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Novel rapid tissue lysis method to evaluate cancer proteins: Correlation between elevated Bcl-X_L expression and colorectal cancer cell proliferation

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Abstract

AIM: We optimized a rapid and efficient tissue lysis method using the MagNA Lyser (Roche, Germany). Using this novel method combined with immunoblot analysis, we investigated the correlation between abnormal Bcl-X_L expression and clinicopathological characteristics in colorectal cancer.

METHODS: Tissue samples from Sprague-Dawley rats were tested to determine optimal lysis conditions for use with MagNA Lyser. We next used the new method to extract tissue proteins from the tumor tissue of a colorectal cancer patient. The availability of extractable tissue proteins for proteomic study was demonstrated by two-dimensional (2D) gel electrophoresis and subsequent matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. In addition, we prepared tissue lysates from paired tumor tissues and adjacent nontumor tissues of 50 colorectal carcinoma patients. Ensuing immunoblot analyses were performed to detect the level of Bcl-X_L expression.

RESULTS: The optimal sample sizes processed were found to be around 200 mg, with oscillation frequency of 6 500 r/min for 80 s. Test of the first human tissue lysate confirmed that the MagNA Lyser method was adequate for protein extraction and subsequent identification by current proteomic protocols. The method was also applicable to immunoblot analysis. Thirty of 50 (60%) colorectal patients exhibited higher level of Bcl-X_L expression in their tumor tissues. Raised level of Bcl-X_L expression correlated with patients' gender and tumor cell proliferation index ($P = 0.037$ and $P<0.001$, respectively), but was independent of clinicopathological characteristics and overall survival.

CONCLUSION: We report a novel tissue lysis method applicable to proteomic and immunoblot analyses, which can facilitate the discovery and detection of cancer protein alterations.

Key words: Tissue lysis; MagNA Lyser; Bcl-X_L; Colorectal cancer

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INTRODUCTION

Cancer is a complicated and heterogeneous disease involving multiple molecular alterations. To globally explore protein alterations in cancer tissues, the first and most essential step is to rapidly and efficiently extract tissue proteins from the study specimens. Conventional methods for tissue lysis usually require grinding, homogenization and sonication to disrupt tissues and cells[1,2]. It is not only tedious, but also carries biohazard concerns for laboratory personnel. Recently, the Roche Company (Penzberg, Germany) developed a machine called the MagNA Lyser, and it could be combined with certain commercially available reagent kits to rapidly isolate DNA or RNA from tissue specimens[3,4]. The basic principle of tissue lysis centers on the ability of the machine to make fast and reciprocal oscillations, which produce collisions between tissue samples and ceramic beads provided in special centrifuge tubes. The whole procedure is simple and quick, and is therefore highly suitable for protein extraction from tissues, even though protein extraction had not been previously published, as a function of the MagNA Lyser. In this study, we have optimized a rapid tissue lysis protocol for use with the MagNA Lyser. The tissue proteins prepared by this new method can be resolved by two-dimensional (2D) gel electrophoresis and further identified by matrix-assisted laser

desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. In addition, this novel method is also applicable to immunoblot analysis. We prepared tissue lysates from the tumor tissues and adjacent non-tumor tissues of 50 colorectal carcinoma patients. The ensuing immunoblot analyses were performed to detect the level of Bcl-X_L expression. Bcl-X_L is an important member of the Bcl-2 family proteins, contributing to the anti-apoptotic characteristic during the progression of tumors[5-7]. Previously, 43-60% of colorectal cancer patients have been reported to exhibit elevated level of Bcl-X_L expression in their tumor tissues by immunohistochemical staining[8,9]. In this study, elevated Bcl-X_L expression in tumor tissues was observed in 60% of colorectal cancer patients using the new tissue lysis method and immunoblot analysis. Raised levels of Bcl-X_L expression correlated with patients' gender and tumor cell proliferation index ($P = 0.037$ and $P<0.001$, respectively), but were independent of patients' age, tumor site, tumor size, grade of tumor cell differentiation, depth of tumor invasion, nodal or distant metastasis, and 5-year overall survival rate.

MATERIALS AND METHODS

Rat tissues

Sprague-Dawley rats weighing approximately 250 g (obtained from National Animal Center, Taiwan) were used in our pilot studies and fed with standard laboratory chow and water *ad libitum*. All procedures were performed in accordance with the Taipei Veterans General Hospital Animal Committee's guidelines for animal care. The rats were killed under anesthesia, and their organs were surgically taken and rinsed with phosphate-buffered saline (PBS). All specimens were cut into pieces, snap-frozen immediately and stored at -80 ℃ until use.

Patients and tumor specimens

Tumor specimens were obtained from 50 patients who received surgical resection for colorectal cancer at Taipei Veterans General Hospital during the time period of 1994-2004. Informed consent was obtained from each patient. Tissue specimens, both tumor and non-tumor parts, were frozen in liquid nitrogen immediately after resection and PBS rinse. All were kept in a -80 ℃ freezer until retrieval for the study. The non-tumor parts, taken from a site at least 10 cm away from the tumor part, were pathologically certified to be free from tumor cells.

Conventional grinder method for tissue lysis

Tissue specimens were ground in mortars with liquid nitrogen. The tissue powder obtained was then incubated overnight at -20 ℃ with 10% trichloroacetic acid plus 0.3% dithiothreitol (DTT) in acetone, then spun down, washed with acetone, and air-dried, followed by the addition of TLysis buffer, which is made up of 30 mmol/L Tris-HCl, pH 9.0, 9 mol/L of urea, 4% of CHAPS, 1% of DTT, 1 mmol/L of phenylmethylsulfonyl fluoride, 10 μg/mL of aprotinin, 10 μg/mL of pepstatin A, and 10 μg/mL of leupeptin. The mixture underwent three 20-s duration of homogenization on ice before further sonication at 10% output for 20 pulses. The lysate was then centrifuged at 100 000 *g* for 1 h at 4 ℃, and the supernatant was saved and assayed for protein concentration by the Lowry method[10].

MagNA Lyser method for tissue lysis

Tissue pieces were put into ceramic beads-containing special centritubes (Roche, Penzberg, Germany), 1 mL of pre-chilled TLysis buffer was immediately added, and subjected to oscillation made by the MagNA Lyser machine at 6 500 r/min for time periods as indicated. The lysate was then centrifuged at 100 000 *g* for 1 h at 4 ℃, and the supernatant was saved and assayed for protein concentration (Lowry method).

Two-dimensional (2D) gel electrophoresis

Prior to 2D gel electrophoresis, 2% of Ampholines/pharmalytes pH 3-10 (Amersham Biosciences, Piscataway, NJ, USA) was added to the tissue lysates. Immobilized linear pH gradient (IPG) strips were rehydrated with tissue lysate samples in the dark at room temperature overnight[11]. Isoelectric focusing was performed using IPG phor apparatus (Amersham Biosciences) for a total of 17 500 Vh at 20 ℃. The strips were then equilibrated for 15 min in 50 mmol/L Tris-HCl, pH 8.8, 6 mol/L urea, 2% (w/v) sodium dodecyl sulfate (SDS), 64 mmol/L DTT, 300 mL/L glycerol, and a trace of Bromophenol blue. Equilibrated IPG strips were transferred onto 12% uniform polyacrylamide gels. Standard SDS-polyacrylamide gel electrophoresis was performed, and the 2D gels were then stained with Coomassie blue and scanned directly using AlphaImager 2000 (Alpha Innotech Co., Avery Dennison, CA, USA). The images were further analyzed using ImageMaster 2-D Elite v3.01 software (Amersham Biosciences).

Protein spot identification

Spots of interest were manually excised from the 2D gels. After washing twice in 25 mmol/L ammonium bicarbonate/50% acetonitrile and once in 100% acetonitrile, gel pieces were vacuum-dried and rehydrated with 10 μg/mL of trypsin (Promega, Madison, WI, USA) in 25 mmol/L ammonium bicarbonate, pH 8.0. Proteins were proteolysed for 16-24 h at 37 ℃. Supernatants were collected, and the peptides in gel pieces were further extracted with 5% trifluoroacetic acid (TFA)/50% acetonitrile. The peptide extracts were vacuum-dried and re-solubilized in 5 μL of 0.1% TFA. The peptide extracts (1 μL for each) were then spotted onto the target plate, and α-cyano-4-hydroxycinnamic acid was added as a matrix (1 μL for each). Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry was performed using M@LDI *R*™ reflector time-of-flight mass spectrometer (Micromass, Manchester, UK). All mass spectra were internally calibrated with ACTH peptide and trypsinized alcohol dehydrogenase peaks. Peptide mass mapping was carried out using the MS-Fit program (Protein Prospector, University of California, San Francisco, CA).

Immunoblot analysis

The expression levels of Bcl-X_L and Ki-67 in human tissue lysates were detected by conventional immunoblot analysis[12]. In brief, 40 μg of protein samples were resolved in 10% SDS-polyacrylamide gels and then electrotransferred onto

polyvinylidene membranes (Amersham Biosciences). After blocking with PBST (PBS plus 0.1% Tween-20) plus 5% non-fat milk, the blots were incubated with anti-Bcl-X_L (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-Ki-67 (NeoMarkers, Fremont, CA, USA) antibody at 4 ℃ for 12 h. The blots were then washed thrice with PBST buffer, and incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Blots were again washed thrice with PBST buffer, and the protein band signals were obtained by enhanced chemiluminescence (ECL, Amersham Biosciences). The levels of β-tubulin in the tissue lysates were also analyzed as internal control. The antibody against β-tubulin was obtained from Santa Cruz Biotechnology.

Statistical analyses

Statistical analyses were performed using the SPSS 11.0 software (SPSS, Chicago, IL, USA). χ^2 or Fisher's exact test was used to analyze the relationship between Bcl-X_L expression and patients' clinicopathological characteristics. Independent sample *t* test was used to compare the ages and tumor sizes between the patients with or without Bcl-X_L overexpression. Overall survival rates were calculated by the Kaplan-Meier method and the statistical comparison was made using the log rank test. The difference was considered significant if the *P* value was less than 0.05 (two-tailed test).

RESULTS

Determination of conditions for tissue lysis using the MagNA Lyser

Different organs derived from the Sprague-Dawley rats were utilized as test tissue materials for a series of pilot studies. First, we intended to determine the optimal tissue size for lysis by MagNA Lyser. For each organ, we used four pieces of tissue specimens ranging from 100 to 600 mg. Tissue lysis was performed by setting the oscillation rate to 6 500 r/min durations of 20, 40, 80, 120, or 180 s. Evaluation of the lysing efficiency was preliminarily based on protein recovery rates. From the data shown in Figure 1, we realized that there was a ceiling limit to the size of tissue specimen tested, because the larger tissue pieces usually resulted in a low protein recovery rate. The big tissue samples were probably

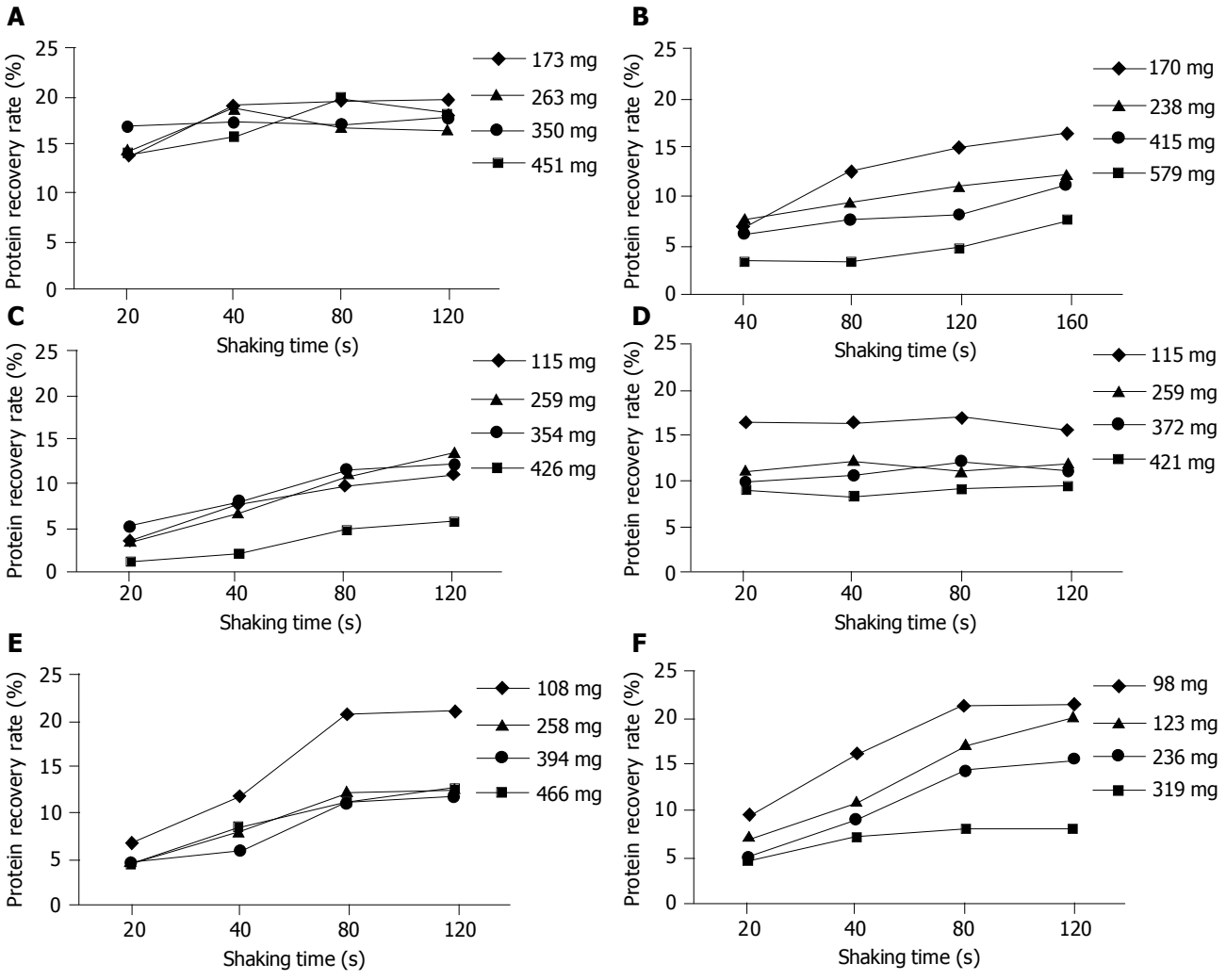

Figure 1 Determination of the optimal specimen size from different organs for tissue lysis by the MagNA Lyser. Four specimen sizes, ranging from 100 to 600 mg, of rat organs were tested for the protein recovery rates from the new tissue lysis method using the MagNA Lyser. The oscillation frequency and duration was set at 6 500 r/min for 20, 40, 80, 120, or 180 s as indicated. Protein concentrations of tissue lysates were assayed by the Lowry method. The protein recovery rate from tissue lysis was calculated as the amount of total protein obtained divided by the original weight of the specimen. **A**: Liver; **B**: Stomach; **C**: Colon; **D**: Kidney; **E**: Spleen; **F**: Heart.

subjected to incomplete lysis and thus were being wasted. Up to 350 mg of liver and colon tissues could be processed efficiently, but in general, the optimal tissue size of each organ for this study should not be more than 200 mg.

Next, the optimal shaking duration was determined. Tissue pieces in sizes ranging from 150 mg to 200 mg were oscillated in MagNA Lyser for different time periods. The protein recovery rate after each time period is shown in Figure 2A. Based on these data, optimal shaking time set at 80 s resulted in almost maximal protein recovery rates from the various organ tissues. When shaking time extends beyond 20 s, the lysates unavoidably became heated. To ascertain the impact of heat on tissue proteins, the tissue lysates prepared after different shaking periods in the MagNA Lyser were further analyzed by 2D gel electrophoresis. As shown in Figure 2B, the 2D profiles of tissue lysates prepared by 80-s oscillation seemed not worse than those prepared from shorter duration oscillations.

MagNA Lyser method vs conventional grinder method

According to the above test results, for optimal protein extraction from tissue samples using the MagNA Lyser, tissue size should be no more than 200 mg, and oscillation frequency should be set at 6 500 r/min for 80 s. Furthermore, in comparing tissue extraction efficiency using the MagNA Lyser or the conventional grinding method, protein recovery rates obtained through the MagNA Lyser were significantly superior at tissue specimens weighing between 150 and 200 mg (Figure 3A). The profiles from 2D gel electrophoresis were similar between tissue lysates prepared through either method (Figure 3B). Some protein spots appear to differ on two profiles, this could not be entirely attributed to the two different methods used for protein extraction, and quality variation during the 2D gel electrophoresis could also contribute to the differences. From our series of quality control pilot studies, we conclude that the MagNA Lyser method is indeed a convenient and efficient method for protein extraction from tissue samples.

MagNA Lyser method compatible with ensuing proteomic and immunoblot analyses

After the previous pilot studies using rat tissues as test material, we attempted protein extraction from human tumor specimen obtained from a colon cancer patient using the MagNA Lyser. The tissue lysate was resolved by 2D gel electrophoreses and several protein spots on the 2D profile were identified after standard procedure, including trypsin digestion, peptide extraction and MALDI-TOF mass spectrometric analyses (Figure 4). Our results confirmed that tissue lysate prepared using the MagNA Lyser method was entirely adequate for subsequent protein identification by the current proteomic protocols.

In order to ascertain whether the novel tissue lysis method is also applicable to immunoblot analysis, we used the MagNA Lyser to prepare tissue lysates from paired tumor and non-tumor tissues obtained from 50 colorectal cancer patients, followed by performing immunoblot analyses to compare the levels of Bcl-X_L expression in these patients. The results from six patients are shown in Figure 5 as representative examples. Only one of 50 colorectal cancer patients exhibited down regulation of Bcl-X_L expression in their tumor tissues, whereas 30 patients (60%) had raised levels of Bcl-X_L expression in the tumor tissues rather than their adjacent non-tumor tissues. Statistical analyses revealed that elevated Bcl-X_L expression correlated with patients' gender and elevated Ki-67 expression (P = 0.037 and P<0.001,

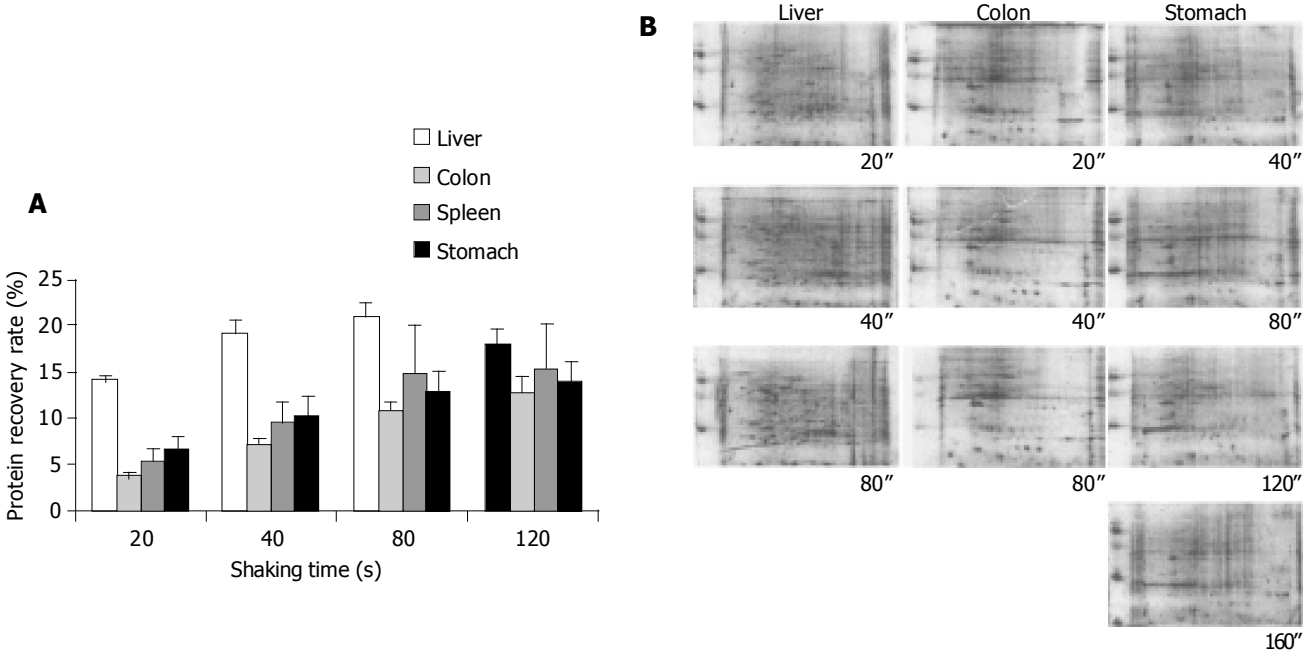

Figure 2 Determination of the optimal shaking time for tissue lysis by the MagNA Lyser. Tissue pieces in sizes ranging from 150 to 200 mg were subjected to reciprocal oscillation by the MagNA Lyser at 6 500 r/min for different time periods. Protein concentrations of tissue lysates were assayed, and protein recovery rates were calculated as above. Additionally, an aliquot (200 μg) of each tissue lysate was resolved on a 7-cm IPG strip, pH 3-10, then run in an 8×9-cm 12% SDS-polyacrylamide gel, and visualized by Alphalmager 2000 after staining with Coomassie blue dye. The optimal shaking time for tissue lysis by MagNA Lyser was determined according to both the protein recovery rates (**A**) and the protein profiling from 2D gel electrophoresis (**B**).

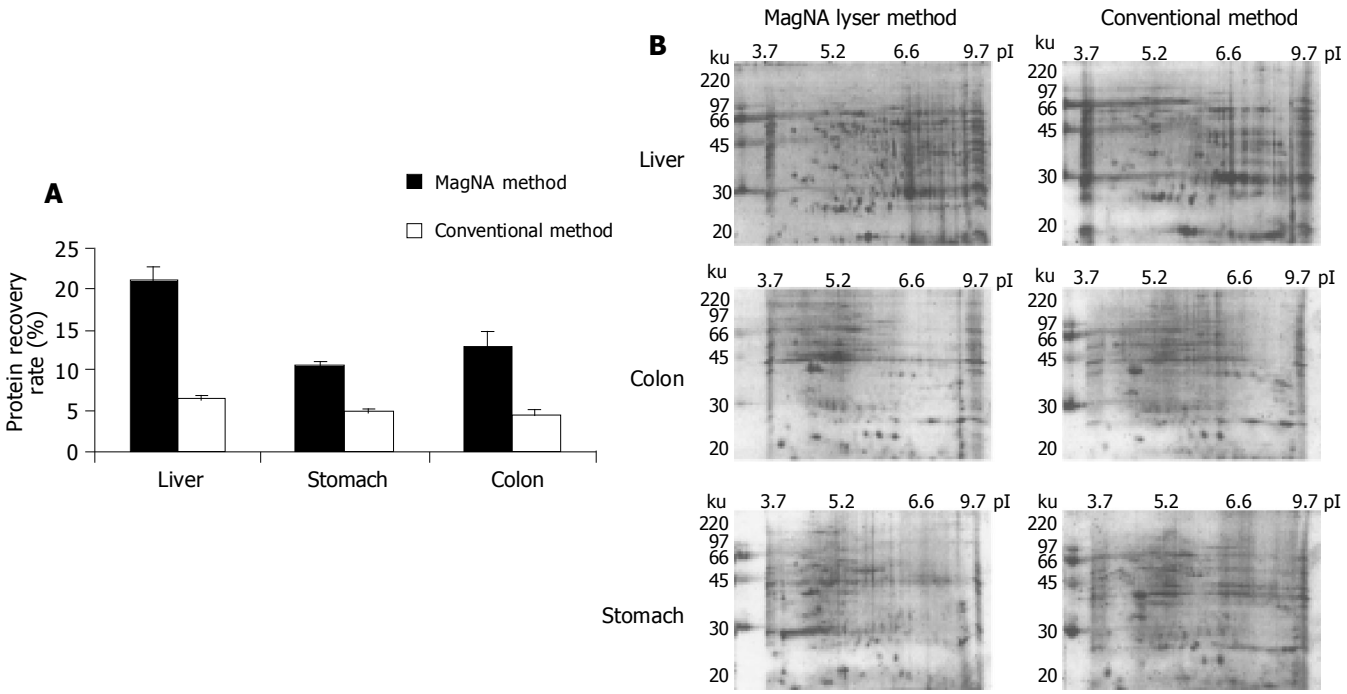

Figure 3 Comparison between conventional tissue lysis with the new method using MagNA Lyser. Tissue specimens ranging from 150 to 200 mg in size were processed using the conventional tissue lysis protocol and the new MagNA Lyser method. The protein recovery rates (**A**) and 2D gel profiling (**B**) were compared for the two methods.

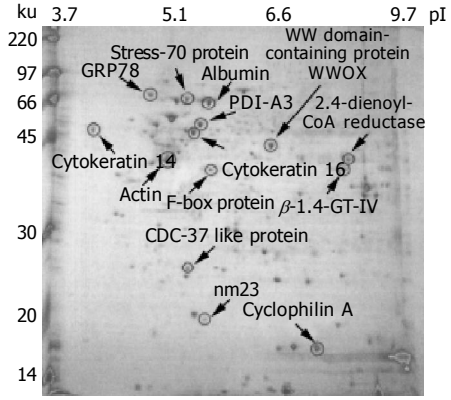

Figure 4 Proteomic analysis of the human colorectal cancer tissue lysate prepared by the MagNA Lyser. Approximately 150 mg of tumor specimen from a colon cancer patient was subjected to tissue lysis by the new MagNA Lyser method. One aliquot (600 μg) of tissue lysate was resolved on a 13-cm IPG strip, pH 3-10, and subsequently run in a 14 cm×15 cm 12% SDS-polyacrylamide gel, then visualized by staining with Coomassie blue dye. Protein spots were cut from the gel, after trypsin digestion and peptide extraction, the spots were identified through MALDI-TOF mass spectrometry, and have been labeled on the gel image.

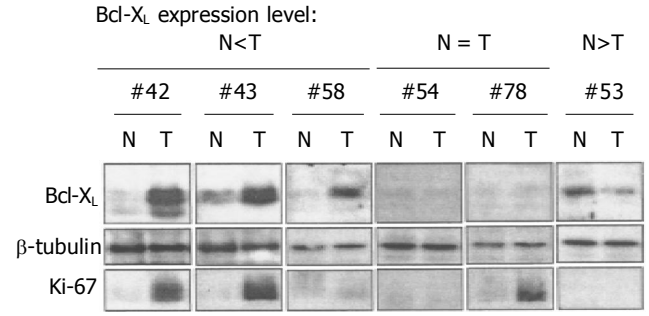

Figure 5 Differential expression levels of Bcl-X_L in colorectal cancer tissue specimens. Immunoblot analyses were performed to analyze the protein levels of Bcl-X_L in the lysates from nontumor tissues (N) and tumor tissues (T) of colorectal cancer patient #42, #43, #53, #54, #58, and #78. The levels of β-tubulin in the same-paired tissue lysates were analyzed as internal control.

respectively), but was not associated with patients' age, tumor site, tumor size, grade of tumor cell differentiation, depth of tumor invasion, lymph node or distant metastasis, and 5-year overall survival rate (Table 1).

DISCUSSION

Conventional methods for protein extraction usually require tedious grinding, homogenization and sonication steps to disrupt tissues and cells. A novel device, the MagNA Lyser (Roche, Germany) was recently designed and optimized to combine with different commercially available kits to facilitate the isolation of DNA and RNA from a variety of tissue samples, although without publication for protein extraction. In this study, we have firmly established the feasibility of using the MagNA Lyser to extract tissue proteins for subsequent proteomic and immunoblot analyses. Firstly, we exploited different organs of Sprague-Dawley rats as test samples in a series of pilot studies to determine the optimal tissue sizes for study as well as the time periods necessary for oscillation. The results suggest that the optimal tissue size should be no more than 200 mg, with oscillation frequency set at 6 500 r/min for 80 s. In addition, our data showed that protein recovery rates of the MagNA Lyser method were superior, when compared with those obtained through the conventional method, and visualization of the protein profiles provided by 2D electrophoreses showed both

Table 1 Relationships between elevated Bcl-X_L expression and clinicopathological characteristics

	Elevated Bcl-X_L expression		
	Yes (n = 30, %)	No (n = 20, %)	P
Age (mean±SD, yr)	68.1±12.1	70.9±9.5	0.383
Gender (male/female)	21/9	19/1	0.037
Tumor size (mean±SD, cm)	4.8±1.8	5.1±2.5	0.631
Tumor site			0.347
Ascending colon	10.0	10.0	
Transverse colon	3.3	5.0	
Descending colon	10.0	5.0	
Sigmoid colon	13.3	40.0	
Rectum or rectosigmoid junction	60.0	35.0	
Multiple sites	3.3	5.0	
Elevated Ki-67 expression	66.7	15.0	0.000
Tumor cell differentiation			1.000
Well differentiated	6.7	5.0	
Moderate differentiation	93.3	95.0	
Poor differentiation	0.0	0.0	
TNM stage			0.640
I	10.0	5.0	
II	33.3	35.0	
III	40.0	30.0	
IV	16.7	30.0	
Tumor invasion depth			0.938
Submucosa	0.0	0.0	
Muscularis propria	13.3	10.0	
Subserosa	76.7	80.0	
Other organs or structures	10.0	10.0	
Lymph node or distant metastasis	56.7	60.0	0.815
Five-year overall survival rate	60.4	60.0	0.701

superior quality as well as quantity in protein extracted.

Completion of pilot studies in rats established preliminary conditions of study for human specimen. Study of the first colon cancer patient identified several proteins by 2D gel electrophoresis and MALDI-TOF mass spectrometry, suggesting that the MagNA Lyser method can foster clinical proteomic studies to assist us in the understanding of diverse processes of carcinogenesis, develop new biomarkers for diagnosis and early detection of cancer, and ultimately determine new therapeutic targets for future therapeutic manipulation. In addition, the MagNA Lyser method is adequate for large-scale tissue lysate preparation, which can be linked with ensuing immunoblot analyses to facilitate the rapid detection of cancer protein alterations in large-scale cancer tissues. As an example, we detected elevated Bcl-X_L expression in colorectal cancer patients. Further statistical analyses revealed that raised level of Bcl-X_L expression correlated with elevated Ki-67 expression (P<0.001), providing clinical evidence that abnormal Bcl-X_L expression contributed to tumor cell proliferation during colorectal cancer progression. The potency of tumor cell proliferation does not only depend on tumor cell growth, but also associates with tumor cell survival (e.g. anti-apoptosis) and cell cycle control[5-7]. However, the close correlation between elevated Bcl-X_L expression and tumor cell proliferation did not extend to enlargement of tumor size (P = 0.631), probably because the formation of tumor mass requires the involvement of other events like angiogenesis.

Bcl-2 family proteins comprise both pro-apoptotic members, e.g. Bax, Bak, BH3-only proteins, as well as anti-apoptotic molecules such as Bcl-2 and Bcl-X_L[5-7]. BH3-only proteins function as mediators of apoptotic response to elicit activation and translocation of Bax or Bak to the mitochondria, thereby facilitating Bax and Bak interaction with mitochondrial channels to release cytochrome c[13]. Bcl-2 and Bcl-X_L sequester Bax or BH3-only proteins and thus block this apoptotic process[14]. Much evidence indicates that induction of apoptosis is accompanied by an increase in Bax or Bak expression and/or a decrease in the expression of Bcl-2 or Bcl-X_L[15-18]. Overexpression of Bcl-2 or Bcl-X_L can prevent apoptotic cell death induced by diverse events including abnormal expression of p53 or c-Myc and exposure to chemotherapeutic agents[19-22]. In contrast, down-regulation of Bcl-2 or Bcl-X_L by antisense, siRNA or ribozymes sufficiently sensitize various types of cells destined for apoptosis[23-26]. Clinically, a characteristic feature of follicular lymphoma is the t(14;18) translocation, which brings *bcl-2* under the control of the immunoglobulin heavy chain gene locus leading to elevated Bcl-2 expression[27]. However, a survey of 30 colorectal carcinoma and 24 adenomatous polyps patients suggested that a transition from Bcl-2 to Bcl-X_L expression might occur during progression of colorectal tumors[8]. Only 10% of colon carcinoma patients had increased levels of Bcl-2 in tumor tissues rather than adjacent normal colonic mucosa, and in fact 83% of patients exhibited down regulation of Bcl-2 expression in their tumors[8]. But instead, the level of Bcl-X_L expression was increased in 18 (60%) of 30 colon carcinoma patients. Besides, there

was another study showing elevation of Bcl-X_L expression in 43% of 58 colorectal carcinoma patients[9]. These results were all obtained by immunohistochemical staining. In this study, we exploited the new tissue lysis method and immunoblot analysis to detect 30 (60%) of 50 colorectal carcinoma patients with elevated Bcl-X_L expression. This data compares well with previous immunohistochemical staining data.

Our study also suggests that elevated Bcl-X_L expression preferentially occurred in female colorectal cancer patients ($P = 0.037$). The underlying pathophysiological basis is unclear, but it could be partly attributed to the findings that Bcl-X_L expression can be regulated by female hormones estrogen and progesterone[28,29]. Further investigation is needed to elucidate the relationship between gender and elevated Bcl-X_L expression.

In conclusion, we report a rapid and efficient tissue lysis method compatible with subsequent proteomic and immunoblot analyses, which can facilitate the discovery or detection of cancer gene alterations at the protein level.

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