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A Proteomic Method For Core Needle Biopsy Sample Characterization

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Abstract Proteomic analysis of core needle biopsy (CNB) sample from patient populations is critical to our understanding of human disease, but has been hindered by its particular small size. Here, we present a method for the proteomic analysis of CNB sample based on the two dimensional electrophoresis. Proteins were extracted directly from 3 rat liver CNB specimens and a human prostate CNB sample, respectively. 24 cm Immobiline DryStrip (pH 3-10NL) and 12.5% SDS-PAGE were introduced to separate the proteins. Interesting spots were analyzed by MALDI TOF/TOF mass spectrometry after tryptic digestion. With this method, consistent electrophoretic patterns of more than 2 500 protein spots were reproducibly obtained after silver staining, from rat liver CNB specimens. Qualitatively and quantitatively reproducible results also yield when the method was applied to a human prostate CNB sample. 57 stochastically selected protein spots were analyzed by MALDI TOF/TOF mass spectrometry, and were identified with high confidence including faint ones. This simple and reproducible approach raises the opportunity of defining key molecular events of human disease pathologies.

Key words core needle biopsy; mass spectrometry; proteomics; two dimensional electrophoresis

一种适合针刺活检样品的蛋白质组学分析方法

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摘要 针刺活检样品是一种重要的临床组织样品, 其蕴涵的蛋白质信息, 对了解人类疾病极为重要. 然而, 由于该样品的体积极小, 其研究受到很大限制. 本文建立和优化了适合针刺活检样品的基于双向电泳的蛋白质组学分析平台: 通过直接抽提获得针刺活检组织的蛋白质样品; 用 24 cm 固定梯度干胶条(pH 3-10NL)等电聚焦及 12.5% SDS-PAGE 分离获得蛋白质样品; 感兴趣的蛋白质点经过胰酶酶解后用 MALDI TOF/TOF 质谱分析. 运用所建立的平台对 3 例来自 3 只不同大鼠的肝脏针刺活检样品进行分析, 获得了多于 2 500 个蛋白质点的高重复性的二维凝胶银染图谱. 应用该方法分析人前列腺针刺活检样品的蛋白质组, 同样获得了高质量、高重复性的结果. 其中随机选取的包括低丰度点在内的 57 个蛋白质点, 经胰酶酶解后进行 MALDI 串联质谱分析, 均获得了高确定性的鉴定结果. 通过建立针刺活检样品的蛋白质组学分析方法, 为研究人类疾病的分子机制提供了必要的前提保障.

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Core needle biopsy (CNB) sample is a unique tissue, and is being used frequently these days for the definitive diagnosis of many diseases such as tumor[1,2], rejection and delayed graft function[3], and pelvic actinomycosis[4]. It together with surgical specimen are reliable clinical materials for studying the pathology or biology of the disease, since human disease occurs in the context of complex tissue microenvironments that may not be adequately reflected either in vitro studies or in non-human animal studies[5]. CNB sample is taken from the patient before the surgical treatment. Analysis performed on CNB sample could be integrated with clinical data obtained from studies of pre-operative treatment of disease, and in fact provides a test of 'in vivo' drug sensitivity, and may allow the identification of potential predictors of response. In addition, the comparison between protein profiles of pre- and post-treatment (surgical) samples may provide useful information about potential molecular mechanisms involved in drug sensitivity or resistance. Therefore, CNB sample presents unique superiority as compare to surgical specimen.

Traditionally, morphology analysis or/and immunohistochemistry is/are performed on CNB sample to make out a clinical diagnosis and subclassification[5]. For morphology analysis, CNBs are formalin fixed, paraffin embedded, sectioned onto microscopic slides and stained. Diagnosis is largely made on the basis of morphology and pattern recognition involving multiple variables including tissue architecture, cellular configurations, pleomorphism, nuclear shape and contour, and staining patterns. Immunohistochemistry has added a significant dimension to clinical diagnostics, as well as the research setting. Panels of antibodies have been developed to aid in the differential diagnosis of diseases. However, many benign reactive conditions can also exhibit similar characteristics. And within tumors bearing the same histologic and immunophenotypic diagnosis there is often a wide range of patient response to treatments. There is a diverse biology of diseases on a molecular level that is not necessarily apparent by outward microscopic morphology or antibody based immunohistochemistry.

Gene microarray has recently been introduced to study molecular characteristics of CNB sample in genomics phase[6], and has successfully elucidated gene expression patterns associated with disease to some extent. However, it gives no indication of the complexity of protein-protein interactions, their localization, or post-translational modifications, while the execution of the disease process occurs through altered protein function. The complex pattern of protein expression and functional state is presumed to contain more important information about the pathologic process taking place in the cells within their tissue microenvironment. Therefore, 2DE-based differential proteomics of healthy and pathological tissues is an attractive alternative. In the case of CNB sample, however, such an approach is difficult to perform due to its small size and the high susceptibility. These impair the detection of low abundant proteins and the reproducibility of proteomic analysis using this scarce sample.

Previously, a method was described to proteomic analyze CNB samples from breast lesions using 7 cm long IPG strips with pH 4—7[7]. Tissue extracts were well resolved and several spots were successfully identified. However, these successes were mainly restricted to high abundant non-alkali proteins. There is a crucial need for reliable method with high resolution and sensitivity to define the proteomic features implicated in CNB specimens and to identify potential molecular targets for disease diagnostics and therapeutics. In this study, we developed a method allowing us to efficiently and reproducibly generate sufficient amounts of protein extracts from just a single CNB specimen and to resolve them with high resolution on 2D gels. This strategy was successfully adapted to analysis the protein profile of human prostate CNB and a series of protein spots were successfully identified.

1 Materials and Methods

1.1 Materials

IPG buffer (pH 3-10NL), 24 cm Immobiline DryStrip (pH 3-10NL), urea, thiourea, CHAPS, Coomassie BB G-250, acrylamide, Bis, Tris, SDS, AP, and TEMED were obtained from Amersham Biosciences. DTT, iodoacetamide, tris (2-carboxyethyl)-phosphine hydrochloride (TCEP), 4-vinylpyridine (4-VP), acetonitrile (ACN), NH_4HCO_3, trifluoroacetic acid (TFA), α-cyano-4-hydroxy cinnamic acid (CHCA), $K_3Fe(CN)_6$, and $Na_2S_2O_3 \cdot 5H_2O$ were from Sigma. Trypsin was purchased from Promega. Mass standard kit for the 4700 proteomics analyzer calibration mixture was from Applied Biosystems. Other analytical grade chemicals used in this study were from domestic sources.

Liver tissues were dissected from three rats and washed extensively in cold PBS for removal of blood cells. CNB samples were taken from these tissues using a biopsy gun with a 14 gauge needle. Briefly, the needle was advanced until it was a small distance from the lesion intend to biopsy. Then the biopsy gun was fired. First, the inner stylet was thrown forward piercing the lesion. Tissue protruded in the tissue notch of the inner stylet. Next the outer sleeve of the needle was forwarded, cutting a core of tissue and securing it in the tissue notch. The

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needle was removed, the tissue collected. A human prostate CNB tissue was donated by a patient who undergone prostate needle biopsy. Samples were immediately snap frozen in liquid nitrogen and stored at − 80℃ until further analysis.

1.2 Sample preparation

CNB sample was homogenized in 400 μl of denatured buffer [7 mol/L urea, 2 mol/L thiourea, 4% CHAPS, 1% DTT, and 2% IPG buffer] with a glass homogenizer on ice. After vigorously agitating for one hour, the extract was centrifuged at 45 000 × g for one hour. Protein concentrations were determined using a modified Bradford method[8]. Supernatant was aliquoted at − 80℃ until further use.

1.3 Two dimensional electrophoresis (2-DE)

Protein samples were mixed with rehydration solution [8 mol/L urea, 2% CHAPS, 0.4% DTT, and 0.5% IPG buffer (pH 3-10NL)], resulting in final protein concentration of 160 μg (or 1 mg for micro-preparative gel) in 450 μl. The mixtures were applied onto each 24-cm IPG strip (pH 3-10NL), and proteins were focused on an IPGphor IEF system (Amersham Biosciences) for a total of 60 kVh (92 kVh for micro-preparative gel). Then the strip was subjected to two-step equilibration in equilibration buffer containing 6 mol/L urea, 30% glycerol, 2 % SDS and 50 mmol/L Tris-HCl (pH 8.8) with 1 % DTT for the first step, and 2.5% iodoacetamide for the second step. The second-dimension separation was performed on 12.5% SDS-PAGE gels (260 mm × 200 mm × 1 mm) using the Ettan DALTtwelve apparatus (Amersham Biosciences). All samples were run at least in triplicate to guarantee reproducibility. After electrophoresis, analytical gels were stained with silver nitrate as described by Yan and coworkers[9]. Micro-preparative gels were stained with Coomassie BB G-250 as described by Neuhoff *et al*.[10].

1.4 Image acquisition and analysis

The stained 2-DE gels were digitized using a D2000 Uniscan scanner (Tsinghua Uniscan, Beijing, China) and analyzed using ImageMaster™ 2D Platinum software (Version 5.0; Amersham Bioscience). Spot detection parameters were chosen to detect the maximum number of actual protein spots. Manual editing was performed on each gel image to eliminate any artifacts arising from dust specks, edge effects or other anomalies in the gel image. Individual spot volume was normalized against total spot volumes. Alignment and matching of the spots was carried out by choosing one gel as reference and manually selecting one common spot as a landmark. Scatter plots and correlation coefficients were calculated to analyze gel similarities or experimental variations.

1.5 Tryptic in-gel digestion

Spots were excised from 2-DE gels of human prostate CNB and destained with a 1∶1 solution of 30 mmol/L $K_3Fe(CN)_6$ and 100 mmol/L $Na_2S_2O_3 \cdot 5H_2O$ for silver stained spots or with 50% (V/V) ACN and 50 mmol/L NH_4HCO_3 for Coomassie BB stained spots. After being dehydrated in 100% (V/V) ACN and dried in a Speed-Vac, the gel pieces were rehydrated on ice for 30 min in 2 μl of 25 mmol/L NH_4HCO_3 containing 10 ng/μl sequencing-grade trypsin. Proteins were digested overnight at 37℃. Peptides were sequentially extracted with 5% TFA and 2.5% TFA/ 50% ACN. Supernatants were pooled and dried in a Speed-Vac. Peptides were then resuspended in 1.5 μl of 0.5% TFA.

1.6 Protein identification

Digested peptide samples (0.3 μl) were applied on a MALDI target using the dried droplet method with CHCA as a matrix. MS and MS/MS spectra were obtained using an ABI 4700 Proteomics Analyzer MALDI-TOF/TOF mass spectrometer (Applied Biosystems). Six external standards (Mass standard kit) were used to calibrate each spectrum to a mass accuracy within 5 ppm in MS Reflector Positive Operating Mode or within 10 ppm in MS-MS 1KV Positive Operating Mode. A combined database search of MS and MS/MS measurements was performed using GPS Explorer TM software (Version 3.5; Applied Biosystems) and MASCOT software (Version 2.0; Matrix Science, London, UK). Peptide mixtures that yielded statistically significant search scores (> 95% C. I., equivalent to MASCOT expect value < 0.05) and accounted for the majority of ions present in the mass spectra were defined as positive identifications.

2 Results

2.1 Establishment of a method for proteome extraction and analysis for core needle biopsy sample

Intrinsic limitations of classical proteomic approaches for studying CNB sample are mostly due to the small size of this tissue, which strongly affects the protein integrity and effectiveness of the proteomic analysis, especially for low abundant proteins. To address this problem, a reliable method for proteome characterization of CNB sample, described in details in Materials and Methods, was designed.

First, CNB sample was lysed directly in a denaturing buffer, thereafter the time interval between surgical removal and sample processing was shortened, and the loss of proteins was reduced. The result 2D maps didn't show obvious inferiority over the maps of large liver tissue samples (Fig. 1). Proteins extracted from a single CNB sample were sufficient for getting reduplicate 2D maps of high quality to fix on, MS identify, and Western blot confirm the exciting spots.

Second, several experiments focused on the reducing agent DTT were performed to optimize the IEF in the alkaline region. In our primary 2D maps (Fig. 2A), poorly resolved protein smears or streaks were present across the basic pH range. Elevating the concentration of DTT in protein extraction buffer from 0.5% to 1.5%

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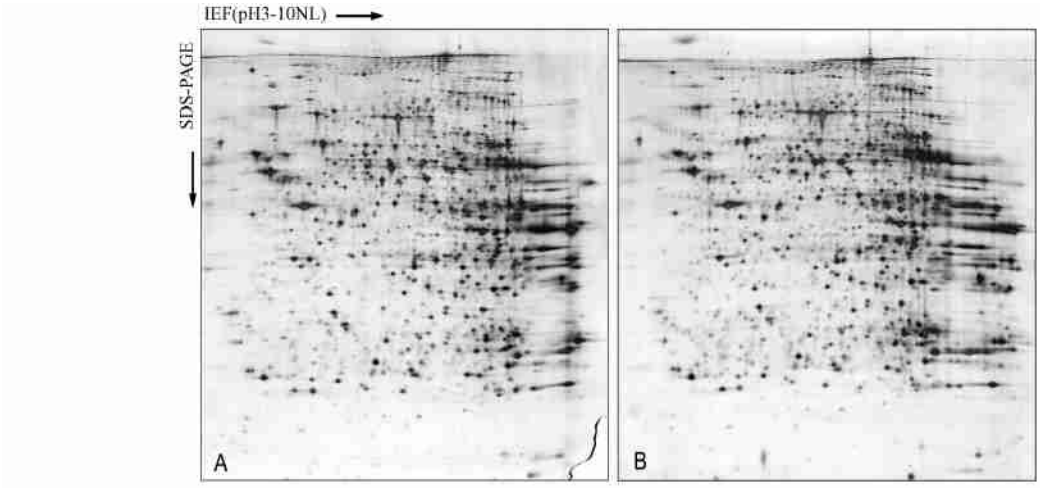

Fig. 1 Comparison of 2-D maps of different rat liver tissue samples

(A) Large tissue sample; (B) Core needle biopsy sample.

The samples were prepared with the same procedure expect that the large tissue samples were ground under liquid nitrogen

(total concentration of DTT in IEF was elevated from 0.42% to 0.57%) seemed to have little use (Fig. 2A, B, and C). However, it optimized the resolution of the acidic proteins (Fig. 2A, B, and C). The problem was addressed to a large extent when the concentration of DTT in rehydration solution was elevated from 0.4% to 0.8% (total concentration of DTT was elevated from 0.49% to 0.91%; Fig. 2B and D). But this also brought about another problem that burrs existed around many spots (Fig. 2D). Reducing the IEF focusing time seemed to be more effectual. As shown in Fig. 3B, high resolution maps with at least 2 500 spots were obtained with few protein smears or streaks, and burrs were also restricted to a minor range when the IEF focusing time was reduced from 65 kVh to 60 kVh. Good results were also obtained by reducing proteins with another reducing agent TCEP in

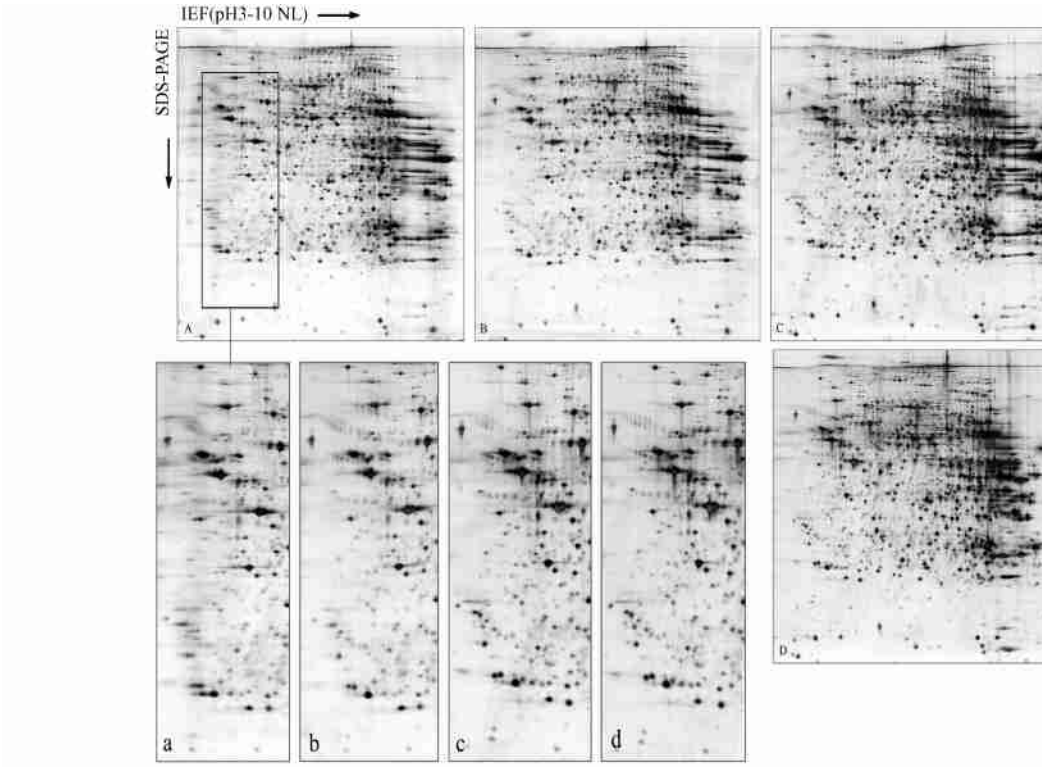

Fig. 2 The effect of reducing agent DTT on protein extraction and resolution

Rat liver CNB proteins (160 μg for each gel) were prepared with an extraction buffer containing: (A) 0.5% DTT, (B and D) 1% DTT, or (C) 1.5% DTT, and isoelectric focused with a rehydration solution containing: (A, B, and C) 0.4% DTT or (D) 0.8% DTT. Inset boxes with corresponding lowercase letters illustrate areas of the gels where comparisons were made

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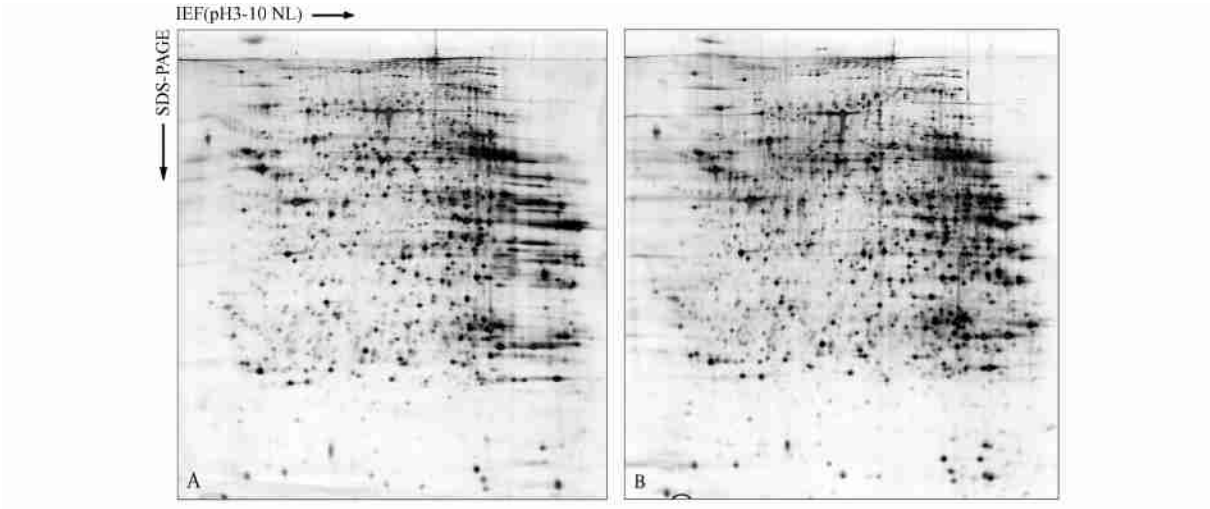

Fig. 3 Comparison of isoelectric focusing conditions

Protein extracts (160 μg) from rat liver CNB samples were isoelectric focused on 24 cm pH 3-10NL IPG strips for (A) 65 kVh or (B) 60 kVh in the first dimension and then separated on 12.5% SDS-PAGE gels. Proteins were visualized by silver staining

stead of DTT and alkylating subsequently with 4-VP in the sample preparation step before the 2-DE analysis (Fig. 4B). This was in agreement with previous observations[11]. However, the resulting patterns were quite different from those obtained with DTT (Fig. 4C), and spots detected were greatly reduced. Scatter polts analysis also confirmed this variation. As shown in Fig. 5A, the correlation coefficient between gels using TCEP and gels using DTT was only 0.446, even much worse than that between silver stained gels and CBB G-250 stained gels (Corr. = 0.799; Fig. 5B). The correlation coefficient was calculated according to the matched spots,

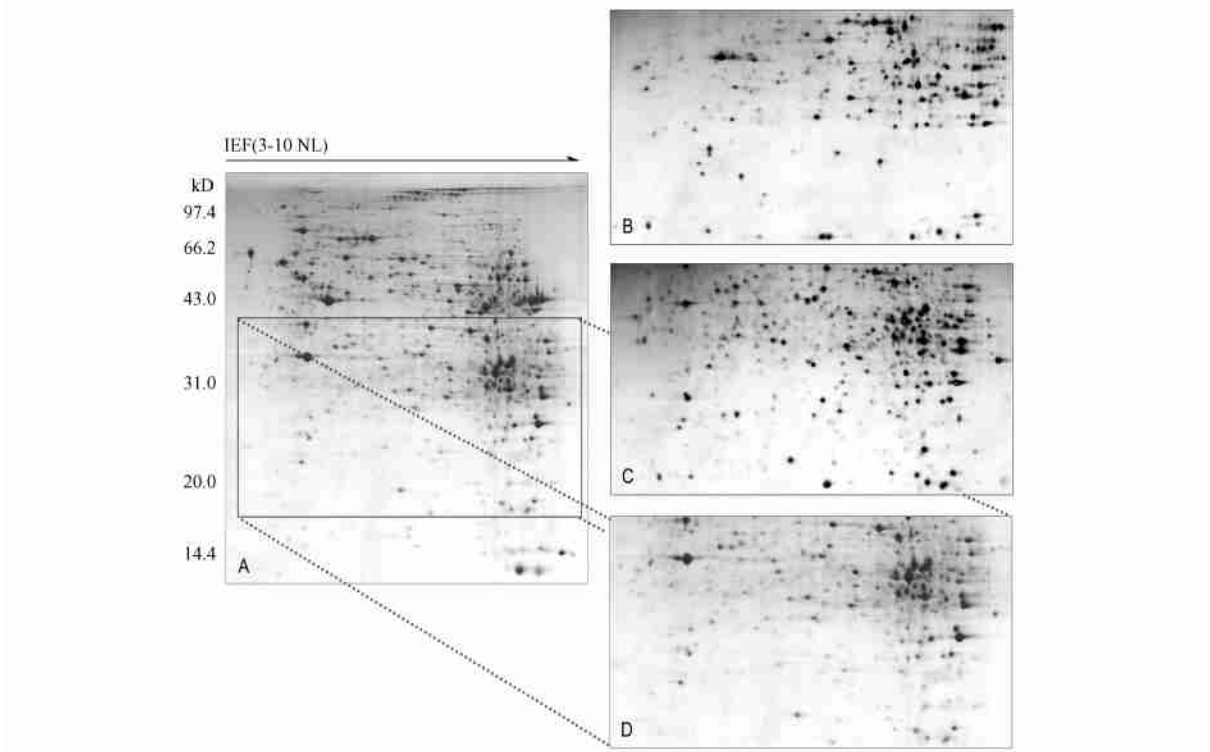

Fig. 4 Comparison of 2-D gel resolution using different reducing agents

(A, C, and D) Rat liver CNB sample was homogenized in an extraction buffer containing 9 mol/L urea, 4% CHAPS, 1% DTT, and 0.5% IPG buffer (pH 3-10NL) or (B) was subjected to the same denaturation, solubilization, and reduction process, except that 10 mmol/L TCEP was used instead of 1% DTT. After reduction with TCEP, alkylation was performed with 400 mmol/L 4-VP for 1 hour while vortexing, and was quenched by the addition of 400 mmol/L DTT. (A) micro-preparative 2D map of rat liver CNB proteome. Inset box illustrates areas of the gels where comparisons were made. Proteins were visualized by CBB G-250 staining (A and D) or silver staining (B and C)

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and obtained automatically by ImageMasterTM 2D Platinum software without any manual matching processing. So a correlation coefficient of 0.799 revealed good match between the gels stained with different methods (also see Fig. 4C and D), while the correlation coefficient of 0.906 between patterns of different CNB samples represented an excellent reproducibility of this established method (Fig. 5C).

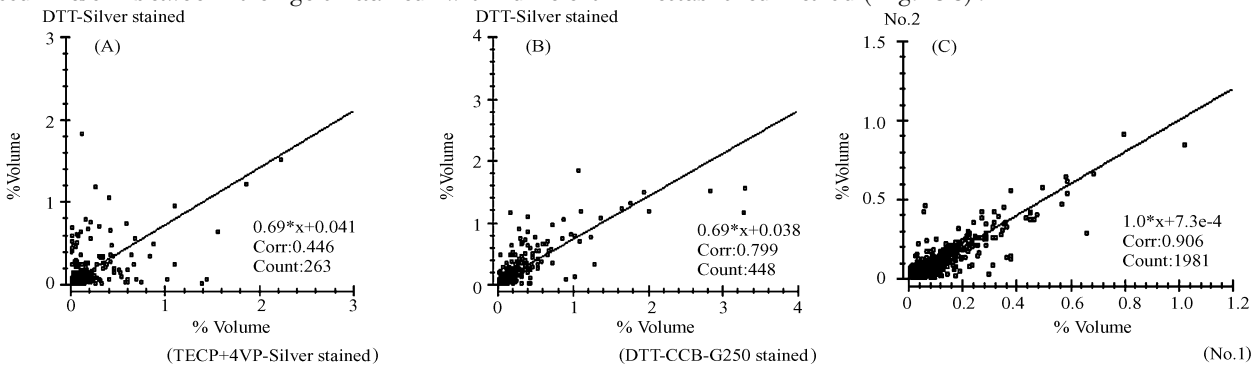

Fig. 5 Scatter plots of values (% Vol) of matched spots obtained from representative 2D maps

(A) The plot obtained from representative 2D maps of samples treated with TCEP and 4-VP, or with DTT, as indicated in Fig. 4B and C. (B) The plot obtained from representative 2D maps of analytical gels and micro-preparative gels. (C) The plot obtained from representative 2D maps of two CNB samples. CNB extracts were separated by 2-DE, scanned and analyzed with ImageMasterTM 2D Platinum software (Version 5.0; Amersham Bioscience)

2.2 Proteomic analysis of human prostate core needle biopsy sample

The established method was then adapted to proteome characterization of a human prostate CNB sample. With little optimization, good results were obtained. As shown in Fig. 6, well-resolved patterns were obtained with mean 1848 spots ($n = 3$). Spots corresponding to 10 protein spots in analytical gels and 47 protein spots in micro-preparative gels were randomly chosen and submitted to MS analysis. Using the tryptic digestion and MS identification procedures described in Materials and Methods, all spots were identified with marked mascot scores ranging from 73 to 646 [a mascot score> 53 was considered significant ($p < 0.05$)], and

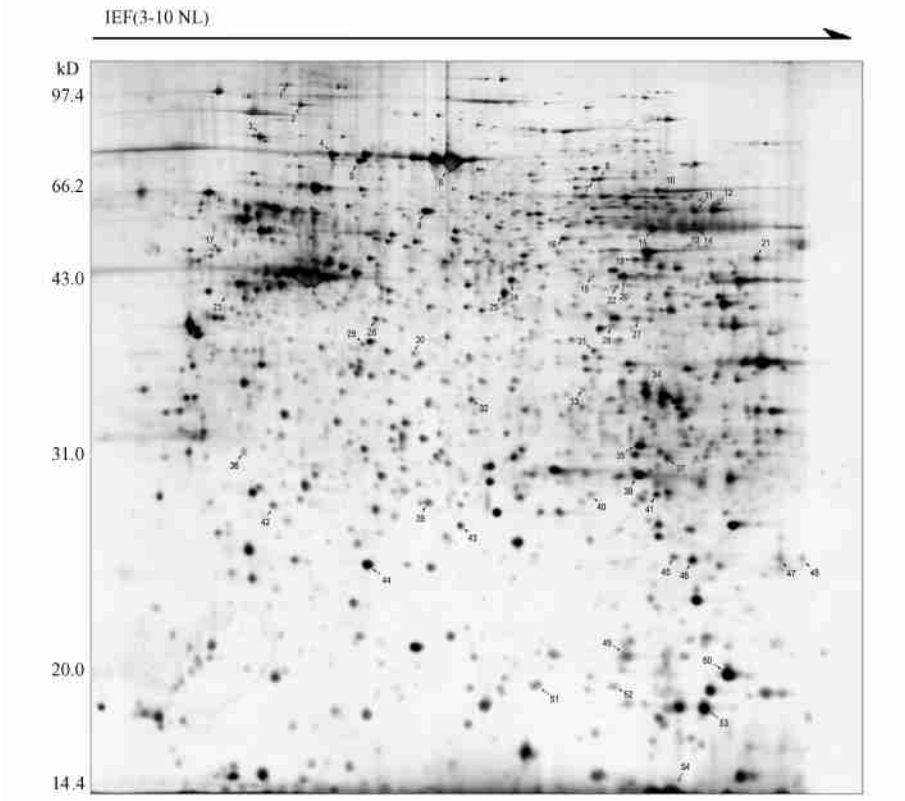

Fig. 6 2-DE protein profile of a prostate CNB specimen

Proteins (160 μg) were separated on the basis of pI (X-axis) and molecular mass (Y-axis) and visualized by silver staining. Identified spots are indicated by spot numbers

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were summarized in Table 1. The identification was greatly promoted by increasing the protein amount (Table 1 and Fig. 7). The identification of low abundant proteins is very important for proteomics. The success in this aspect (Fig. 8) further indicated that the method described here is reliable for proteome characterization of human prostate CNB sample.

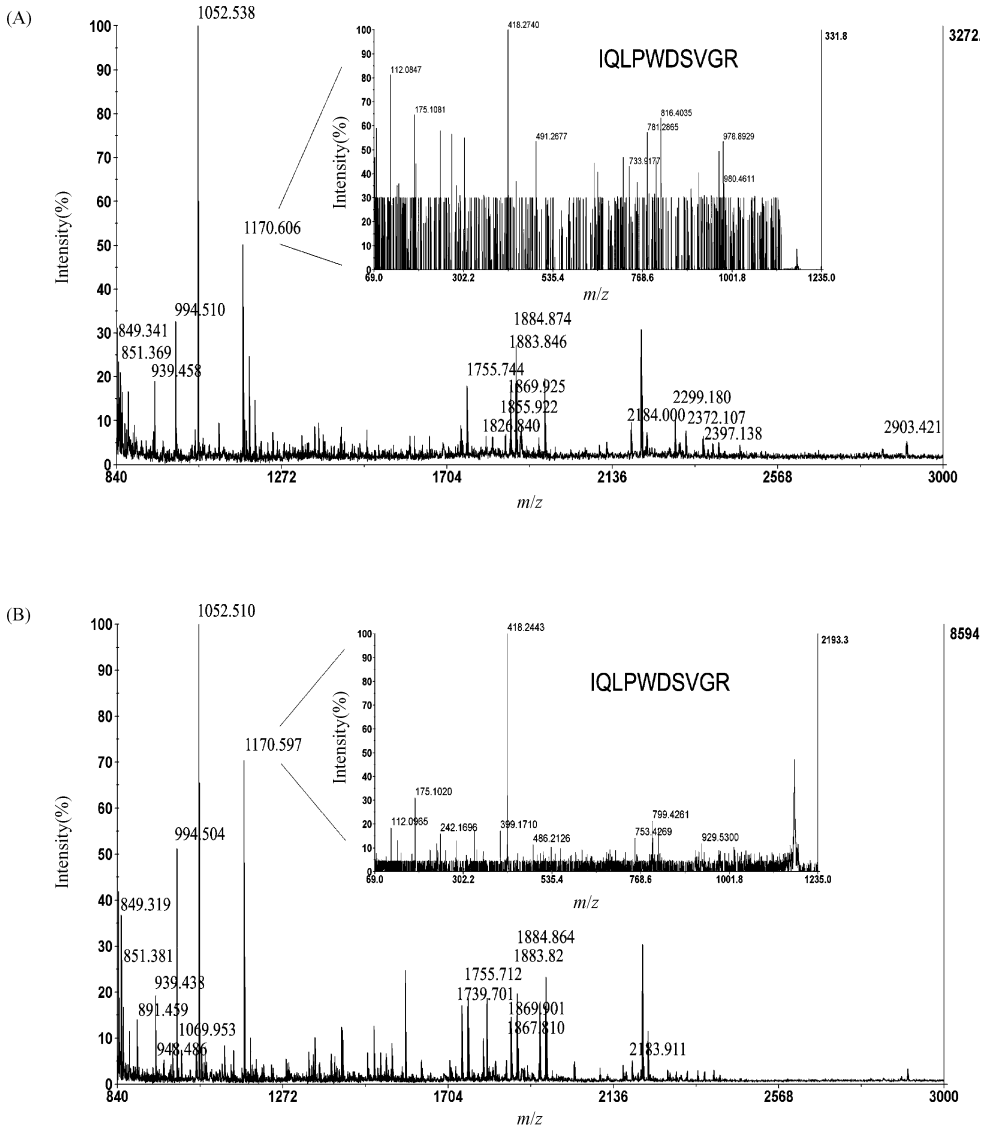

Fig. 7 MALDI TOF MS/MS spectra of spot 18

(A) From analytical gels. (B) From micro-preparative gels.

The subsequent database searching identified spot 18 as hydroxymethylglutaryl-CoA synthase with a mascot score of 116 or 150, respectively. Matched peptide peaks are labeled with mass values, and the peptide m/z 1 170.6 sequence was confirmed from the labeled ions

3 Discussion

Despite an increasing need for large scale characterization of the CNB sample proteome, classical proteomic approaches have been impractical until recently, due to the particular small size and the susceptibility of this unique tissue. Here we describe a reliable method allowing 2D gel-based proteomic characterization of the CNB sample. It was found qualitatively and quantitatively reproducible not only for rat liver CNB model, but also for human prostate CNB clinical sample.

Traditionally, large tissue samples were ground under liquid nitrogen using a mortar and pestle and then solubilized in lysis buffer. TCA/acetone precipitation is often performed to remove impurities. However, it is difficult to perform these treatments on small tissue specimens. In this method, proteins were directly extracted from the CNB without any adjuvant treatment such as impurity cleanup. Though this complicated the composition of the exact and impaired the 2DE separation, it ensured to obtain creditable information to

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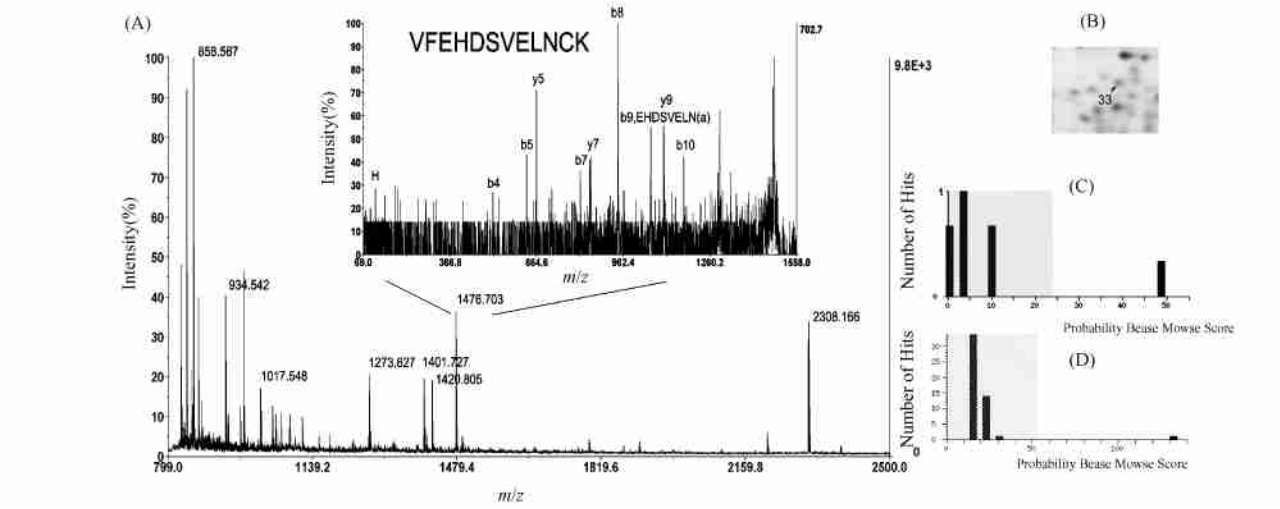

Fig. 8 Identification of faint spot 33 as Esterase D by MALDI-TOF MS/MS

(A) Matched peptide peaks are labeled with mass values, and the peptide m/z 1 476.703 sequence was confirmed from the labeled ions. (B) Enlarged region of the 2-DE profile of esterase D. (C) The MASCOT Peptide and (D) Protein summary report for esterase D

Table 1 Proteins identified by MALDI-TOF MS/MS

Spot no.[a]	Protein name	Acc. no.[b]	Theor. M_r/ pI[c]	Mascot Score[d]
1	Heat shock 70 kD protein 4	P34932	94240.3/5.18	260
2*	Transitional endoplasmic reticulum ATPase	P55072	89134.7/5.14	239
3	78 kD glucose-regulated protein[Precursor]	P11021	72288.4/5.07	646
4	Heat shock cognate 71 kD protein	P11142	70854.2/5.37	586
5	Heat shock 70 kDa protein 1	P08107	70009.0/5.48	532
6	Serum albumin precursor	P02768	69321.5/5.92	640
7	Bifunctional purine biosynthesis protein PURH	P31939	64575.3/6.27	257
8	Stress-induced-phosphoprotein 1	P31948	62599.4/6.4	324
9	Protein disulfide-isomerase A3 [Precursor]	P30101	56746.8/5.98	464
10	UDP-glucose 6-dehydrogenase	O60701	54989.2/6.73	278
11*	Methylmalonate-semialdehyde dehydrogenase [acylating], mitochondrial[precursor]	Q02252	57802.6/8.72	235
12*	Methylmalonate-semialdehyde dehydrogenase [acylating], mitochondrial[precursor]	Q02252	57802.6/8.72	230
13	Fibrinogen beta chain precursor	P02675	55892.3/8.54	456
14	Fibrinogen beta chain precursor	P02675	55892.3/8.54	488
15	Glutamate dehydrogenase 1, mitochondrial precursor	P00367	61359.2/7.66	594
16	Lipoamide acyltransferase component of branched-chain alpha-keto acid dehydrogenase complex, mitochondrial [Precursor]	P11182	53452.9/8.71	266
17	Vimentin	P08670	53488/5.06	417
17*	Vimentin	P08670	53488/5.06	204
18	Hydroxymethylglutaryl-CoA synthase, mitochondrial precursor	P54868	56599.4/8.4	150
18*	Hydroxymethylglutaryl-CoA synthase, mitochondrial precursor	P54868	56599.4/8.4	116
19	Isocitrate dehydrogenase[NADP] cytoplasmic	O75874	46623.5/6.53	323
20	Isocitrate dehydrogenase[NADP] cytoplasmic	O75874	46623.5/6.53	379
21	Elongation factor 1-alpha 1	P68104	50109.1/9.1	80
22	Acyl-CoA dehydrogenase, long-chain specific, mitochondrial [Precursor]	P28330	47639.3/7.28	94
23	Desmin	P17661	53372.1/5.21	146
24	Septin 2	Q15019	41461.2/6.15	303
25	Prostatic acid phosphatase precursor	P15309	44537.5/5.83	158

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(Continued)

Spot no.[a]	Protein name	Acc. no.[b]	Theor. M_r/ pI[c]	Mascot Score[d]
26*	Arginase-2, mitochondrial[Precursor]	P78540	38553.9/6.00	192
27	Poly(rC)-binding protein 1	Q15365	37473.9/6.66	116
28	Fructose-1,6-bisphosphatase 1	P09467	36659.8/6.6	223
29	Eukaryotic translation initiation factor 3 subunit 2	Q13347	36478.6/5.38	205
30	Transaldolase	P37837	37516.5/6.36	97
31	Acetyl-CoA acetyltransferase, cytosolic	Q9BWD1	41324.4/6.47	165
32	Annexin A4	P09525	35729.1/5.85	306
33	Esterase D	P10768	31442.5/6.54	132
34	Prostate-specific antigen[Precursor]	P07288	28722.6/7.62	214
35*	Carbonic anhydrase 1	P00915	28721.3/6.63	338
36	Calpain small subunit 1	P04632	28297.7/5.05	101
37*	Hemoglobin subunit beta	P68871	15857.2/6.81	329
38	Triosephosphate isomerase	P60174	26521.7/6.51	606
38*	Triosephosphate isomerase	P60174	26521.7/6.51	373
39	26S proteasome non-ATPase regulatory subunit 10	O75832	24412.4/5.71	161
40	Ubiquinol-cytochrome c reductase iron-sulfur subunit, mitochondrial[Precursor]	P47985	29633.4/8.55	209
41	GTP-binding nuclear protein Ran	P62826	24407.6/7.01	225
42	Rho GDP-dissociation inhibitor 2	P52566	22973.6/5.1	73
43	Abhydrolase domain-containing protein 14B	Q96IU4	22331.6/5.94	316
44	Peroxiredoxin 2	P32119	21878.2/5.66	585
45	Transgelin	Q01995	22465.4/8.88	229
46*	Flavin reductase	P30043	21974.4/7.31	145
47	Transgelin	Q01995	22465.4/8.88	322
48	Transgelin	Q01995	22465.4/8.88	264
49	Transgelin	Q01995	22465.4/8.88	310
50	Cofilin-1	P23528	18359.6/8.26	268
51	Transgelin	Q01995	22465.4/8.88	260
52	Transgelin	Q01995	22465.4/8.88	322
53	Peptidyl-prolyl cis-trans isomerase A	P62937	17869.8/7.82	390
54	Hemoglobin subunit beta	P68871	15857.2/6.81	414

a) Spot no. was defined according to spot positions in 2-D gel indicated as in Fig. 6.

b) Acc. no. Swiss-Prot database accession number.

c) Theor. Mr(Da)/pI. Theoretical molecular weight of the matched protein/Theoretical isoelectric point of the matched protein.

d) Mascot scores were taken from the search results using GPS Explorer TM software (Version 3.5). In this program, a mascot score> 53 was considered significant ($p < 0.05$).

* Spots excided from analytical gels

the greatest extent with this scarce clinical material. However, an obvious limitation is implicated in this protocol that the high heterogeneity of the CNB in terms of cell types and pathology largely affects the veracity of the proteomic analysis. This problem can be resolved using laser capture microdissection (LCM) which allows the microdissection and extraction of a microscopic homogeneous cellular sub-population from its complex tissue milieu under direct microscopic visualization[12,13]. In the case of CNB, however, the low protein content obtained by this approach from a single CNB specimen can't offer typical proteomic profiles[5]. And this prolongs the sample processing time and may bring about uncertain impacts on this susceptible tissue. There are two approaches, morphology assessment performed on part of the CNB sample and immunoblot analysis with disease specific antibody conducted on the whole protein exacts, may compensate this limitation to some extent.

Low-abundance proteins are of great interest in proteomic research, and are the special challenges for 2-D PAGE. Though high protein loads, pre-fractionation methods, or liquid-phase isoelectric focusing devices can increase the relative amounts of low-abundance proteins in the sample[14], they are impractical for the CNB for its small size. Extend the separation distance, and optimize other parameters of 2DE seem to be more feasible to give

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access to increased numbers of proteins through increased resolution of protein separation. Using 24 cm long IPG strip with pH 3-10 NL in IEF can largely improve the resolution of complex protein patterns. However, it also brings about a series of problems such as horizontal streaks in the alkaline region[15]. These streaks are the proteins cross-linked through the formation of intra- and inter-molecular disulfide bridges following the oxidation of the cysteinyl thiol groups (-SH). It is primarily due to the depletion of the reducing agent, such as DTT (a weak acid with a pK> 8) in the basic pH range during the first dimension IEF[16-18]. To address this problem which also presented in our primary results, and get insight into the mechanism of the 2DE technique, four strategies were introduced and compared including elevating the concentration of DTT, shortening IEF duration, using the reducing agent TCEP and alkylating agent 4-VP in sample preparation, and anodic cup-loading (data not shown). Although all these approaches have achieved acceptable results, shortening IEF duration to the homeostasis range, in which proteins were focused adequately before the inordinate depletion of the DTT, seemed to the most effective. Because elevating the concentration of DTT was only effective when the concentration reached to a certain high level, and the high concentration of DTT was inclined to distort the protein patterns; Using TCEP and 4-VP complicated the sample preparation procedure and reduced the spots detected in the 2DE gels; Though cup-loading brought about higher resolution, it suffered from worse reproducibility and was highly experience-relied.

Identification of interesting protein spots is the essential goal of proteomic analysis. The method presented in this study ensured successes in MS identification of 57 spots with marked confidences including the faint spots. Whereas, the good correlations between analytical gels, or analytical gels and micro-preparative gels, have implicated that combing spots corresponding to the same protein spot from several gels, or using the spot from micro-preparative gels can be adopted to improve the identification of low abundant proteins.

In summary, we have outlined here a method to the analysis of core needle biopsy sample. It is achievable from low quantities of starting material, and was found reproducible with high resolution and sensitivity. Also, it allows convenient practice. Through this methodology, an in-depth proteomic analysis of CNB can be pursued.

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