Application of sensitive fluorescent dyes in linkage of laser microdissection and two-dimensional gel electrophoresis as a cancer proteomic study tool

The combination of laser microdissection and two-dimensional gel electrophoresis (2-D PAGE) has been developed to perform proteomic analysis on specific populations of cells in cancer tissues. However, as conventional low sensitivity silver staining was used for spot detection, the microdissection required to obtain an adequate amount of protein for 2-D PAGE is laborious and only a restricted number of protein spots could be visualized. As a consequence, this technology was impractical for direct clinical applications and had a limited impact on cancer studies. To solve these problems, we developed an application in which fluorescent dyes label the proteins extracted from microdissected tissues prior to 2-D PAGE separation. In this application, a small amount of protein, less than 6.6 µg, was enough to generate a 2-D profile with approximately 1500 protein spots. This technique was applied to compare the proteome of normal intestinal epithelium with that of adenoma in Min mice. Thirty-seven protein spots reproducibly showed significant differences in intensities. Mass spectrometric analysis and Western blotting identified eight of them, including prohibitin, 14-3-3zeta, tropomyosin 3 and Hsp84. These results indicate that fluorescence labeling of proteins from microdissected tissues prior to 2-D PAGE is a powerful cancer proteomic study tool.

Keywords: Adenoma / Fluorescence two-dimensional difference gel electrophoresis / Laser microdissection / Min mice / Proteome PRO 0531

1 Introduction

Many lines of evidence have revealed that genetic alterations cause the transformation of cells. Small genetic mutations activate oncogenes or inactivate tumor suppressor genes, resulting in changes in a series of signal transduction pathways, DNA repair, apoptosis, and cell mobility, ultimately causing normal cells to develop into fully malignant tumor cells. Although the initial changes occur at the DNA level, the effects are expressed as the protein content of the cells and the proteins are entities reflecting the genetic alterations. mRNA expression is often studied to follow changes in cells, in an effort to understand the mechanisms behind cancer progression. However, a recent global comparative study of mRNA and protein expression in lung cancer revealed that only a subset of mRNA exhibited a significant correlation with protein abundance [1]. In addition, many DNA mutations result in changes in post-translational modifications, which play important roles in cancer biology and these alterations cannot be predicted from mRNA information. Therefore, monitoring protein expressions in a global manner, namely conducting a proteomic study, is the optimal means of following changes in the cells associated with cancer development.

Cancer tissues are composed of multiple subpopulations including nontumor cells such as normal epithelial counterparts of cancer cells, stromal cells, inflammatory cells and angiogenic elements. Each population has its own gene and protein expression profiles that change during the course of cancer development with dynamic environmental interactions. To perform accurate expression analysis of tumor cells and their counterparts, isolation of individual cell populations is considered very important to assess mechanisms of cancer biology and to develop diagnostic markers. Laser microdissection (LMD) was developed to collect specific populations of cells under direct microscopic observation. LMD has been coupled with many proteomic technologies including antibody array [2], reverse phase array [3], surface enhanced laser
desorption/ionization-time of flight mass spectrometry (SELDI-TOF MS) [4–6], immunoassay of biomarkers [7–10] and two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) [4, 11–15]. Among these proteomic technologies, 2-D PAGE has unique advantages that make it possible to examine hundreds of proteins simultaneously estimating their expression and post-translational modifications quantitatively. In addition, mass spectrometric analysis enables identification of interesting protein spots directly from 2-D gels [16] and bio-informatic tools allow integrating 2-D images information to databases [17, 18]. Although the combination of LMD and subsequent proteomic analysis by 2-D PAGE appears to be a promising technology for cancer proteomic study, the limited sensitivity of silver staining used for protein visualization has seriously hindered the efficiency of data output. In a typical proteomic study using LMD followed by 2-D PAGE separation and silver staining, laser captured microdissection (LCM) (Arcturus Engineering, Mountain View, CA, USA) is employed to isolate specific populations of cells. While LCM allows the collection of a single cell from heterogeneous tissues [19], obtaining an adequate amount of protein for 2-D PAGE from LCM was tremendously time-consuming, taking from 3 h up to 4 d, and the total number of protein spots was between 470 and 930, depending on tissue type [11]. These workflow features are far from the current necessity of high throughput analysis and make direct clinical application impractical. More sensitive methods to visualize the protein spots would decrease the required amount of protein for 2-D PAGE and diminish the time consumed for microdissection. Recently, Zhou et al. [20] labeled the protein extracted from microdissected tissues with fluorescent dyes manufactured by Amersham Biosciences (Little Chalfont, Buckinghamshire, UK), which react with lysine residues in proteins, prior to 2-D PAGE. However, because the sensitivity of the dyes was essentially equal to that of silver staining, the total number of protein spots obtained remained less than 1000, and the dissection time was not shortened according to the reported number of laser shots.

In the present report, to solve these problems, we applied sensitive fluorescent dyes, which react with thiol groups of cysteine residues, to label the proteins extracted from microdissected tissues. LCM was replaced by a noncapture type of microdissector, the Leica microdissection system (Leica, Milton Keynes, UK). While Leica’s LMD does not isolate a single cell, it facilitates collection of larger areas of tissue, which is required for proteomic study. This application enabled instant microdissection for 2-D PAGE and raised the total number of protein spots to 1500. Proteomes of normal intestinal epithelium and adenoma tissues in Min mice were compared, and mass spectrometric analysis and/or specific antibodies identified the spots of interest. We conclude the application of highly sensitive fluorescent dyes to the linkage of LMD and 2-D PAGE to be a powerful cancer proteomic study tool.

2 Materials and methods

2.1 Tissue preparation and staining

Min mice were sacrificed and intestinal tissue with adenoma was immediately embedded in OCT compound (Sakura Finetechnical, Tokyo, Japan), snap frozen in liquid nitrogen and stored at −80°C until use. The OCT embedded tissue blocks were cut into 10 μm thick sections using a Leica CM 3050 S (Leica, Milton Keynes, UK). The sectioned tissues were placed on a membrane-coated slide glass (Leica) pretreated with tissue adhesive solution, 0.1% poly-L-lysine (Sigma, St. Louis, MO, USA). The steps of eosin staining and xylene dehydration for standard H&E staining were omitted to improve the recovery of proteins from dissected tissues. The sectioned tissues were fixed with 95% ethanol for 30 s and washed briefly in water. After soaking in Mayer’s hematoxylin (Muto Pure Chemicals, Tokyo, Japan) diluted 10 times with water for 1 min, they were washed twice in 95% ethanol and with water for 10 s. The neighboring section was occasionally stained with standard H&E to confirm the diagnosis. All staining procedures were carried out on ice. The area of tissue to be microdissected was identified under microscopic observation and recorded using Leica Laser Microdissection V3.1.0.0 (Leica) and laser microdissection was then performed using a Leica AS LMD.

2.2 Protein extraction and fluorescent dye labeling

Microdissected normal epithelium and adenoma tissues were solubilized in 50 μL of lysis buffer (LB) containing 6 M urea, 2 M thiourea, 3% CHAPS and 1% Triton X-100. The concentration of proteins was measured by ProteinAssay kit (Bio-Rad, Hercules, CA, USA). The protein lysates were labeled with Cy3 or Cy5 dyes, which were designated as saturation cysteine dye (Amersham Biosciences). The pH of the protein lysates was adjusted to 8.0 using 50 mM Tris-HCl and the proteins were then reduced by incubation with 40 mM Tris-(2-carboxyethyl) phosphine hydrochloride (TCEP)(Sigma) for 60 min at 37°C. Each sample was divided into two tubes and labeled with 40 nM each of Cy3 or Cy5 dyes for 30 min at 37°C. Cy3-labeled samples of normal and adenoma
Figure 1. Schematic workflow of sample preparation for quantitative analysis. Protein lysates from microdissected tissues are labeled with fluorescent dyes of different wavelengths of excitation and emission. Cy3-labeled samples are simultaneously mixed and divided into Cy5-labeled samples. Then, mixtures of Cy3- and Cy5-labeled lysates are coseparated by 2-D PAGE. The gel is scanned with two wavelengths, each specific either for Cy3- or Cy5-dye.

2.3 Two-dimensional gel electrophoresis

The first dimension separation was performed using immobilized pH gradient (IPG) gels (Amersham Biosciences) as recommended by the manufacturer. Briefly, IPG gels (length 24 cm and pI range 3–10) were rehydrated with Cy-labeled samples prepared as described in Section 2.2 at 20°C for 12 h. IEF was performed using IPGphor (Amersham Biosciences) for a total of 80 KVh at 20°C. The IPG gels were then equilibrated for 15 min in 50 mM Tris-HCl, pH 8.8, 3 mM urea, 30% v/v glycerol, 1% SDS containing 0.5% DTT and transferred onto 9–16% gradient gels, 25 cm × 20 cm in size, made between low-fluorescence glass plates. The IPG gels were then sealed with 0.5% w/v low melting point agarose (Amersham Biosciences) in running buffer containing bromphenol blue. Gels were run in an Ettan Dalt II (Amersham Biosciences) at 18 W for 12 gels at 18°C for 15 h and 20 min. Each sample was run on triplicate gels and the average spot intensities were calculated for quantitative comparison.

2.4 Scanning and image analysis

2-D PAGE gels were scanned directly between glass plates with the appropriate wavelength for each CyDye using a 2-D 2920 MasterImager (Amersham Biosciences). The spots on the images were merged and quantified by Differential In-gel Analysis (DIA) mode of DeCyder software (Amersham Biosciences) and quantitative comparison was carried out by biological variation analysis (BVA) mode. Cy3 images generated from the mixture of Cy3-labeled samples were used as a control for quantification.

2.5 In-gel digestion and protein identification by MS

Mouse fibroblast cells, NIH 3T3, were maintained in MEM with 10% fetal bovine serum. Protein samples were extracted from the cells with LB. Five hundred μL of NIH 3T3 cell protein extract were labeled with Cy5 dye and subjected to 2-D PAGE separation. The gels were scanned at an appropriate wavelength for Cy5 dye and the spots were detected by the DIA-mode of DeCyder software as described in Section 2.5. The spots of interest in the analyzed gels were matched to the corresponding spots in the preparative gels. An automated spot cutter, SpotPicker (Amersham Biosciences), collected the spots of interest. For in-gel digestion, the gel plugs were washed with water twice for 5 min and incubated with 100% acetonitrile for 10 min. Then, the gels were dried completely for at least 30 min. The protein in the gel was digested by treatment with 100 ng TPCK-treated trypsin (Promega, Southampton, UK) in 50 mM ammonium bicarbonate at 37°C overnight with gentle agitation. After digestion, peptides were extracted with 50 μL of 50% acetonitrile/0.1% TFA twice, concentrated and extensively treated with ZipTip (Millipore, Bedford, MA, USA). Then trypsin digests were mixed with an equal volume of matrix solution, comprised of saturated dihydroxybenzonic acid (Sigma) in 50% acetonitrile/0.1% TFA. The mixture was spotted onto a MALDI target plate and subjected to mass spectrometric analysis. Mass spectrometric analysis of tryptic digests was performed using a Q-STAR Pulser-i equipped with an oMALDI ion source (Applied Biosystems, Framingham, MA, USA). Peptide mass finger printing was carried out using the Analyst QS program (Applied Biosystems).
2.6 Protein identification by Western blotting

One hundred μg of Cy5-labeled proteins were separated by 2-D PAGE and the gels were scanned with appropriate excitation and emission spectra for Cy5, as in Section 2.4. The proteins were transferred onto nitrocellulose membranes (Amersham Biosciences) and blotted with antibodies against prohibitin (Oncogene Research, San Diego, CA, USA), 14-3-3zeta (Santa Cruz Biotechnology, Santa Cruz, CA, USA), 14-3-3 (IBL, Fujioka, Gunma, Japan), tropomyosin (Chemicon, Temecula, CA, USA), and Hsp84 (Affinity BioReagents, Golden, CO, USA). The blots were detected using an ECL system (Amersham Biosciences) according to the manufacturer’s instructions. The ECL signal was detected using a LAS-1000 (Fujifilm, Tokyo Japan). The identification of spots that reacted with a specific antibody was confirmed by comparing the 2-D PAGE image generated by Cy5 fluor with that generated by the ECL signal.

3 Results

3.1 Strategy for quantitative comparison of protein profiles

We designed the experiment so that each spot could be quantitatively analyzed by running two samples in the same gel (Fig. 1). First, protein lysates of microdissected normal epithelial and adenoma tissue were labeled with saturation fluorescent dyes of Cy3 and Cy5 in separate tubes. Cy3-labeled protein extracts of normal epithelial and adenoma tissues were mixed to create the pooled standard sample and subsequently added to Cy5-labeled samples. The mixture of Cy3- or Cy5-labeled proteins was then coseparated in the same gel and 2-D profiles were obtained by scanning the gels at an appropriate wavelength for excitation and emission of each CyDye. The fluorescent dyes used were engineered so that the migration of a given protein labeled with Cy3- or Cy5-dye were identical in 2-D gels. For every spot, intensity differences observed in Cy3 images of different 2-D gels were considered to be artificial and attributable to electrophoresis, because Cy3 images were produced with a common Cy3-labeled protein mixture. The Cy5 image spot intensity was normalized by the intensity of the corresponding Cy3 image spot in the same gel. This approach allowed detection of small alterations in spot intensity, e.g. less than 20%, in a reproducible manner (manuscript in preparation). In addition, because the comparison was carried out on the spots that were labeled with the same dye, we avoided the effects of fluorescence resonance energy transfer, by which the fluorescence intensity of Cy3 and Cy5 is enhanced or quenched, respectively, for certain proteins when high density labeling is attained [21].

3.2 LMD and protein quantification in microdissected adenoma tissues

Frozen small intestinal tissues were sectioned, stained with hematoxylin and subsequently microdissected using the laser. Figure 2A shows adenoma tissue with standard

![Figure 2. LMD of adenoma tissues in Min mice. Sectioned adenoma tissues before and after laser-assisted microdissection. Adenoma tissue was stained with standard H&E (A) and a neighboring section was stained with hematoxylin for protein extraction (B). Adenoma tissue was microdissected for an area of approximately 2.9 mm² (C), which produced three 2-D PAGE images with approximately 1500 protein spots. (D) The amount of protein was measured in a certain area of sectioned tissue. Amounts of proteins contained in 1 mm² of microdissected tissue ranged from 2.7–6.6 μg.](image_url)
H&E staining. Neighboring sections were stained with hematoxylin, showing a clear border between normal and adenoma tissues (Fig. 2B). Figure 2C shows adenoma tissue after LMD. The area of microdissected tissue was 2.9 mm² and the time required for microdissection was less than 1 min. The amount of protein in the microdissected adenoma tissues was measured for various size areas (Fig. 2D), and showed that a 1 mm² area of microdissected adenoma tissue contained 2.7–6.6 μg of protein. Because the 2-D pattern generated from a 1 mm² area of microdissected tissue had a number of protein spots similar to that obtained from a larger area (approx. 1500, data not shown), we collected at least a 1 mm² area of microdissected tissue per one 2-D image for further studies.

3.3 Detection of altered protein spots in adenoma

Figure 3A is a representative 2-D gel pattern with Cy5-labeled proteins from a 1 mm² area of microdissected adenoma tissue. Approximately 1500 spots were detected and quantified by the DIA-mode of DeCyder software. Each sample was run on triplicate gels and average spot intensity in three gels, which were normalized to Cy3 images of the same gels, was compared in five experiments. The spots with altered intensity in at least four out of five independent experiments were taken as being significantly changed. Computer-assisted comparative analysis using the BVA mode of DeCyder software revealed ten spots to have decreased (marked by circles) and 27 spots increased (marked by hexagons) in intensity in adenoma tissues, compared with their normal counterparts (Fig. 3A). Boxed areas in Fig. 3A were enlarged (Fig. 3B for box X and Fig. 3C for box Y) to show spots marked by hexagons to be obviously increased in adenoma tissues (panel A in Fig. 3B and Fig. 3C) compared with their normal counterparts (panel N in Fig. 3B and Fig. 3C). The spots identified by mass spectrometric analysis and/or Western blotting are numbered in Fig. 3. The fold differences of the protein spots, which were identified by mass spectrometric analysis and/or Western blotting, between normal epithelium and adenoma tissues are summarized in Table 1.

3.4 Identification of protein spots by MS

Peptide mapping by mass spectrometric analysis was carried out for identification of protein spots. As the amount of protein from microdissected tissues was limited and insufficient for reliable mass spectrometric identifications, the protein spots of interest were matched to the corresponding spots in 2-D gels prepared from a large amount of protein from a mouse cultured cell line, NIH3T3 cells. With the assistance of computational processing, 2-D images were matched and the protein spots were extracted for mass spectrometric analysis. A mass spectrum of trypsin digests of spot 1 is presented in Figure 4A and peptide sequences matched to the amino acid...
Table 1. Identification of protein spots

<table>
<thead>
<tr>
<th>Spot no.</th>
<th>Protein name (Acc. No.)</th>
<th>Fold differences</th>
<th>Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Prohibition (P24142)</td>
<td>3.7</td>
<td>MS, WB</td>
</tr>
<tr>
<td>2</td>
<td>14-3-3</td>
<td>2.1</td>
<td>WB</td>
</tr>
<tr>
<td>3</td>
<td>14-3-3</td>
<td>1.4</td>
<td>WB</td>
</tr>
<tr>
<td>4</td>
<td>14-3-3z (P35215)</td>
<td>1.6</td>
<td>MS, WB</td>
</tr>
<tr>
<td>5</td>
<td>14-3-3z (P35215)</td>
<td>2.1</td>
<td>WB</td>
</tr>
<tr>
<td>6</td>
<td>14-3-3</td>
<td>1.9</td>
<td>WB</td>
</tr>
<tr>
<td>7</td>
<td>Tropomyosin 3 (P21107)</td>
<td>1.7</td>
<td>MS, WB</td>
</tr>
<tr>
<td>8</td>
<td>Hsp84 (P11499)</td>
<td>2.1</td>
<td>MS, WB</td>
</tr>
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Mass spectrometric analysis (MS) and Western blotting with specific antibodies (WB) resulted in identification of protein spots differing in intensity between normal epithelial and adenoma tissues. The spot numbers referred to are those in Fig. 3. The ratios of spot intensity between normal and adenoma tissues were calculated from five independent experiments using the BVA-mode of DeCyder software.

3.5 Identification of protein spots by Western blotting

Western blotting analysis was also performed to confirm the identification by MS and identify the remaining interesting spots. Boxed area X in Fig. 3 was enlarged in Fig. 5A, and the corresponding 2-D image area of NIH3T3 cells is shown in Fig. 5B. All protein spots identified as being altered in adenoma tissues were detected in spots from NIH3T3 cells (marked by hexagons) and additional spots were observed in the 2-D image of NIH3T3 cells (spot A in Fig. 5B). Mass spectrometric analysis identified spot 4 as 14-3-3zeta (data not shown) and specific antibody against 14-3-3zeta protein reacted with spot 4 and the additional spot, namely spot 5 (Fig. 5C). Antibody generated against peptides common to seven isoforms of 14-3-3 protein [22] reacted with spots 2, 3, 4 and 6 (Fig. 5D). Spot 5 may have had post-translational modifications, which changed reactivity for the antibody raised to 14-3-3 protein. The antibody used for 14-3-3 protein also reacted with the spot, namely spot A, which was present in NIH3T3 cells (Fig. 5B) but was absent from adenoma tissues (Fig. 5A).
The antibodies against prohibitin, tropomyosin and Hsp84 reacted with single spots, which were identified by mass spectrometric analysis (data not shown). All identified spots and the fold differences are presented in Table 1.

4 Discussion

We applied sensitive fluorescent dyes to the linkage of LCM and 2-D. We recognized two advantages of our application compared with silver staining. First, as considerably less protein is required for 2-D PAGE, microdissection time is dramatically shortened. In a previous report in which colon cancer tissues were subjected to linkage of LCM and 2-D PAGE, 25–50 μg of protein was separated for LCM samples in IPG gels (7 cm, pI 3-10NL) [14]. The resultant 2-D gels, stained with silver, apparently had far fewer spots than ours. With our application, because only 2.7–6.6 μg of total protein was sufficient to generate one 2-D image with 1500 spots, the dissection time is shortened regardless of tissue type even when LCM is used instead of Leica’s LMD. This means that multiple gels can be run from small amounts of sample to distinguish biological alterations from artifacts even for silver staining. Because spot detection is based on fluorescence signals and the scan time was adjusted to ensure that the intensity of the most abundant spot was not saturated, linearity of spot intensities can be obtained over a broad dynamic range. In addition, by running the sample with a pooled control in the same gel, each gel contains a common control image for quantitative normalization and rapid spot matching on the different gels can be performed. Moreover, time-consuming staining procedures were replaced by simple scanning of the gels between the glass plates, which took about 15 min for one 2-D image, 24 × 20 cm in size. The workflow of this application will allow high throughput analysis and facilitate clinical proteomic studies.

Fluorescent dyes used in this study label the thiol group of cysteine residues of proteins. Therefore, the proteins with multiple cysteine residues will have a stronger fluorescent signal and the degree of spot intensity will reflect both the actual amount of protein and the number of cysteine residues of the protein. As a consequence, the appearance of 2-D images of proteins labeled with these dyes is distinct from those of unlabeled proteins (data not shown). Saturation dyes may make it possible to study a novel aspect of the proteome, one which has not been studied previously. This idea is supported by our present study results. Previous studies using 2-D PAGE did not identify prohibitin, 14-3-3zeta, tropomyosin 3 (TM3) and Hsp84 as proteins that changed in amount in adenomas. On the other hand, the proteins reported to show altered expression in colorectal polyps such as S100A6 [23] and numatrin [24] using 2-D PAGE were not identified as altered proteins in our study. Further spot identification will reveal how these two types of 2-D PAGE, 2-D of fluorescence-labeled proteins or that based on colorimetric staining, can complement each other.

Mass spectrometric analysis of proteins labeled with fluorescent dyes was successfully performed. Work by Yan et al. (personal communication) indicates that similar peptide coverage is obtained with MALDI-MS analysis of both unlabeled and saturation labeled model proteins and cell lysates. However, in our experience, the number of ion peaks from labeled protein spots was less than those from unlabeled proteins and the ability to identify the individual proteins with MS appeared to be affected, although sufficient peptide coverage was achieved to obtain positive protein identification in five of the protein spots identified. The CyDye fluoros may have suppressive effects on ionization of peptides. Besides the mass spectrometer equipped with a MALDI ion source, which we used in this study, other types of mass spectrometry such as ESI, which can ionize certain peptides more efficiently, are worth examining. As clinical materials often do not contain an adequate amount of protein for preparative gels and 2-D patterns of CyDye fluor-labeled proteins are distinct from those visualized by conventional silver staining, development of a new 2-D database for saturation dye labeled proteins will play an important role in rapid identification of interesting spots in clinical studies.

In this report, 37 protein spots showed expression level changes in adenoma tissues and eight of them were identified by mass spectrometric analysis and/or Western blotting. Prohibitin is a putative tumor suppressor, which interacts with retinoblastoma protein and regulates E2F functions [25], with inhibitory effects on cell proliferation exerted by blocking the transition between the G1 and S phases of the cell cycle [26]. A recent transcriptome study, based on serial analysis of gene expression, revealed prohibitin to be a direct target of c-myc, the oncogeneic transcription factor [27]. Prohibitin is located in region 17q21, a region in which allelic loss has frequently been observed in colon cancer [28]. While prohibitin has not been extensively studied in colon cancer, mutation and single nucleotide polymorphism in the 3’ untranslated region of prohibitin have been used in assessing the risk of breast cancer [29–31]. In addition to cell cycle regula-
tion, the functions of prohibitin include a component of mitochondrial respiratory chain complexes [32, 33], signal transduction [34, 35], cellular immortalization [36, 37], cellular senescence [38, 39] and apoptosis [40]. The increased expression of prohibitin in adenoma tissues might involve mechanisms of a negative feedback loop in proliferating adenoma cells.

14-3-3zeta is a multifunctional protein. It re-organizes the actin cytoskeleton by interacting with the actin-depolymerizing factor cofilin and its regulatory kinase, LIM-kinase 1 [41]. 14-3-3zeta functions as a protein kinase B/Akt substrate suggesting its possible involvement in cell cycle regulation [42]. A recent study using the yeast two-hybrid system revealed a complex formation of 14-3-3zeta with tumor suppressor gene products, hamartin and tuberin and subsequent experiments suggested the involvement of 14-3-3zeta in the PI3K/Akt signaling pathway [43]. 14-3-3zeta overexpression may play functional roles in mechanisms of the adenoma-carcinoma sequence of colorectal cancer.

We also recognized an increase in TM3 in adenoma tissues. While tropomyosin isoforms are known to function in muscle contraction, the functional significance of multiple isoforms in nonmuscle cells are largely unknown. Several reports have discussed possible implications of altered expression of tropomyosin isoforms at an early step in carcinogenesis. Transformation with Rous sarcoma virus resulted in the up-regulation of TM3 in chicken embryo fibroblast cells [44]. Tropomyosin isoforms including TM3 were down-regulated in breast cancer cells [45] and experimentally transformed fibroblasts [46–48]. As the altered expression of TM3 is observed in many cases of cancerous transformation in vitro and in vivo, this alteration reflects some common phenotypic changes which occur during the course of cancer development.

Hsp84 is a heat shock induced protein and is known to activate the transcription factor MyoD1 [49]. While the functional roles of Hsp84 in cancerous tissues are unknown, as Hsp84 forms a complex with mutant p53 and probably contributes to increasing the stability of mutant p53 protein [50], its increased expression in adenomas and carcinomas in the colon may influence the regulation of cell cycle development.

5 Concluding remarks

In summary, we developed a novel application of fluorescent dyes with LMD and 2-D PAGE and demonstrated this method to shorten the dissection time and increase the number of protein spots observed. Using this application, we identified four proteins, the expressions of which differed between adenoma and normal epithelial tissues. As they are multifunctional proteins and play essential roles in the cell, further study of these proteins in cancer tissues will elucidate adenoma biology and facilitate the development of diagnostic markers.

Received January 24, 2003

6 References