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Differential proteomic analysis of HeLa cells treated with Honokiol using a quantitative proteomic strategy

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Summary. Honokiol (HNK) is an active component purified from Magnolia officinalis. HNK exhibits antitumor effects by inducing apoptosis and inhibiting the growth of many cancer cell lines, while proteins involved in antitumor effects in proteomic level are still unclear. In our study, HNK could inhibit HeLa cell proliferation and induce apoptosis in a concentration- and time-dependent manner. We utilized a quantitative proteomic technique termed SILAC (Stable isotope labeling with amino acids in cell culture)-MS (mass spectrometry) to study the differential proteomic profiling of HeLa cells treated by HNK. A total of 85 proteins were changed after HeLa cells were treated with 12 μg/ml HNK for 8 h, and 8 proteins showed up-regulation while 77 proteins down-regulated. The changed proteins were classified into 9 different categories, which covered a broad variety of cellular functions. In conclusion, HNK performs cytotoxicity to HeLa cells through co-operating of many proteins and different pathways.

Keywords: Honokiol – Stable isotope labeling with amino acids in cell culture (SILAC) – Differential proteomic profile – HeLa cells

Abbreviations: ACN, acetonitrile; DMSO, dimethyl sulfoxide; 2-D PAGE, 2-dimensional polyacrylamide gel electrophoresis; FCM, Flow cytometry; HNK, honokiol; LC-MS/MS, liquid chromatography followed by tandem mass spectrometry; Leu-d0, normal leucine; Leu-d3, deuterated leucine; MALDI-TOF, matrix assisted laser desorption ion-time of flight; MS, mass spectrometry; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered-saline; PBST, phosphate-buffered-saline with 1% Tween; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; 14-3-3 sigma, 14-3-3σ; SILAC, Stable isotope labeling with amino acids in cell culture; TFA, trifluoroacetic acid

1. Introduction

Honokiol (HNK) is an active natural product extracted from the bark of Houpu (Magnolia officinalis), which is widely used in Chinese traditional drugs (Squires et al., 1999). HNK demonstrated antitumor effects by inducing apoptosis and inhibiting the growth of many cancer cell lines (Hibasami et al., 1998; Yang et al., 2002; Chen et al., 2004; Kenji et al., 2005), and HNK is a promising chemotherapy candidate in treating several cancers in clinic in future (Nacht et al., 1999; Squires et al., 1999; Yang et al., 2002; Chen et al., 2004). The conventional approach to study HNK action mechanism was focused on expression changes and a signaling pathway of single or several genes. However, the mechanism of apoptosis and growth inhibition by HNK and the target proteins in proteomic levels are still unclear by now.

Stable isotope labeling with amino acids in cell culture (SILAC) (Ong et al., 2002) has shown great advantages as a valuable quantitative proteomic technique and has been applied in many fields (Blagoev et al., 2003; Foster et al., 2003; Ong et al., 2003; Albrecht et al., 2005). Protein expression level in cancer has been mostly analyzed by using two-dimensional (2D) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) coupling with matrix assisted laser desorption ion-time of flight (MALDI-TOF) mass spectrometry (MS). But 2D-PAGE provides a relatively small amount of data on protein identification and quantification compared with LC/MS coupled with SILAC, because it has low sensitivity in identifying proteins with their PH values too low or too high or their molecular masses too small or too large (Edwin et al., 2005). With SILAC method to study the B cell differentiation process at the proteome level, the amount of proteins identified and quantified was 10 times

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more than that of 2D-PAGE (Edwin et al., 2005). Similarly, Patrick et al. (2004) compared the expression levels for more than 440 proteins in the microsomal fractions of prostate cancer cells with varying metastatic potential, and 60 proteins were elevated in highly metastatic cells, whereas 22 proteins were reduced.

Using SILAC, we can incorporate one non-radioactive, deuterated leucine (Leu-d3) which replaced normal leucine (Leu-d0) into cells. When cells are cultured in medium with Leu-d0 or Leu-d3, the proteins in these two groups of cells have Leu-containing peptides incorporated either all Leu-d0 or all Leu-d3, respectively. Along with SDS-PAGE and LC-MS/MS, many of identified proteins can be quantified the expression levels based on the ratio of a pair of isotope peaks (Ong et al., 2002).

Here we applied SILAC-MS technique to study the changed proteomic profiling of HeLa cells treated by HNK. Among 85 changed proteins after HeLa cells treated with 12 μg/ml HNK for 8 h. The changed proteins were classified into 9 different categories, which covered a broad variety of cellular functions. Studying the differential proteins changed by HNK may be a starting point for further revealing general action mechanism of HNK.

2. Materials and methods

2.1 Stable isotope labeling with Leu-d3 amino acid in HeLa cell culture

Normal HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM). The labeling HeLa cells were cultured in Leu-d3-containing DMEM with 10% dialyzed fetal bovine serum (GIBCO), which was supplemented with Leu-d3 (5,5,5-D3,98%)(Cambridge Isotope Laboratories, Inc, U.K.) replaced normal L-leucine(Leu-d0). The labeling HeLa cells grew at least for seven passages in media containing Leu-d3 to completely labeling before treatment with HNK.

HNK (Si Ke Hua company, Chengdu, China, 99.9%) was dissolved in dimethyl sulfoxide (DMSO) at the stock concentration of 20 mg/ml,which was further diluted in culture medium at the final DMSO concentration <0.1%.

2.2 Measurement of HeLa cell proliferation

Cells were seeded into 96-well plates at a density of 2×10^3/well in triplicate. On the next day, the medium was replaced with fresh medium at various concentrations of HNK in a final volume of 200 μl, after incubation at 37 °C for 6, 12 and 24 h, respectively, 20 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5 mg/mL in PBS) (Sigma) was added to each well for an additional incubation for 4 h, then the medium was removed and 100 μl DMSO was added in to dissolve MTT formazan precipitate. After shaking for 10 min, the absorbance at 570 nm was detected on a spectra max MS (MDC, Sunnyvale, CA, U.S.A.).

2.3 Flow cytometry analysis

Flow cytometry (FCM) was used to detect the apoptotic rate of HeLa cells treated by HNK. Cells were seeded into 6-well plates at a density of 2×10^6/well in triplicate and incubated overnight. Cells were treated with HNK at various concentrations and time points. After the treatment, cells were harvested, washed, suspended in cold phosphate-buffered-saline (PBS), and fixed in 75% ethanol at 4 °C overnight. Then cell samples were stained with 5 μg/ml propidium iodide and analyzed on a FACScan flow cytometry system (Becton Dickinson, San Jose, CA, U.S.A.). Data were analyzed using winMDIv2.8 software. Results were tested for statistical significance by Dunnett-*t* test. Significance was defined as $p < 0.05$.

2.4 Protein preparation, separation and in-gel digestion

Two kinds of cell samples (untreated and treated by HNK) were collected, washed three times with ice-cold PBS, and total protein was extracted using RIPA lysis buffer. Protein concentration was determined using the Protein Assay Kit (Bio-Rad, Hercules, CA, U.S.A.). After the concentration was determined, same amount of proteins from two groups of cells were mixed at 1:1 ratio, then separated on a 12% SDS-PAGE and Coomassie-stained to visualize the gel bands. The gels were excised and subjected to in-gel tryptic digestion using mass spectrometry grade trypsin (Promega, Madison, WI, U.S.A.) mainly as described previously (Patrick et al., 2004).

2.5 Protein identification

The incorporation of Leu-d3 into cellular proteins was monitored by MALDI-TOF MS analysis on a Voyager DE-STR mass spectrometer (Waters Corporation, Holland, USA). For protein identification, peptides were re-dissolved with 50% ACN/0.1% TFA and analyzed with reverse liquid chromatography followed by tandem mass spectrometry (LC-MS/MS), then searched either by using Protein Lynx Global Server 2.0 (PLGS2.0) (Micromass, Waters Corporation, Holland, U.S.A.) or Mascot search algorithm (Matrix Science, London, U.K.) against human database from NCBI or Swiss-Prot. Proteins were successfully identified by probability-based Mascot scores exceeding their threshold.

2.6 Protein quantification

Identified proteins were quantified by tracking pairs of labeled and unlabeled peptides from the MS spectra, and it requires at least a leucine-containing peptide to quantify. Protein abundance was calculated as ratios of the peak intensity of the fragment ions from the labeled versus the unlabeled peptides. Ratios were calculated from the average of all quantified peptides of a single protein. A changed protein was defined as a real regulated one only when the average ratio of that protein was significantly higher or lower than the average standard variation of all quantified proteins.

2.7 Semiquantitative RT-PCR

Total RNA was extracted from two kinds of cell samples using Trizol regent (Invitrogen). The amount of RNA was measured by spectrophotometer. The cDNAs were synthesized from 1 μg total RNA by a ThermoScript™ RT-PCR system (Invitrogen) and then used as templates for the subsequent PCR assay with 0.4 pM of both forward and reverse primers. The PCR reaction was the first cycle of 94 °C for 2 min, 30 cycles were followed by denaturation at 94 °C for 30 sec, annealing at 55 °C for 1 min and extension at 72 °C for 1 min, then the final extension at 72 °C for 10 min and holding at 4 °C. The house-keeping gene β-actin was taken as the control.

2.8 Western blotting

The whole cell extraction was separated on 12% SDS-PAGE after quantification and transferred to nitrocellulose membrane. Membranes were blocked with 5% nonfat dry milk in PBS/0.1% Tween 20 (PBST) overnight at 4 °C, and incubated for 1 h at room temperature with antibodies indicated (14-3-3σ, 1:100; annexin A1, 1:1600) (abcam), then the mem-

branes were washed for 5 min in PBST for 3 times, followed by incubation with goat anti-mouse/rabbit at 1/5000 dilution (abcam) in PBST for 1 h. After washing 3 times with PBST and PBS for 5 min, the bands were developed using the enhanced chemiluminescence (ECL) detection system (Pierce Biotech Inc., Rockford, IL) according to the manufacturer's instruction.

3. Results

3.1 Honokiol inhibits HeLa cell proliferation

Honokiol inhibits HeLa cell growth in a concentration- and time-dependent manner through MTT analysis. With the treatment by HNK for 12 h, the concentration leading to a 50% decrease in cell number (IC50) was about 12 μg/ml. During the cell proliferation there was no difference between the DMSO experiment group and control group (data not shown).

Based on the MTT result, HeLa cells were exposed to increase doses of HNK (6–18 μg/ml) for 12 h, and all the cells were harvested to measure apoptosis level by FCM. Even low dose of 6 μg/ml HNK could cause 3.9% apoptosis on HeLa cells ($p < 0.05$), and high dose of HNK could cause 8.13%, 15.70%, 21.44% and 28.0% apoptosis. These data indicated that HNK had cytotoxicity on HeLa cells in a concentration-dependent manner. According to the above results, 12 μg/ml HNK was used to treat HeLa cells for 8 h in the following experiments.

3.2 Leu-d3 labeling detection

Figure 1 clearly showed that Leu-d3 was gradually incorporated into proteins from cell passage 1–7, and after about seven passages, the incorporation of Leu-d3 was

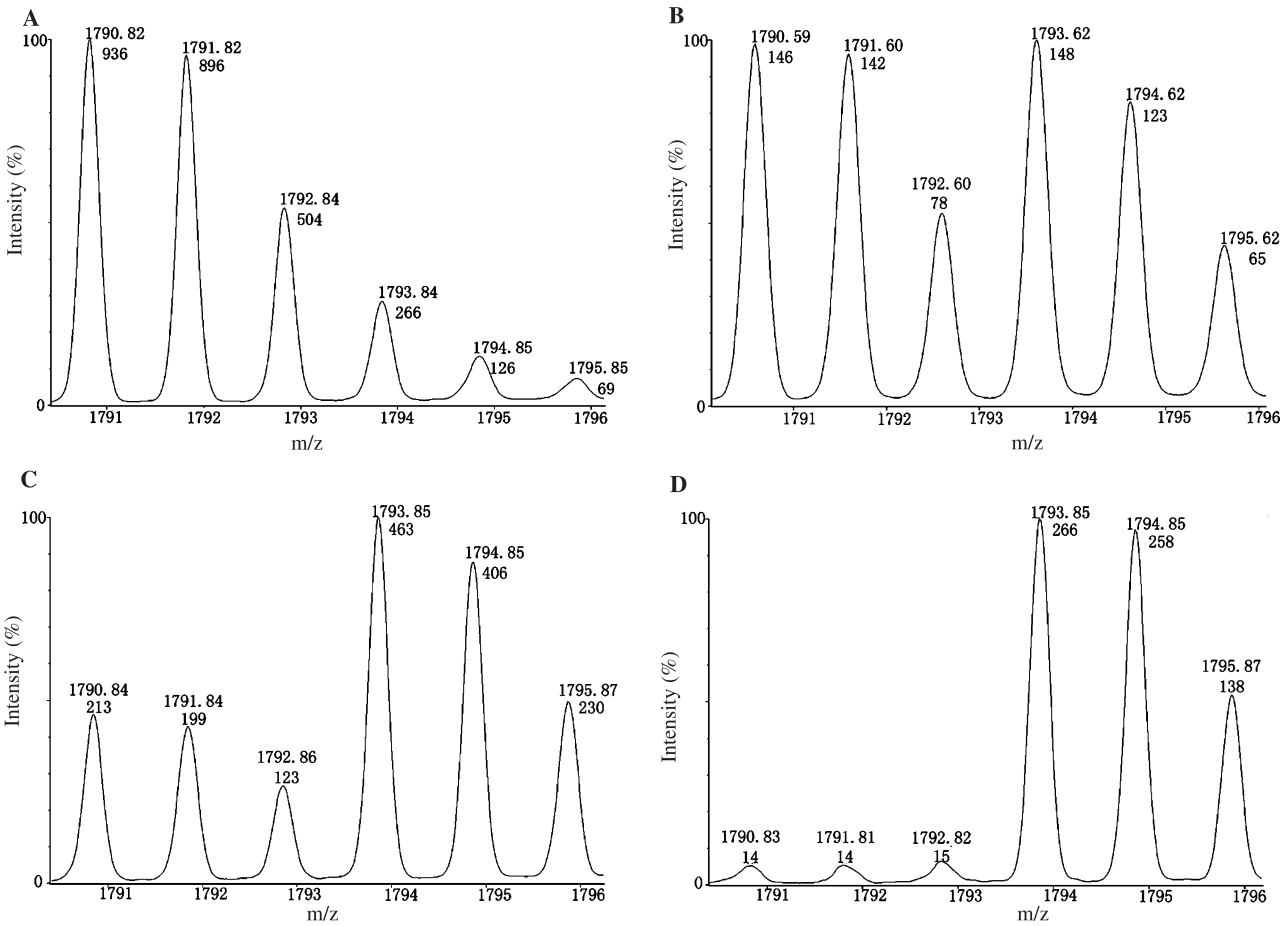

Fig. 1. Incorporation of Leu-d3 in β-actin proteins at various time points. Leu-d3 containing medium was introduced to HeLa cells already adapted to Leu-d0 at the initial time point. Samples were harvested at 1, 3, 5, and 7 cell passages, the cellular lysates were resolved by SDS-PAGE. The band of β-actin was excised and digested, and its same peptide SYE*L*PDGQVITIGNER was analyzed at different time points by MALDI-TOF MS, the peptide pair differs in mass by 3 Da because of the incorporation of one Leu-d3. As shown (**A**–**D**), Leu-d0 is replaced in the protein with Leu-d3, the abundance of the Leu-d0-containing peptide decreases while the Leu-d3-labeling peptide increases, and the incorporation rate was steadily increased. After seven passages, the incorporation of Leu-d3 was accessed to above 95%

accessed to above 95%. All cellular lysate extracted from HeLa cells for SILAC-MS analysis was harvested after seven passages.

3.3 Protein identification and quantification

The strategy of our research is summarized in Fig. 2. Using this method, 348 proteins were successfully identified by LC-ESI/MS, and the MS/MS tolerance is 0.3, and 306 proteins were quantified by Leu-containing peptides, and 85 proteins were changed significantly after HeLa cells treated by HNK based on our stringent standard. The average standard deviation (SD) of the isotopic intensity ratios was 0.13 for all 306 quantified proteins, while the real changed protein was defined as the one whose significant threshold p value was lower than 0.01 ($p<0.01$), and the SD was 2.5 times higher than the average SD. In other words, only the quantified proteins with $p<0.01$, whose upregulation rate was over 1.3 and the downregulation rate was below 0.7, were defined as the significantly changed proteins.

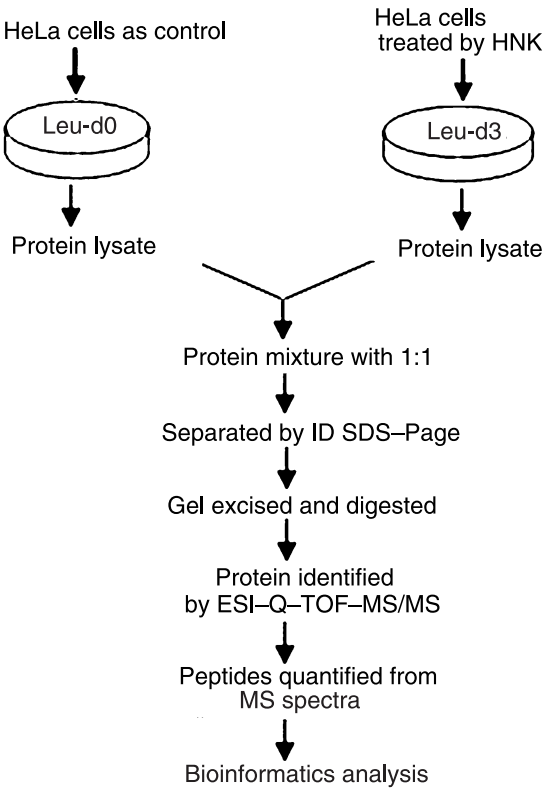

Fig. 2. Schematic strategy of SILAC-MS method to study differential proteome from HNK-treated HeLa cells. HeLa cells untreated and treated by HNK were cultured in DMEM containing Leu-d0 and Leu-d3, respectively. After protein lysate was isolated, equal amounts of each protein sample were mixed, resolved by SDS-PAGE. The entire gel lane was divided into nearly 20 sections, then each part was digested and subjected to MS for identification and quantification

Because the mass of Leu-d3 is 3 Da heavier than that of Leu-d0, the mass difference of fragment ions between unlabeled and labeled peptides is shifted by +3 Da (+1 Leu-d3), +6 Da (+2 Leu-d3) or +9 Da (+3 Leu-d3), and so on.

Table 1 shows the SILAC ratios for partial quantified proteins, and the representative quantified isotope peptides are illustrated in Fig. 3. All the ratios of changed proteins after HNK-treated could be seen in supplemental Table 1. As Fig. 4A showed, after 12 μg/ml HNK treated for 8 h, about 2% (8/348) proteins were up-regulated and 22% (77/348) proteins were down-regulated, while 76% proteins remained invariable or could not be quantified. As for 85 changed proteins, the functional and location categories of these changed proteins are illustrated in Fig. 4B and C. And function annotation used in the clustering analysis was referred to genome and protein databases, they were classified into 9 different categories, which covered a broad variety of cellular functions. Among these different functions, the largest functional group (31%) was the cluster of post-translational modification, protein turnover, chaperones. According to the subcellular location of ExPASy, seven categories were made for the changed proteins, and most of them located in cytoplasm and nucleus, which was high about 63%.

Table 1. Representative examples of quantified proteins by SILAC-MS

Protein expression	NCBI Acc. No.	Protein name	Leu-containing peptides	SILAC ratio
Up-regulation	gi|16924265	peroxisomal	1	2.34
Unvaried	gi|83753120	chain B, vinculin	4	1.01
Down-regulation	gi|56757569	tubulin beta-2 chain	7	0.63
	gi|187302	14-3-3σ	2	0.59
	gi|55959290	annexin A1	3	0.61

Ratios were calculated from the average of all quantified peptides for a single protein

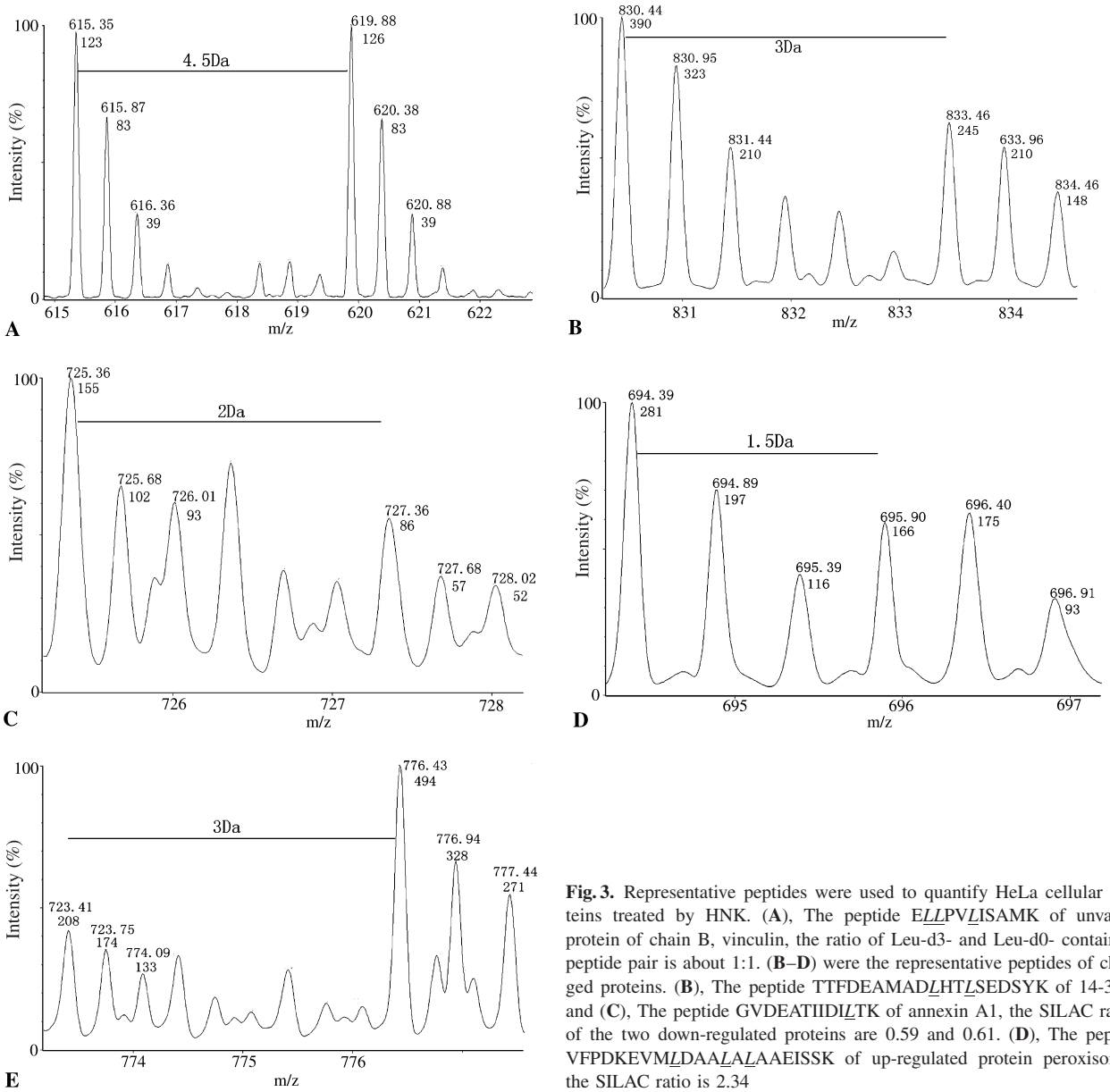

Fig. 3. Representative peptides were used to quantify HeLa cellular proteins treated by HNK. (**A**), The peptide E*LL*PV*L*ISAMK of unvaried protein of chain B, vinculin, the ratio of Leu-d3- and Leu-d0- containing peptide pair is about 1:1. (**B**–**D**) were the representative peptides of changed proteins. (**B**), The peptide TTFDEAMAD*L*HT*L*SEDSYK of 14-3-3σ and (**C**), The peptide GVDEATIIDI*L*TK of annexin A1, the SILAC ratios of the two down-regulated proteins are 0.59 and 0.61. (**D**), The peptide VFPDKEVM*L*DAA*L*A*L*AAEISSK of up-regulated protein peroxisomal, the SILAC ratio is 2.34

3.4 Confirmation of quantitative proteins

To confirm the quantitative proteins, two down-regulated proteins, 14-3-3 sigma and annexin A1, were chosen for further validation by semi-quantitative RT-PCR and Western blotting analysis. As shown in Fig. 5A, after cells treated by same concentration of HNK (12 μg/ml) in different time points, the RNA expression of 14-3-3σ and annexin A1 was inhibited by HNK in a time-dependent manner. The mRNA expression of the two proteins were consistent with the above SILAC results.

The two proteins were further examined by Western blotting to verify the protein expression level. The changes of protein level were also the same in a time-dependent manner as the above semi-quantitative RT-PCR results (Fig. 5B). The confirmation results from RT-PCR and Western blot assays demonstrated our quantification for changed proteins by SILAC-MS strategy was correct.

4. Discussion

The number of peptide pairs used for quantification ranged from 1 to ∼20 per protein. Only nearly 12% identified proteins could not be quantified because the detected peptides for identification lacked leucine amino acids, which indicated the labeling efficiency of leucine was very high. For primarily confirming our

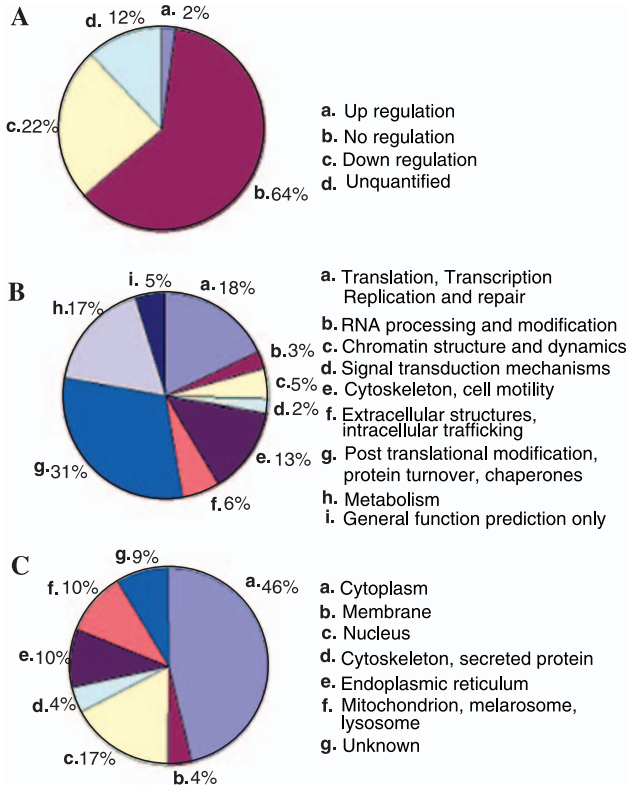

Fig. 4. The functional and cellular location profile of identified proteins. (**A**) Classifications of protein identified. This chart was generated with all identified proteins, about 12% of which were not quantified. **B** and **C** were generated only with regulated proteins. (**B**) Functional classification. The cluster of post-translational modification, protein turnover, chaperones is the biggest one. (**C**) Cellular location classification. Most of the regulated proteins are located in cytoplasm and nucleus (For an interpretation of the reference to colour in this figure, the reader is referred to the online version of this paper under www.springerlink.com)

SILAC results, both the levels of mRNA and protein are coincident with the SILAC ratios, which indicated that SILAC (using Leu-d3) technique is an excellent method in quantitative proteomics. Furthermore, the advantages of high-throughput, fast-speed and easy-manipulation of SILAC method were obvious compared with that of 2D-PAGE and other conventional Western blot analysis.

Of these regulated proteins, 14-3-3σ protein has pivotal roles with oncogenesis. Processes relevant to cancer biology and regulated by 14-3-3σ protein interactions include cell-cycle progression, apoptosis, mitogenic signaling and MAP kinase activity (Anne et al., 2005). 14-3-3σ was regulated by p53 and down-regulation in breast cancer cells (Nacht et al., 1999), prostate cancer cells (Lodygin et al., 2004), basal cell carcinoma (Lodygin et al., 2003), human hepatocellular carcinoma (Iwata et al., 2000), while 14-3-3σ was up-regulated in cervical cancer (Sano et al., 2004), lung cancer (Qi et al., 2005) and pancreatic cancer (Ahmed et al., 2004). In different cancers, the expressions of 14-3-3σ are different, which may be due to tissue specificity. In our study, 14-3-3σ was down-regulated in HeLa cells after HNK treated, which was consistent with that 14-3-3σ is up-regulated in cervical cancer (Sano et al., 2004). In order to further investigate 14-3-3σ functions on honokiol biological effects, we will study whether the methylation of 14-3-3σ is in HeLa cells and what's the relation between 14-3-3σ down-regulation treated by honokiol and its interacting proteins.

Annexin A1, a down-regulated protein in our experiments, is over-expression in hairy cell leukaemia (Falini et al., 2004) and cervical carcinogenesis (Moon et al., 2006), involved in signal transduction, tissue growth and differentiation. On cell proliferation, annexin A1 could be linked to its MAPK inhibitory effect (Debret et al., 2003), and on apoptosis, it could activate the caspase-3 protease (Solito et al., 2001), through tumor necrosis factor-related apoptosis inducing ligand (TRAIL)-induced apoptosis (Petrella et al., 2005). Our data indicated HNK probably performs biological effects through annexin A1 – invovled several pathways to regulate HeLa cell growth and apoptosis.

Another protein, ubiquitin, is also an important one related to cancer. Some cancer-related proteins may lose their

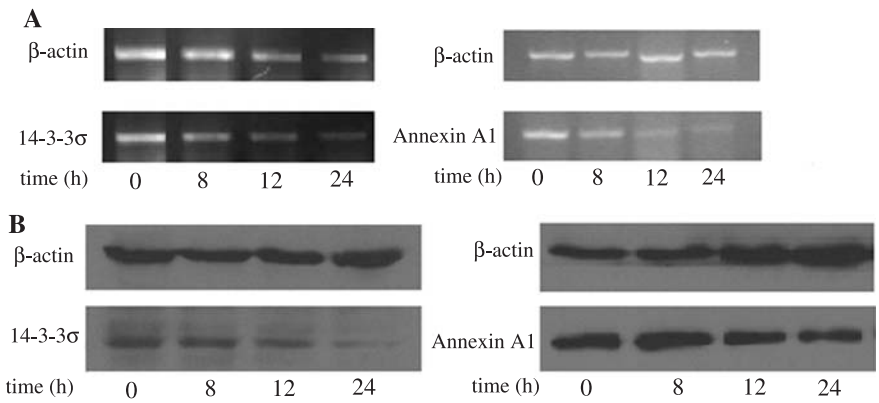

Fig. 5. The expression confirmation of 14-3-3σ and annexin A1 by semi-quantitative RT-PCR and Western blotting analysis. HeLa cells were exposed to 12 μg/ml HNK at different time points. β-actin was used as equal loading control. As shown, HNK treatment inhibited the mRNA (**A**) and protein expression (**B**) of 14-3-3σ and annexin A1 in a time-dependent manner

functions by protein degradation, and a major pathway for protein degradation is the ubiquitin-proteasome pathway (Ishii et al., 2007). Targeting this pathway with proteasome inhibitors has been proved as a rational strategy against hematologic malignancies (Dees and Orlowski, 2006). Ubiquitin was down-regulated after treated by HNK, so HeLa cell apoptosis induced by HNK probably involved in the ubiquitin-proteasome pathway and its regulation.

Except the above proteins, many other regulated proteins have important roles in carcinogenic process, such as ezrin, protein disulfide-isomerase precursor (p55), and so on. Ezrin and p55 have been found be relative to cancer metastasis (Khanna et al., 2004; Kitakata et al., 2004). They may have different effects in the antitumor mechanism of HNK.

Previous studies had discussed several kinds of possible antitumor mechanisms for HNK. For example, HNK can induce apoptosis in human squamous lung cancer CH27 cells through the modulation of Bcl-X_L and Bad proteins, release of mitochondrial cytochrome c and activation of caspase-3 (Yang et al., 2002). Hsp27 and Hsp70 were found significantly down-regulated expression after HNK treated, which were consistent with other studies (Kenji et al., 2005). Although some reported proteins including Bcl-X_L and Bad proteins were not detected by our method, it was probably due to different cell lines, different treatment dose and time by HNK, and so on.

In summary, HNK exhibits cytotoxicity to HeLa cells and induces apoptosis through co-operation of many proteins and multi-pathways including regulating cell growth, proliferation, protein translation and degradation, multidrug resistance, etc. Among these changed proteins, several candidate HNK-targeted proteins including 14-3-3σ, annexin A1, etc. play important role in carcinogenesis and tumor development. It needs to further validate these candidate target proteins of HNK action in vivo.

Acknowledgments

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