The Proteomics Approach to Find Biomarkers in Gastric Cancer

Gastric cancer is a very serious disease and is naturally resistant to many anticancer drugs. To reduce the mortality and improve the effectiveness of therapy, many studies have tried to find key biomarkers. Proteomic technologies are providing the tools needed to discover and identify disease-associated biomarkers. The proteomic study of gastric cancer establishes any specific events that lead to cancer, and it provides a direct way to define the true function of genes. Using two dimensional (2-D) electrophoresis of the stomach cancer tissue, we have gained about 1,500 spots in each gel, and 140 protein spots also were identified. Among the identified proteins, there were seven over-expressed proteins in stomach cancer tissue: NSP3, transgelin, prohibitin, heat shock protein (hsp) 27 and variant, protein disulfide isomerase A3, unnamed protein product and glucose regulated protein. There were also seven under-expressed proteins in stomach cancer: Apolipoprotein A-1, p20, nucleoside diphosphate isomerase A, alpha 1 antitrypsin, desmin, serum albumin and serotransferrin.

Key Words: Proteomics; Stomach Neoplasms; Electrophoresis, Gel, Two-Dimensional

INTRODUCTION

Gastric cancer is the most common cancer in Korea. It is an unfortunate reality for many Koreans. It has been a very serious disease and is naturally resistant to many anticancer drugs, so the results after surgery and chemotherapy have been disappointing. To reduce the mortality and improve the effectiveness of therapy, many studies have tried to find key biomarkers. Biomarkers are important molecular signposts of the biologic state of a cell at a specific condition. Active genes, their respective protein products, and other organic chemicals made by the cell create these signposts. Proteomic technologies are providing the tools needed to discover and identify disease associated-biomarkers.

After the completion of human genome project, the practice of medicine will be altered fundamentally, which includes the identification of genes (genomics) that are involved in the appearance, progression, and treatment of cancer, the answers to what those specific genes do and how they interact in communication networks (functional genomics), and the roles played by their protein products (proteomics) in molecular pathways.

There have been many studies including analysis of prognostic factors, development of chemotherapeutic agents, and the search for the early detecting molecules, but there are currently very few molecular markers that are clinically in use. Proteomics is one of the technologies that rapidly change our approach to cancer research. The conventional proteomics has been the high resolution 2-D gel electrophoresis (1) followed by computational image analysis and protein identification using mass spectrometry. The proteomic studies could lead to the molecular characterization of cellular events associated with cancer progression, signaling, and developmental stages (2, 3), and these studies with clinical tumor samples have led to the defining of cancer-specific protein markers which will be the basis for developing new methods for early diagnosis and detection and clues to understand the molecular characterization of cancer progression (3-6).

In this study, we tried to find biomarkers in human gastric cancer tissue via proteomics as high throughput method.

MATERIALS AND METHODS

Materials

Eleven human stomach tissue samples were prepared from resection materials of cancer patients in Dankook University

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Received : 8 December 2002
Accepted : 4 April 2003

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*This paper was supported in part by the grant of Bio-medical research center of Medical College of Dankook University (to JW Ryu).
Hospital. Resections were examined by a pathologist and normal tissue samples were prepared from noncancerous regions. The margin tissue samples were prepared from noncancerous regions 5 cm apart from primary tumor. Their clinico-pathologic characters were described in Table 1.

The power supply, the 2D electrophoresis system (PROTEAN IEF cell, PROTEAN II Xi cell), the Trans-Blot Cell, CHAPS, DTT, SDS, iodoacetamide, ampholyte, and IPG (Immobilized pH gradient) and strip were purchased from Bio-Rad (Richmond, CA, U.S.A.). DNase and the protein pI marker were from Amersham-Pharmacia (Bucks, U.K.). Acrylamide, N,N'-methylene-bis-acrylamide, TEMED, ammonium persulfate, Trisma base, glycine, methanol, glacial acetic acid, glycerol, potassium chloride, urea, thiourea, phenylmethyl-sulfonylfluoride (PMSF), ethylenedinitrilotetraacetic acid disodium salt (EDTA), benzamidine, and brilliant blue R-250 were from Sigma-Aldrich (Missouri, U.S.A.).

**Sample Preparation**

Proteins were extracted from human stomach tissue as instructed by German Heart Center method (http://userpage.chemie.fu-berlin.de/~pleiss/tissue.html). Stomach tissue was washed in 50 mM Tris-HCl pH 7.1, 100 mM KCl, 60 mM EDTA, 5.8 mM benzamidine and 0.2 mM PMSF, and was lyophilized for 1hr. Samples of 30 mg were crushed by liquid nitrogen and were solubilized in 100 µL buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 100 mM DTT, 25 mM tris-HCl pH 7.1, 50 mM KCl, and 0.2% Bio-Lyte 3/10 ampholyte. DNase was added to 210 U and was incubated at room temperature for 30 min. Insoluble materials were removed by centrifugation at 13,000 rpm for 20 min at 10°C. Protein concentration was determined using a commercial Bradford reagent (Bio-Rad) and the samples were stored at -70°C until use.

**Two-dimensional Electrophoresis**

The first-dimensional gel separation was carried out with 17 cm pH 4-7 IPG strips following the manufacturer's protocol (Bio-Rad) with minor modifications. 1 mg of the protein was loaded and Iso-electric focusing (IEF) was performed using PROTEAN IEF Cell for a total of 145 kVh at 20°C. After IEF, strips were equilibrated for 15 min in 6 M urea, 2% SDS, 0.05 M Tris-HCl pH 8.8, and 20% glycerol containing 2% DTT, and then equilibrated again for 15 min in the same buffer containing 2.5% iodoacetamide. Equilibrated IPG strips were transferred onto 13% uniform polyacrylamide gels and then were run in PROTEAN II xi cell tank at 30 mA per gel. The gels were visualized using the Coomassie brilliant blue R250 staining method.

After the staining, 2D gels were imaged using Powerlook 1,100 (UMAX, Fremont, CA, U.S.A.) and the images were analyzed using Melanie III (Genebio, Geneva, Switzerland).

**Protein Identification**

Proteins that were differentially expressed in the tumor tissue were identified using MALDI-TOF mass spectrometry (Yonsei Proteome Research Center) and database comparisons (Gyeongsang National Univ). Proteins were identified using MALDI-TOF mass spectrometry (Yonsei Proteome Research Center) and database comparisons (Gyeongsang National Univ).
RESULTS

Two-dimensional electrophoresis of the stomach cancer tissue produced about 1,500 spots each. A electrophoresis feature is shown in Fig. 1. In this figure, all proteins that have been identified to date have been marked with arrows and accession number. Spots marked with circles and arrows are differentially expressed proteins in normal, margin, and cancer tissues (Fig. 2). In this study, we compared cancer tissue with normal tissue to obtain definite differential expressions of protein between them. A comparison of stomach cancer tissue with normal tissue in same patients showed the seven proteins, namely: NSP3, transgelin, prohibitin, heat shock protein (hsp) 27 and variant, protein disulfide isomerase A3, unnamed protein product, and glucose-regulated protein were over-expressed (Table 2). The seven proteins, namely: apolipoprotein A-1, p20, nucleoside diphosphate isomerase A, alpha 1 antitrypsin, desmin, serum albumin, and serotransfemin were under-expressed (Table 3).

Table 2. Over-expressed Proteins in Human Stomach Cancer Tissue

| Acc. #      | Apparent pI/Mw (kDa) | Protein Identified | Abundance Level | Calculated pI/Mw (kDa) |
|-------------|----------------------|--------------------|----------------|-----------------------|
| BAA84968    | 5.22/35.74           | NSP3              | 2.7            | 4.66/34               |
| Q01995      | 8.90/22.60           | Transgelin (SM22-alpha) | 2.2           | 4.79/36               |
| P35232      | 5.57/29.89           | Prohibitin         | 2.0            | 5.67/31               |
| P04792      | 7.83/22.33           | Heat-shock 27kDa protein | 5.3           | 6.19/30               |
| P30101      | 6.00/56.78           | Protein disulfide isomerase A3 | 3.2           | 6.07/29               |
| BAB70985    | 7.23/42.45           | Unnamed protein product | 2.5           | 5.47/66               |
| NP_053955   | 6.87/28.44           | Glucose regulated protein, 58 kDa | 2.5       | 6.05/64               |

Acc.#: accession numbers from SWISS-PROT database.

Table 3. Under-expressed Proteins in Human Stomach Cancer Tissue

| Acc. #      | Apparent pI/Mw (kDa) | Protein Identified | Abundance Level | Calculated pI/Mw (kDa) |
|-------------|----------------------|--------------------|----------------|-----------------------|
| P02647      | 5.56/30.78           | Apolipoprotein A-1 | 2.4            | 5.43/28               |
| B53814      | 6.00/17.17           | P20                | 1.4            | 6.43/22               |
| P15531      | 6.00/22.00           | Nucleoside diphosphate isomerase A | 3.3       | 5.38/17               |
| P01009      | 5.37/46.74           | Alpha-1-antitrypsin | 1.7           | 5.10/62               |
| P17661      | 5.21/53.39           | Desmin             | 3.2            | 5.52/59               |
| P02768      | 6.18/80.00           | Serum albumin      | 6.4            | 5.92/69               |
| P02787      | 6.81/77.05           | Serotransfemin     | 4.2            | 6.59/91               |

Acc.#: accession numbers from SWISS-PROT database.

Fig. 2. 2DE patterns showing the under-expression of desmoplakin I (indicated by arrows) and up-regulation of prohibitin (indicated by circles) in stomach cancer tissue samples (G-I) as compared to normal (A-C) and margin stomach tissue samples (D-F).
ferrin were under-expressed (Table 3).

**DISCUSSION**

Recent advances in genomics and proteomics hold great potential for diagnostic, prognostic, and therapeutic applications (7). Genes work at the protein level, and comparisons of transcripts and corresponding protein expressions have shown that mRNA and protein levels are not necessarily highly correlated. The proteomic study includes post-translational modifications such as acetylation, ubiquitination, phosphorylation, or glycosylation (8, 9). Proteomic methods detect the functioning units of expressed genes (10) using protein fingerprint (11). The proteomic results express both the intrinsic genetic effect on cell and the impact of its environment, so it is very valuable to determine biomarkers.

Today, it has become common to read that proteins are the main functional outputs of genes, and the proteomics will lead biology and medicine beyond genomics. The molecular events into neoplastic progression are complex and diverse, and they remain incompletely characterized. The identification, quantification, classification, and functional assignment of proteins will be essential to the full understanding of these molecular events. Such information will likely prove to be crucial in cancer prognosis, diagnosis, prevention and therapy with the ultimate goods being therapeutic target discovery, rational drug design and the identification of early detection surrogate biomarkers (12, 13).

In this study, we have evaluated our 2-D data from reference 2-D map (from Gyeongsang National University, Jinju, Korea). The over- and under-expressed proteins were confirmed by MALDI-TOF mass spectrometry (Yonsei Proteome Research Center, Seoul, Korea). There are useful URLs for other cancer, bladder, colorectal, breast, liver, and leukemia, but there is little data for stomach cancer (14). We obtained seven over-expressed proteins (NSP3, transgelin, prohibitin, HSP27, protein disulfide isomerase A3, unnamed protein product and glucose regulated protein) in cancer. In thermoresistant human gastric cancer cell line study, stratifin, cytokeratin 7-9, calreticulin, glucose regulated protein 78, and heat shock proteins (hsp27, hsp70 variant, hsp 27 variant) were identified as over-expressed protein (15). They declared that the development of thermoresistance in stomach cancer cell and their chemo-resistance variants is a complex phenomenon that is not only associated with the expression of HSPs but of molecular chaperones at every subcellular level. In our study, the cytoplasmic proteins (transgelin, prohibitin, and HSP 27) were identified as over-expressed proteins which are molecular chaperones. Also, we obtained seven under-expressed proteins (apo-lipoprotein A-1, p20, nucleoside diphosphate isomerase A, alpha-1-antitrypsin, desmin, serum albumin, and serotransferrin) in human gastric cancer tissue. The extracellular proteins (serum albumin, serotransferrin, and alpha-1-antitrypsin) were identified as under-expressed proteins in cancer tissue.

In thermoresistant cancer cell line study, eight proteins (14-3-3, aldehyde dehydrogenase 1, annexin 1, lipocortin 1 variant, initiation factor-5A, initiation factor-5A variant, reticulocalbin, and vimentin) were reported as under-expressed proteins (15).

We analyzed primary tumor biopsies and normal tissues in same patients. The primary tumor biopsies may contain several different cell types other than carcinoma cells, so the exact defining for major cellular changes during the conversion of normal to malignant stomach is limited. It is related to defects in a multiplicity of 2-D features. On the contrary cell line in culture, being free of any tissue components may have multiplicity, but may not represent their exact feature in vivo counterpart. Clearly, more work and data are required to understand and clarify the exact role of proteins for progression of carcinoma.

In this study, we observed the over- and under-expressed proteins in human gastric cancer tissue. We hope that these results shall be correlated with clinical data in recent forthcoming day to define clinically useful biomarkers. The identification of functioning protein is essentially needed to provide more effective therapy for patients suffering from gastric carcinoma.

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