms may be involved in the modulation of serum deprivation-induced apoptosis (17). However, in the present study, the effects of serum deprivation on cell proliferation and apoptosis were not observed in the Tca-8113 cells, even following a long duration (72 h) of treatment (Fig. 1). The proteomic analysis showed that a 24 h period of serum deprivation induced significant expression changes of numerous mitochondrial proteins, including prohibitin, peroxiredoxin, glyoxalase, caspase and ATP synthase (Table II). This is consistent with a previous study, in which mitochondrial function was readily disrupted by alterations of the cultivation environment through serum deprivation, which affects mitochondrial viability in several fibroblast lines (18). **However, the detailed molecular mechanism linking culture conditions and mitochondrial activity remains to be fully elucidated.** The results of the present study provided novel insight into the mechanism by which Tca-8113 cells subjected to serum deprivation avoid apoptosis.

Although the mechanism by which serum deprivation stimulates reactive oxygen species (ROS) production remains to be elucidated, studies have shown that ROS resulting from serum deprivation can induce programmed cell death (7,19). Oxidative stress has a wide range of effects on cellular proliferation, differentiation, migration and other physiological processes (20). Several antioxidant proteins are involved in alleviating oxidative stress by removing ROS. The present study found that peroxiredoxin-2 (Prx2) was upregulated, whereas the associated Prx6 was downregulated following

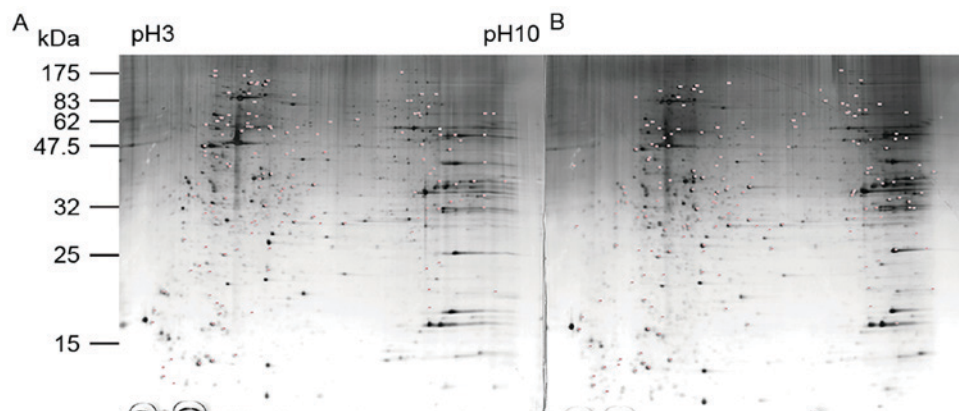


Figure 2. Typical silver-stained 2-DE polyacrylamide gel. Numbers refer to differentially expressed protein spots in Tca-8113 cells subjected to serum deprivation for (A) 0 h and (B) 24 h.

Table II. List of differentially expressed proteins during serum-deprivation treatment.

Spot ID	Percentage of total volume of all spots ^a			Protein number	Protein name	Sequence coverage (%)	Score	Mr/pI
	Control (0 h)	Serum-deprivation (24 h)	Protein number					
Upregulated								
59	0.000±0.000	0.086±0.005	gi12250700	KNP-I α protein	62	138	23,806/8.61	
489	0.000±0.000	0.002±0.001	gi14505773	Prohibitin	58	197	29,843/5.57	
497	0.000±0.000	0.153±0.005	gi128931	β -subunit (AA 1-312)	42	95	34,026/4.90	
566	0.001±0.001	0.110±0.015	gi1194376310	Actin, β , partial	34	174	38,950/5.19	
343	0.007±0.004	0.214±0.030	gi1194375974	Peroxiredoxin-2	57	193	20,209/8.90	
70	0.016±0.010	0.156±0.016	gi120150093	Chain B, crystal structure of caspase-7 complexed with xiap	63	143	12,244/5.73	
220	0.010±0.003	0.090±0.004	gi155959240	Nicotinamide nucleotide adenylyltransferase 1	66	132	18,413/8.99	
103	0.078±0.023	0.417±0.035	gi11362965	T cell receptor M β 11 β chain	48	109	15,697/6.56	
376	0.076±0.024	0.399±0.017	gi15822016	Chain C, erythropoietin complexed with extracellular domains of erythropoietin receptor	66	165	18,655/9.28	
549	0.065±0.019	0.250±0.024	gi121667979	Oxysterol binding protein-related protein 3 isoform 2d	40	123	35,786/5.11	
390	0.456±0.037	1.119±0.024	gi1662841	Heat shock protein 27	39	111	22,427/7.83	
560	0.439±0.035	1.011±0.071	gi14502101	Annexin A1	52	215	38,918/6.57	
392	0.856±0.067	1.734±0.087	gi11871210	T-complex protein 1, β subunit (TCP-1- β)	63	154	22,924/5.88	
Downregulated								
358	1.744±0.043	1.021±0.067	gi12329338	Glyoxalase-I	42	121	20,861/5.12	
775	0.852±0.030	0.456±0.067	gi1301030821	c-Myc promoter-binding protein 1	42	100	36,874/6.17	
1184	0.416±0.007	0.174±0.012	gi1194388088	Heat shock 70 kDa protein 1A/1B	34	205	64,170/5.39	
1162	0.120±0.004	0.050±0.011	gi1194384236	Heat shock 70 kDa protein 1A/1B	19	87	60,091/5.39	
1194	0.774±0.028	0.311±0.029	gi162897129	Heat shock 70 kDa protein 8 isoform 1 variant	17	78	71,083/5.28	
885	1.450±0.087	0.476±0.039	gi192911770	XTP3TPA-transactivated protein 1	50	146	23,896/6.42	
714	0.822±0.156	0.233±0.076	gi1296211572	Tubulin α -1B chain isoform 2	30	110	46,797/4.96	
204	0.327±0.035	0.057±0.018	gi156605994	CDGSH iron-sulfur domain-containing protein 2	82	177	15,497/9.66	
1077	0.055±0.005	0.005±0.003	gi132189394	ATP synthase subunit β , mitochondrial precursor	46	208	56,525/5.26	
198	0.161±0.022	0.000±0.000	gi157162210	CDC14 cell division cycle 14 homolog B (<i>S. cerevisiae</i>)	47	126	31,655/8.33	
206	0.107±0.012	0.000±0.000	gi151479152	ATP synthase subunit d, mitochondrial isoform b	48	89	15,820/6.60	
245	0.052±0.010	0.000±0.000	gi1119595056	Glutathione S-transferase pi, isoform CRA_c	24	41	19,577/4.84	
314	0.054±0.009	0.000±0.000	gi1306517833	MHC class I antigen	48	109	21,098/6.64	
405	0.356±0.006	0.000±0.000	gi14758638	Peroxiredoxin-6	41	133	25,133/6.00	
419	0.267±0.034	0.000±0.000	gi13329384	Translation initiation factor 4e	55	131	27,481/10.00	

Table II. Continued.

Spot ID	Percentage of total volume of all spots ^a			Protein name	Protein number	Sequence coverage (%)	Score	Mr/pI
	Control (0 h)	Serum-deprivation (24 h)	Mr/pI					
772	0.076±0.004	0.000±0.000	gil7705592	Mediator of RNA polymerase II transcription subunit 31	61	201	15,966/8.72	
845	0.131±0.003	0.000±0.000	gil89574029	Mitochondrial ATP synthase, H+ transporting F1 complex β subunit	39	134	48,083/4.95	
1000	0.235±0.047	0.000±0.000	gil48146259	CCT2	31	159	57,766/6.01	
1006	0.173±0.035	0.000±0.000	gil220702506	Chain A, TapasinERP57 HETERODIMER	34	174	54,541/5.61	
1080	0.052±0.003	0.000±0.000	gil169404695	Chain A, pyruvate kinase M2, phosphotyrosine binding protein	29	153	57,091/8.00	
1189	0.063±0.010	0.000±0.000	gil14718539	HIC-3	15	82	65,103/5.89	
1222	0.186±0.005	0.000±0.000	gil386758	GRP78 precursor, partial	32	169	72,185/5.03	
1230	0.035±0.001	0.000±0.000	gil15293595	Seven transmembrane helix receptor	43	85	24,812/8.87	

^aData are presented as the mean ± standard deviation. Mr/pI, molecular mass/isoelectric point.

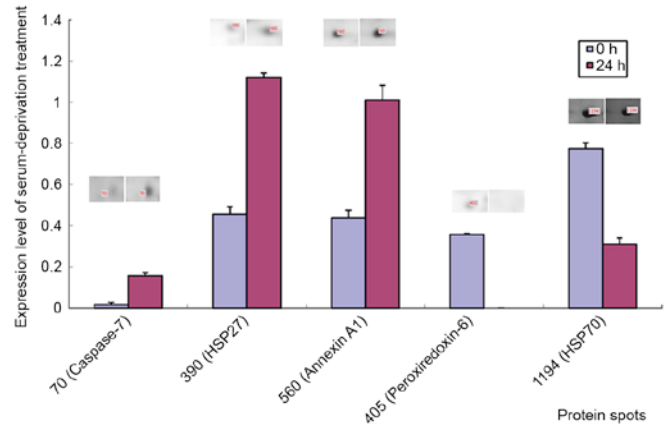


Figure 3. Mean relative abundance of five selected differentially expressed proteins (data extracted from Table II and Fig. 2).

serum deprivation. Prx enzymes have six isoforms, and they are located at sites of energy production and cellular motion, where ROS can be produced in abundance, particularly in mitochondria and at the plasma membrane. The peroxiredoxins are known for their function in removing endogenous peroxides. Therefore, the upregulation of peroxiredoxins is linked to high levels of oxidative stress (21). Prx2 is the major Prx enzyme in the cytoplasm, where it reduces peroxynitrite and hydroperoxides via a rapid, diffusion-controlled reaction (22). Prx2 is also important in the mechanism of H₂O₂-induced redox signalling (23). The overexpression of Prx2 has been associated with the progression of squamous cell carcinoma, and it may be an underlying biomarker for radiotherapy in colorectal cancer (24) lung cancer (25) and bladder cancer (26). Similarly, the expression of Prx6 has been associated with high rates of metastasis of breast cancer cell lines, and the overexpression of Prx6 occurs in malignant mesothelioma, breast cancer, oesophageal carcinoma and oligodendroglioma (27). A previous study revealed a marked upregulation of Prx2 following demethylation in gefitinib-resistant A549 cells (28). The present results suggested that Tca-8113 cells may resist the serum deprivation-induced apoptosis by upregulating Prx2, which may serve as a potent target for assisting in anti-angiogenic drug treatment in human TSCC.

Annexin A1 (ANXA1) is a well-characterised apoptosis-associated membrane protein, which is regulated by the epidermal growth factor receptor, insulin receptor, TRPM7 channel kinase 1, protein kinase C and protein kinase A. ANXA1 has also been linked with carcinogenesis and metastasis in various types of tumour (29). As an essential protein in tumourigenesis and apoptosis, the overexpression of ANXA1 is linked to carcinogenesis and high pathological differentiation grade (30,31). There is accumulating evidence suggesting that ANXA1 may be regulated by pro-inflammatory proteins, including lipopolysaccharide and interleukin-6, and it is reported to act as a brake to control the inflammatory response (32). Kang *et al* found that ANXA1 was positively upregulated during serum deprivation-induced autophagic degradation in HCT116 cells, indicating a potential role in autophagy by downregulating the inflammatory response (33). The overexpression of ANXA1 can also inhibit the aberrant dysregulation of cytoskeletal proteins and proliferation of

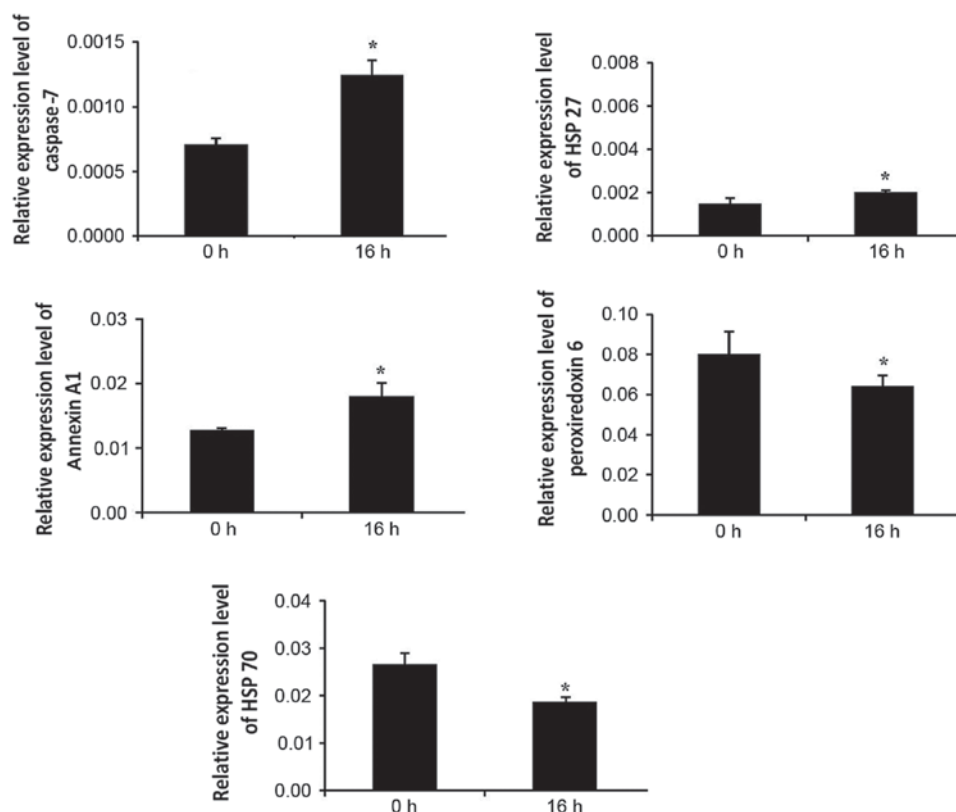


Figure 4. Verification of five selected differentially expressed proteins by reverse transcription-polymerase chain reaction analysis of mRNA levels following 16 h serum-deprivation. The relative expression levels of the mRNA were measured with GAPDH as reference. Experiments were repeated three times with similar results. * $P < 0.05$ vs. 0 h.

pulmonary microvascular endothelial cells (34), which is consistent with the observed morphology, static proliferation and upregulation of actin in the serum deprived Tca-8113 cells in the present study. The results of the present study showed that ANXA1 was upregulated at the mRNA and protein levels following serum deprivation, suggesting that autophagy, and not apoptosis, was a factor in Tca-8113 cells; autophagy protected the cells against serum deprivation by promoting cell survival.

Autophagy and apoptosis are the two major cell death pathways, and are tightly regulated at the genetic level to ensure that tissue and organ development proceeds correctly, in addition to other pathophysiological processes (35). A hallmark of autophagy is lysosomal degradation, which performs an adaptive function to protect organisms against infections, cancer and ageing (36). Apoptosis is a programmed process for killing and removing certain cells during morphological development, which also assists in maintaining tissue homeostasis in multicellular organisms. The aberrant regulation of apoptosis has been consistently linked with human proliferative diseases, including malignant tumours (37). Apoptosis involves a large number of molecules, including cysteine proteases in the form of caspases, which are synthesised as pro-forms and become activated by cleavage at aspartate residues. Initiator caspases, including caspase-1, 2, 4, 5, 8, 9, 10, 11 and 12, integrate molecular signals and activate downstream effector caspases, including caspase-3, 6, 7 and 14. As caspases can cleave and activate each other, triggering of the caspase cascade initiates a rapidly amplified

signal, which ensures accurate apoptotic cell death. Caspases are also able to cleave several other substrates, including nuclear lamins and cytoskeletal proteins, thereby inducing typical morphological characteristics associated with apoptosis. Caspase-7 is most closely linked with caspase-3, and these enzymes are activated by mitochondria-induced apoptosis.

Effector caspases are in charge of initiating DNA fragmentation, cell shrinkage and membrane blebbing (38). Brentnall *et al* (39) showed that caspase-9 and caspase-3 are involved in remodeling of mitochondria and ROS production. Caspase-7 has no significant effect on sensitivity to intrinsic cell death, but is involved in ROS production and cell detachment. In the present study, serum deprivation upregulated the level of caspase-7 in Tca-8113 cells, which underwent shrinkage and membrane blebbing, suggesting that serum deprivation stimulated TSCC differentiation and inhibited cancer growth.

HSPs are also closely linked with stress responses, including the response to serum deprivation. HSP70 is a major stress-inducible protein, which can fulfil multiple roles depending on its location. The upregulation of intracellular HSP70 is involved in the stress associated with recovery from clinical treatment, including radiotherapy or chemotherapy, whereas extracellular HSP70 stimulates the innate immune system to initiate antitumour immunity (40). In the present study, the serum deprivation-induced downregulation of HSP70 may assist TSCCs to escape and evade attacks from natural killer (NK) cells, however, the

downregulation of major histocompatibility complex-I can compensate for this and ensure activation of the NK cells. HSP27 is primarily involved in proteasome-mediated protein degradation and the regulation of apoptosis. A previous study revealed that HSP27 mediates apoptotic programming in pancreatic cancer cells (41), and clinical immunohistochemical evidence demonstrates that the overexpression of HSP27 causes high-grade differentiation in TSCCs (42). Therefore, the serum deprivation-induced upregulation of HSP27 may be correlated with the morphology and differentiation of TSCCs, consistent with the expression of caspase-7.

In conclusion, the present investigative preclinical study found that Tca-8113 TSCC cells resisted apoptosis induced by serum deprivation. The changes in proteins were primarily involved in the oxidative stress process in the mitochondrion, which indicated that ROS may be important in serum deprivation-induced oxidative stress. In the future, further detailed investigations on the signalling pathway and clinical verification are required.

Acknowledgements

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