

Utilization of LCM Technique in Sample Preparation for Proteomics Study of Lung Cancer Tissue*

NAN Yan-dong¹, YANG Shuan-ying², TIAN Ying-xuan², LI Wang-ping¹,
SUN Rui-lin¹, FU En-qing¹, LANG Hong-jue¹, JIN Fa-guang¹

(1. Department of Respiratory Disease, Tangdu Hospital, Fourth Military Medical University, Xi'an 710038;

2. Department of Respiratory Medicine, Second Affiliated Hospital of Medical School, Xi'an Jiaotong University, Xi'an 710004)

Abstract: Objective Protein-based study plays a very important role in exploring tumor pathogenesis in the post-genomic era. However, tissue heterogeneity presents a major problem in the proteomics study of lung cancer. The aim of this study was to explore the methods of sample preparation for protein-based study of lung cancer tissue using laser capture microdissection (LCM) technique. **Methods** Six patients with lung squamous cell carcinoma (SQCC), six patients with lung adenocarcinoma (AD) and six normal lung tissues including 2 cases matched with SQCC and 4 cases matched with AD (from at least 5 cm away the edge of the tumors were included in this study. Tissue sample firstly were cut with a cryostat sections and stained with the improved hematoxylin and eosin (H&E). LCM using the Arcturus PixCell II system was performed to enrich specific cell populations. The protein was quantitated by Bradford assay after protein extraction from LCM cells. One-way analysis of variance (ANOVA) was used to compare the differences of cell number and protein quantity among three groups. **Results** We found that fresh frozen material was more desirable in protein-based studies. The improved H&E was a feasible staining methods for recognizing cells of interest under light microscope. 56, 60, 63 LCM caps in lung SQCC, lung AD and normal lung were respectively collected. Cell number of normal lung tissue was significantly less than that of lung SQCC and lung AD under visual field of microscope ($P < 0.05$). Total protein of lung SQCC, lung AD and normal lung group were respectively 248.20 μg , 261.64 μg and 183.88 μg . **Conclusion** The results indicated LCM was a powerful tool in facilitating the molecular biology analysis of lung cancer tissue. It could effectively overcome the heterogeneous problem of lung cancer tissue and provide a more meaningful insight into tumor pathogenesis.

Key words: laser capture microdissection; proteomics; lung cancer; pathogenesis; heterogeneity

INTRODUCTION

Lung cancer is still the most common malignant cancer up to now, representing the first major cause of cancer related deaths for both men and women worldwide^[1]. Lung cancer continues to carry a poor prognosis, with a low 5-year survival rate ranging from 6% to 15%^[2-3]. Moreover, lung cancer carcinogenesis is a multiple step process and the molecular mechanisms of this process remain unclear. Recently, proteomic technology has been successfully applied to identify tumor-associated proteins in various cancers originating from different

* **Acknowledgements:** This work was supported by grants from the National Natural Science Foundation of China (No. 30570795) and Program for New Century Excellent Talents in University (No. NCET06-0845).

NAN Yan-dong (1975-), male, Ph.D., lecturer of Department of Respiratory Disease, Tangdu Hospital, Fourth Military Medical University; research fields: pathogenesis and early diagnosis of lung cancer.

organs, for example liver^[4], esophagus^[5], breast^[6], ect. However, reports about lung cancer tissue proteomics in public database are relatively rare so far mainly due to its marked heterogeneity and complex pathology. In addition, as an opening organ, lung is vulnerable to be infected by microorganisms and to be invaded by physical and chemical factors. It is not ideally suitable for lung cancer tissue to be directly identified candidate tumor markers. The complexity of lung cancer tissue means that studies of whole tissue sample provide insufficient information about its pathogenesis. Therefore much attention has focused on strategies designed to enrich for selected cell from tissue samples to facilitate marker or target discovery^[7]. Cultured cells can very efficiently amplify a desired cell population, but there has been considerable debate about its representative. For cancer proteomics studies using the cultured cells as a model system, the biggest challenge is to unravel the molecular complexity of the tissue microenvironment, and thus the proteomic analysis of cells cultured in vitro may not truly reflect the expression pattern of cells in vivo^[8]. Laser capture microdissection (LCM), a revolutionary technology in tumor study that was developed during the mid 1990s by Dr Emmert-Buck, etc, at the National Institutes of Health (NIH), USA^[9] can exactly separate single cell or the same kind of cell populations from complex tissues and effectively solve the problem of tissue heterogeneity in experiments^[10].

In this preliminary study, we tested in detail the staining methods of the improved hematoxylin and eosin (H &E) of frozen sections. We also systematically described the LCM process for sample preparation. In addition, we further compared the cell number and protein quantity among lung squamous cell carcinoma (SQCC), adenocarcinoma (AD) and normal lung and evaluate the advantage and limitation of LCM as a tool in global protein expression profiling.

MATERIALS AND METHODS

1. Clinical and Pathological Materials

Informed consents were received from all subjects before study in this experiment. All patients did not undergo chemotherapy or radiotherapy before operation. Complete clinical and pathological information for each case was available. Twelve patients with primary lung cancer enrolled in this study were divided into SQCC and AD groups according to their histological type. SQCC group included five male and one female patient, whose average age was 60.7 ± 8.8 years (47-70 years). The P-TNM stages (UICC, 2007) were that 1 case was stage Ia, 2 stage Ib, 1 stage IIa, 2 stage IIB. Distributions of tumor differentiation were that 1 cases were well, 3 moderate, and 2 poor. In AD group, there were 3 male and 3 female patients with an average age of 58.2 ± 8.0 years (45-69 years). Stage distribution was as follows: 1 stage Ia, 2 stage Ib, 2 stage IIa and 1 stage IIB. Their differentiations were diagnosed as 2 poor, 3 moderate and 1 well. The six normal lung tissues from at least 5 cm away the edge of the tumors as control group were paired with the 2 cases of SQCC and 4 cases of AD. The averaged 60.8 ± 8.3 years (45-69 years), and 3 were males and 3 females.

2. Tissue Preparation and Staining

The tissue samples were obtained at the time of surgery, repeatedly washed using 0.9% sodium chloride to remove blood and excrescent tissues at once and then were cooled by liquid nitrogen and stored at -80°C until use. Frozen samples were retrieved from the tissue bank and placed into a standard cryostat (MICROM/HM5000, GERMAN) at -25°C . After embedding in optimal cutting temperature (OCT) compound, tissue blocks firstly were cut with a cryostat into $10\mu\text{m}$ sections and stained with routine H&E. Histology of tissue sections was carefully examined by a veteran pathologist under light microscope. The tissues were excluded if there was clear necrosis,

inflammation and connective tissue. Secondly, a 8 μm thickness of cryostat section was made and stained with the slightly improved H&E method for LCM. Frozen sections on plain untreated glass slides were stained with hematoxylin for 1s and washed in deionized water for 10s; then dehydrated with 85% ethanol for 10s, 90% ethanol for 10s, and two 10s with 100% ethanol. Finally, the slides were placed in xylene I for 2min and xylene II 2min.

3. LCM Process

LCM was performed to enrich specific cell populations. The Arcturus PixCell II system incorporates an Olympus IX-50 microscope containing a microscope slide stage that is moved by a joystick^[11]. The system employs a pulsed infrared laser to activate a thermoplastic film placed over the cells of interest, causing the film to become fused to the cells. The energy of Laser was set 80 mW and duration time was maintained 15.5ms. Laser shots were repeated until all cells of interest were collected onto a film-coated plastic cap. The cap was then lifted away from the tissue and the plastic-fused cells were removed from the tissue specimen. Cell samples were frozen at -80°C immediately. To minimize degradation of proteins, the LCM cell procurement time was always less than 40min.

4. Cell Lyses and Protein Extraction

The protocol of cell lyses and protein extraction from LCM caps were essentially as described by us.^[10] Briefly, every LCM cap sample was thawed on ice for 30min-1h. Ten microliters of cell buffer (9.5 M urea, 65 mM DTT, 4% CHAPS, 0.2% IPG buffer) was added to sample on LCM cap. All caps were enclosed, inverted and incubated for approximately 30min ensuring the buffer was adequately dispersed to cover the surface of the sample, followed by agitating on a platform shaker at 4°C for 30min. Solution then was transferred into a sterile microfuge tube and cells were further crushed with ultrasound for 50s. Extraction solution was recovered by centrifugation at 15,000 revolutions per minute for 45min. The supernatant was taken and added 5 folds of acetone to remove staining reagents.

5. Protein Quantitation

The precipitation was dissolved in lyses buffer (8 M urea, 4% CHAPS, 40 mM Tris, 65 mM DTT), and quantitated by Bradford protein assay as described previously by Kruger N. J.^[12] Bovine serum albumin (BSA) at a concentration of 1 mg/ml in distilled water was used as a standard protein solution. Bradford work solution was made by dissolving 15 ml of 95% ethanol, 30 ml of 88% phosphoric acid and 30 ml Bradford stock solution in 425 ml distilled water. The solution should be filtered through Whatman No. 1 filter paper and then stored in an amber bottle at room temperature. Standard samples were prepared according to Table 1. Standard protein solution, ultrapure water and Bradford work solution were added in 105ml total volume into test tube 1-7, respectively (Table. 1). The A595 values of the standard samples were in turn measured for three times within 10min. The standard curve was made according to the corresponding mean value of A595 as X-axis and quantitation of BSA as Y-axis (Fig. 1). Different detecting samples solutions of extraction protein from normal lung, AD and SQCC and 5ml Bradford work solution were added into centrifuge tube. Ultrapure water was made up to final volume of 105ml and mixed gently and thoroughly. The A595 of each sample was measured between 2 and 60 min and further calculate protein concentration depending on the standard curve.

Table 1 Standard sample preparation

Tube No.	1	2	3	4	5	6	7
Standard protein solution (ml)	0	10	20	40	60	80	100
Ultrapure water (ml)	100	90	80	60	40	20	0
Bradford work solution (ml)	5	5	5	5	5	5	5

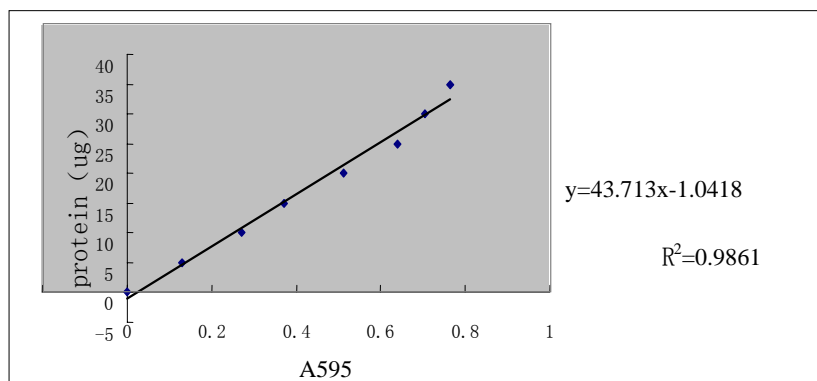


Fig. 1 Protein standard curve

6. Statistics Analysis

We use SPSS 13.0 software for statistical analysis. One-way analysis of variance (ANOVA) of the mean values was used to analyze the difference of cell number and protein quantity among three groups. SNK-q test were further used for multiple comparison between any two groups. $P<0.05$ was considered statistically significant.

RESULTS

1. LCM

Partial normal lung tissues from at least 5 cm away the edges of the tumors were prepared. 10 μm thickness of cryostat section was stained with routine H&E to further confirm the diagnosis. By careful observation, we chose the tissues without obvious necrosis and inflammation for next step analysis. 8 μm thickness of cryostat section with slightly improved H&E staining were prepared for LCM (Fig. 2A). To assure the homogeneities of specific cell populations procured by LCM, we adjust the joystick to position the interested cells under the smallest laser beam diameter that focused in 7.5 μm (Fig. 2B). After LCM, interstitial tissue was remained on section (Fig. 2C) and the interested cells were collected on LCM cap (Fig. 2D).

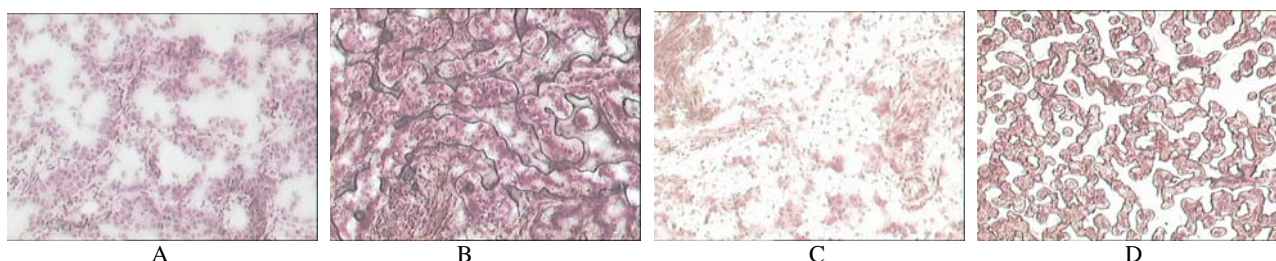


Fig. 2 (A) 8 μm thick tumor cryosections were fixed, stained with improved H&E; (B) Cells of interest were identified and microdissected by LCM; (C) Interstitial tissue remained on section after LCM; (D) Interested cells collected on LCM cap

2. Estimation of Specific Cell

We respectively collected 56, 60, 63 caps in total of 6 cases of normal lung, lung SQCC and lung AD. Approximately 3500-4000 laser shots hit to each LCM cap in lung SQCC and lung AD. 3000-3500 laser shots hit to each LCM cap in normal lung. Comparisons of the cell number among three groups by one-way ANOVA showed statistical difference (Table 2). Surprisingly, multiple comparison by SNK-q test showed that cell number in lung SQCC and lung AD were significantly higher than that of normal lung ($P < 0.05$), while differences between lung AD and lung SQCC had not statistically significant ($P > 0.05$). Total cells of normal lung, lung AD and lung SQCC were about 36,500, 73,600 and 71,800 respectively. We further observed the morphology of each type of cell population and estimated proportion of other components on LCM caps by microscopic visualization. Homogeneity of specific cells was to be over 95 percentages each group.

Table 2 Comparison of LCM interest cells among three groups

Group	Total cap	Cells/cap ($\bar{X} \pm s$)	Total cells
Lung SQCC	60	11,031 \pm 2,214*	71,800
Lung AD	63	11,355 \pm 2,457*	73,600
Normal lung	56	7,311 \pm 1,058	36,500

Note: * Significantly higher than normal groups by SNK-q test.

3. Protein Quantitation

We quantified the expression protein of interest cells microdissection from lung SQCC, lung AD and normal lung using Bradford methods. As showed in Table. 3, quantity of protein extracting from normal lung were significantly lower than those of lung AD and lung SQCC before acetone precipitate ($P < 0.05$). Further comparisons between any two groups after acetone precipitate, protein quantity in lung AD were markedly exceeding those of lung SQCC ($P < 0.05$) and normal lung ($P < 0.01$), meanwhile difference between lung SQCC and normal lung were also statistically significant ($P < 0.05$). Total protein of all LCM caps in lung SQCC, lung AD and normal lung group were respectively 248.20 μ g, 261.64 μ g and 183.88 μ g before acetone precipitate. When 5 folds of acetone was add in order to precipitate protein and remove staining reagents, the quantity of protein in the three groups were 101.89 μ g, 149.42 μ g and 80.38 μ g respectively.

Table 3 Protein quantitation of the three groups before and after acetone precipitate

Group	Acetone precipitate	Protein quantity (μ g/cap) ($\bar{X} \pm s$)	Total protein (μ g)
Lung SQCC	Before	4.14 \pm 0.36	248.20
	After	1.69 \pm 0.12	101.89
Lung AD	Before	4.15 \pm 0.28	261.64
	After	2.37 \pm 0.09**	149.42
Normal lung	Before	3.28 \pm 0.26*	183.88
	After	1.08 \pm 0.11	80.38

Notes: * Significantly lower than the respective values in lung SQCC and lung AD groups before acetone precipitate; ** Significantly higher than the respective values in lung SQCC and normal lung groups after acetone precipitate.

DISCUSSION

The LCM system was firstly reported during the mid 1990s by Dr Emmert-Buck and his colleagues at the NIH, and was commercially developed through a Collaborative Research and Development Agreement

Partnership with Arcturus Engineering Inc^[9]. The principle of this technique is utilization of a cap coated with a heat-sensitive transparent ethylene vinyl acetate layer film that is placed in contact with a stained tissue section being visualized using an inverted light microscope. A focused laser beam of variable diameter 7.5 μm , 15 μm and 30 μm is used to produce localized melting of the film over selected cells such that the underlying tissue becomes fused to the cap and is selectively removed when the cap is lifted^[7].

To date, several reports have published relating to the successful application of LCM technique to obtain specific cells in cancer genomics^[13-16]. However, the application of microdissection to protein-based studies is much more challenging than RNA- or DNA-based studies^[17]. Studies of expression protein profiling about tumor pathogenesis must solve the problem of tissue heterogeneity. The accuracy to identify biomarkers that give information about cancer diagnosis or treatment target spot largely depends on the selection of appropriate study materials in the experiments. In order to clearly recognize homogeneous cell under microscope, it is essential to consider the appropriate methods of fixation and staining for LCM in the first instance. In pre-experiment, we performed a comparison between frozen samples and paraffin-embedded samples. Surprisingly, protein quantity in paraffin-embedded samples was obvious decrease. As it was recognized that expression protein was vulnerable to rapid degradation during formalin fixation, we concluded that fresh frozen material was more desirable for protein-based studies.

At present there are usually several different kinds of staining methods, for example, hematoxylin, eosin, methyl green, toluidine blue and etc. Craven, etc^[18], showed that staining with a single stain was better than with two stains simultaneously; methyl green and toluidine blue staining were both compatible with the analysis of proteins by two-dimensional gel electrophoresis (2-DE). However, some reports^[19-21] showed that the stain of hematoxylin coupled with eosin was easy, rapid and convenient method for morphologically monitoring specific cell population from complex tissue. In our pre-experiment, different staining methods were repeatedly compared for optimizing selection. Ultimately, the slightly improved routine H&E was successfully used in our sample preparation. Morphology of tissue sections (Fig. 2A) showed that cell membrane and cell nuclear was completely maintained and parenchyma and interstitial tissue was easily recognized. Same staining was also adopted in our recent article about proteomics analysis of lung cancer tissue^[10]. The results illustrated their well compatibilities with proteomic analysis.

As the architecture of lung tissue is looser than that of other tissue, the thickness of tissue section should be considered. We routinely used 8-10 μm thick sections of frozen tissue for LCM in this study. We found if the sections thickness was less than 6 μm , it was easy to be broken; while exceeding 15 μm thickness section appeared notable “background noise”. In general terms, the thicker the tissue section, the greater the laser “power” required to dissect cells. Another practical influent factor for LCM may be that it requires a long time to pick up sufficient cells for one experiment^[22]. In order to effectively prevent protein degradation in room temperature, we strictly limited the time of LCM process for sample preparation. In this study, we respectively collected 56, 60, 63 caps in lung SQCC, lung AD and normal lung. We found that cell number of normal lung tissue was significantly less than that of lung cancer under visual field of microscope ($P < 0.05$), while differences between lung AD and lung SQCC had not statistically significant ($P > 0.05$), which suggested that relatively more caps should be acquired to ensure enough protein amount for next step analysis.

In addition, unlike polymerase chain reaction (PCR) amplification could be used on small number of cells for genome-based studies, extracting protein quantity must be considered to use for subsequent isolation and identification analysis. We used Bradford methods to respectively quantify the extracting protein of three groups.

Total protein of lung SQCC, lung AD and normal lung groups were respectively 248.20 µg, 261.64 µg and 183.88 µg. In order to improve the purity of the protein, we tried to remove the staining reagents using 5 folds of acetone precipitation prior to digestion. The results showed that staining reagents might lead to loss a small quantity of protein. Moreover, comparisons of average protein quantity among three groups might be implied variation of expression level in different disease conditions.

The utilization of LCM technique in combination with the traditional proteomics techniques such as 2-DE and matrix assistance laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS) is tissue-dependent. It usually requires at least microgram quantities of tissue. Clearly, obtaining such quantities using LCM from lung cancer tissue is time and investment consuming. Multiple-dimensional liquid chromatography and tandem mass spectrometry (MD-LC-MS/MS) approaches may significantly improve the dynamic range of protein detection and identification in comparison with 2-DE^[23]. It is now sensitive enough to analyze only a few cells, making it compatible with LCM^[24]. Therefore, we think that LCM sample from lung cancer tissues is more suitable to be analyzed by high-through proteomics methods.

In conclusion, LCM is a powerful tool in facilitating the molecular biology analysis of lung cancer tissue in the post-genomic era. It not only can effectively overcome the heterogeneous problem, but also can specifically analyze certain cell population, which provide a more meaningful insight into tumor pathogenesis.

REFERENCES

- [1] Jemal, A., Siegel, R., Ward, E., et al. Cancer statistics, 2007. *CA Cancer J Clin*, 2007, 57(1): 43-66.
- [2] Coleman, M. P., Rachet, B., Woods, L. M., et al. Trends and socioeconomic inequalities in cancer survival in England and Wales up to 2001. *Br J Cancer*, 2004, 90(7): 1367-1373.
- [3] Parkin, D. M., Bray, F., Ferlay, J., et al. Global cancer statistics, 2002. *CA Cancer J Clin*, 2005, 55(2): 74-108.
- [4] Yoon, S. K. Recent advances in tumor markers of human hepatocellular carcinoma. *Intervirolgy*, 2008, 51(Suppl. 1): 34-41.
- [5] Nishimori, T., Tomonaga, T., Matsushita, K., et al. Proteomic analysis of primary esophageal squamous cell carcinoma reveals downregulation of a cell adhesion protein, periplakin. *Proteomics*, 2006, 6(3): 1011-1018.
- [6] Bertucci, F., Goncalves, A. Clinical proteomics and breast cancer: Strategies for diagnostic and therapeutic biomarker discovery. *Future Oncol*, 2008, 4(2): 271-287.
- [7] Craven, R. A., Banks, R. E. Laser capture microdissection and proteomics: Possibilities and limitation. *Proteomics*, 2001, 1(10): 1200-1204.
- [8] ZANG L., Palmer, T. D., Hancock, W. S., et al. Proteomic analysis of ductal carcinoma of the breast using laser capture microdissection, LC-MS, and 16O/18O isotopic labeling. *J Proteome Res*.
- [9] Emmert-Buck, M. R., Bonner, R. F., Smith, P. D., et al. Laser capture microdissection. *Science*, 1996, 274(5289): 998-1001.
- [10] YANG S., NAN Y., TIAN Y., et al. Study of distinct protein profiles for early diagnosis of NSCLC using LCM and SELDI-TOF-MS. *Med Oncol*, 2008, 25(4): 380-386.
- [11] Bonner, R. F., Emmert-Buck, M. R., Cole, K. A., et al. Laser capture microdissection: Molecular analysis of tissue. *Science*, 1997, 278(5342): 1481-1483.
- [12] Kruger, N. J. The Bradford method for protein quantitation. *Methods Mol Biol*, 1994, 32: 9-15.
- [13] Curran, S., Murray, G. I. Tissue microdissection and its applications in pathology. *Current Diagnostic Pathology*, 2002, 8(3): 183-192.
- [14] Kosari, F., Asmann, Y. W., Chevillie, J. C., et al. Cysteine-rich secretory protein-3: A potential Biomarker for prostate cancer. *Cancer Epidemiology Biomarkers Prev*, 2002, 11(11): 1419-1426.
- [15] NIE Y., LIAO J., ZHAO X., et al. Detection of multiple gene hypermethylation in the development of esophageal squamous cell carcinoma. *Carcinogenesis*, 2002, 23(10): 1713-1720.
- [16] CHEN J. Z., Gokden, N., Greene, G. F., et al. Extensive somatic mitochondrial mutations in primary prostate cancer using laser capture microdissection. *Cancer Res*, 2002, 62(22): 6470-6474.
- [17] Kaserer, K., Knezevic, V., Pichlhöfer, B., et al. Construction of cDNA libraries from microdissected benign and malignant

thyroid tissue. *Lab Invest*, 2002, 82(12): 1707-1714.

- [18] Craven, R. A., Totty, N., Harnden, P., et al. Laser capture microdissection and two-dimensional polyacrylamide gel electrophoresis: Evaluation of tissue preparation and sample limitations. *Am J Pathol*, 2002, 160(3): 815-822.
- [19] CHENG Y., ZHANG J., LI Y., WANG Y., GONG J. Proteome analysis of human gastric cardia adenocarcinoma by laser capture microdissection. *BMC Cancer*, 2007, 7: 191.
- [20] Shekouh, A. R., Thompson, C. C., Prime, W., et al. Application of laser capture microdissection combined with two-dimensional electrophoresis for the discovery of differentially regulated proteins in pancreatic ductal adenocarcinoma. *Proteomics*, 2003, 3(10): 1988-2001.
- [21] Banks, R. E., Dunn, M. J., Forbes, M. A., et al. The potential use of laser capture microdissection to selectively obtain distinct populations of cells for proteomic analysis—Preliminary findings. *Electrophoresis*, 1999, 20(4-5): 689-700.
- [22] Seow, T. K., Liang, R. C., Leow, C. K., et al. Hepatocellular carcinoma: From bedside to proteomics. *Proteomics*, 2001, 1(10): 1249-1263.
- [23] WU S. L., Amato, H., Biringer, R., et al. Targeted proteomics of low-level proteins in human plasma by LC/MSn: Using human growth hormone as a model system. *J Proteome Res*, 2002, 1(5): 459-465.
- [24] Baker, H., Patel, V., Molinolo, A. A., et al. Proteome-wide analysis of head and neck squamous cell carcinomas using laser-capture microdissection and tandem mass spectrometry. *Oral Oncol*, 2005, 41(2): 183-199.

(Edited by Jane Chen)