Altered profiles of nuclear matrix proteins during the differentiation of human gastric mucous adenocarcinoma MGc80-3 cells

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AIM: To find and identify specific nuclear matrix proteins associated with proliferation and differentiation of carcinoma cells, which will be potential markers for cancer diagnosis and targets in cancer therapy.

METHODS: Nuclear matrix proteins were selectively extracted from MGc80-3 cells treated with or without hexamethylamine bisacetamide (HMBA), and subjected to 2-D gel electrophoresis. The resulted protein patterns were analyzed by Melanie software. Spots of nuclear matrix proteins differentially expressed were excised and subjected to in situ digestion with trypsin. Peptide masses were obtained by matrix-assisted laser-desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS) analysis and submitted for database searching using Mascot tool.

RESULTS: The MGc80-3 cells were induced into differentiation by HMBA. There were 22 protein spots which changed remarkably in the nuclear matrix, from differentiation of MGc80-3 cells compared to control. Eleven of which were identified. Seven proteins - actin, prohibitin, porin 31HL, heterogeneous nuclear ribonucleoprotein A2/B1, vimentin, ATP synthase, and heat shock protein 60 were downregulated, whereas three proteins - heat shock protein gp96, heat shock protein 90-beta, and valosin-containing protein were upregulated, and the oxygen-regulated protein was only found in the differentiated MGc80-3 cells.

CONCLUSION: The induced differentiation of carcinoma cells is accompanied by the changes of nuclear matrix proteins. Further characterization of those proteins will show the mechanism of cellular proliferation and differentiation, as well as cancer differentiation.

Key words: Nuclear matrix proteins; Cell differentiation; Human gastric mucous adenocarcinoma MGc80-3; Hexamethylamine bisacetamide

INTRODUCTION

Nuclear matrix (nuclear skeleton) is the filamentous protein framework in eukaryotic cell nucleus. Nuclear matrix plays an important role in life activities such as maintaining the cell morphology, dimensional localization, DNA replication and transcription. Thereby, it is intensely associated with cell proliferation and differentiation, as well as carcinogenesis[5-9].

The role of nuclear matrix in cell activities has drawn increasing attention recently. Most of its components, besides the fibrins, are proteins and/or enzyme in DNA replication, transcription and gene expression[10-12]. The nuclear matrix in cancer cells is not only abnormal in morphology, but also apparently different in its composition. Tumor-associated nuclear matrix proteins have been identified in cancers of the breast, colon, bone, bladder, and larynx using 2-D gel electrophoresis[13-9]. But nuclear matrix proteins associated with the differentiation of carcinoma cells were unexplored.

Previously, we found that the morphology of nuclear matrix in differentiated human gastric cancer cells, induced by hexamethylamine bisacetamide (HMBA) and retinoic acid[10], showed similar characteristics with that in normal cells. It is implied that further study on the differential expressed nuclear matrix proteins in response to differentiation reagent will be able to reveal the mechanism of carcinogenesis and phenotypic reversion, as well as cell growth and differentiation.

This study was designed to find and identify specific nuclear matrix proteins, associated with the proliferation and differentiation of carcinoma cells. The human gastric mucous adenocarcinoma MGc80-3 cells was selected as a subject to undergo differentiation induced by HMBA. The nuclear matrix proteins from MGc80-3 cells were selectively extracted, and subjected to 2-D gel electrophoresis. The changed nuclear matrix proteins during the differentiation process were identified by matrix-assisted laser-desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS) combined with database searching.
MATERIALS AND METHODS

Materials

HMBA (Sigma Chemical Company) were used to induce the differentiation of MGc80-3 cells. Sequence grade, modified trypsin (Promega) and iodoacetamide (Sigma) were used in the in-gel digestion. ReadyStrip IPG strips (pH 3-10, 11 cm) and IPG buffer pH 3-10 were from Amersham Biosciences. Other reagents used in 2-D gel electrophoresis and Coomassie blue R250 were from Shanghai Sangon Biological Engineering Technology and Service Co., Ltd.

Cell culture

MGc80-3 cells were cultured in the RPMI-1640 supplemented with 15% new born bovine serum at 37 °C. The cells were seeded in a constant density overnight, and treated with 5 mmol/L HMBA for 7 d. Fresh culture media were added to the cells every 48 h, and cells were harvested at subconfluency. The obtained cells were then stored at -80 °C.

Purification of nuclear matrix proteins

The nuclear matrix proteins were extracted by a method of Fey et al.[11]. The MGc80-3 cells were washed with PBS and extracted with cytoskeleton buffer (CSK100) (10 mmol/L PIPES pH 6.8, 300 mmol/L sucrose, 100 mmol/L NaCl, 4 mmol/L CaCl₂, 1.0 mmol/L PMSF, 0.5% Triton X-100) at 0 °C for 10 min. The nuclei were sheared through a 16-gauge needle, and subjected to centrifugation for 5 min at 400 r/min. The deposition was washed twice with CSK50 (10 mmol/L PIPES pH 6.8, 300 mmol/L sucrose, 50 mmol/L NaCl, 4 mmol/L CaCl₂, 1.0 mmol/L PMSF, 0.5% Triton X-100) and digested for 30 min at 25 °C in the same buffer containing 500 U/mL DNase I. One mole per liter ammonium sulfate was added dropwise to a final concentration of 0.25 mmol/L. After incubation for 15 min, the nuclear matrix proteins were pelleted by centrifugation at 1 000 r/min for 5 min, and washed once with the CSK50 buffer, then stored at -80 °C. Protein concentrations were determined by Bradford’s method.

Two-dimensional gel electrophoresis

To solubilize nuclear matrix proteins, the pellet of purified nuclei was resuspended in 2-D buffer containing 7 mol/L urea, 2 mol/L thiourea, 4% CHAPS, 50 mmol/L DTT and ultrasonicated for 2 min. The supernatant was centrifuged for 30 min at 16 000 g at 4 °C. IEF was performed in ReadyStrip IPG strips. ReadyStrip IPG strips were rehydrated overnight in a reswelling tray with 2-D buffer containing 0.5% IPG buffer pH 3-10 and nuclear matrix proteins in a final volume of 250 µL (200 µg). IEF was carried out on a Protean IEF Cell (Investigator) at 19 °C with a maximum current setting of 80 mA/strip. Focusing was performed for a total of 70 000 V·h.

Before carrying out second dimensional SDS-PAGE, the strips were equilibrated in an equilibration buffer consisting of 20% glycerol, 2% SDS, 65 mmol/L Tris-HCl, pH 6.8 and 20 mmol/L DTT for 10 min at room temperature, then transferred into the second equilibration buffer consisting of 20% glycerol, 2% SDS, 65 mmol/L Tris-HCl, pH 6.8 and 2.5% iodoacetamide. The strips were transferred onto 1-mm thick SDS-PAGE gels and sealed in place with 1% agarose. SDS-PAGE was performed on a 12.5% acrylamide/bisacrylamide gel at 50 V for 30 min followed by 170 V for 8 h. The gels were run in the following electrode buffer: 25 mmol/L Tris, 192 mmol/L glycine, 0.1% SDS. SDS-PAGE standards were used for gel calibration. The gels were stained with Coomassie blue R250 and destained with 5% methanol, 7.5% acetic acid. 2-DE maps of nuclear matrix proteins were subjected to analysis with Melanie software. Protein spots were manually excised for mass spectrometry.

Protein identification by matrix-assisted laser-desorption/ ionization time of flight mass spectrometry (MALDI-TOF-MS) analysis

In-gel digestion Spots were cut into about 1 cm x 1 cm pieces, and washed twice with 80 µL of 25 mmol/L NH₄HCO₃, 50% acetonitrile for 5 min, then dehydrated with 80 µL of acetonitrile. Gel pieces were completely dried. Reduction was achieved by 1 h treatment with 10 mmol/L DTT/100 mmol/L NH₄HCO₃ at 57 °C. Alkylation was performed with 25 mmol/L iodoacetamide/100 mmol/L NH₄HCO₃ for 45 min in the dark at 25 °C. Finally, gel pieces were washed thrice for 5 min alternatively with 100 mmol/L ammonium carbonate and acetonitrile, and then completely dried with a Speed Vac before trypsin digestion. Appropriate volumes of trypsin (12.5 ng/µL, freshly diluted in 50 mmol/L NH₄HCO₃) were added to the dried gel pieces. The digestion was performed at 37 °C overnight. After being centrifuged for 5 min in a Speed Vac, the gel pieces were incubated in 10 µL 20 mmol/L NH₄HCO₃ for 20 min at room temperature, and then incubated in 10 µL 5% TFA/50% acetonitrile for 5 min at room temperature. The supernatant was mixed, and completely dried with a Speed Vac.

MALDI-TOF-MS analysis For MALDI-TOF-MS analysis, samples were dissolved in 2 µL 0.1% TFA. Mass measurements were carried out on a Bruker ULTRAFLEX™TOF/TOF mass spectrometer. This instrument was used at a maximum accelerating potential of 20 kV (in positive mode) and was operated in reflector mode. 0.5 µL of saturated solution of α-cyano-4-hydroxy cinnamic acid in 0.1% TFA/30% acetonitrile was mixed with 0.5 µL sample solution, and added to the target. Internal calibration was performed with tryptic peptides coming from autodigestion of trypsin (monoisotopic masses at m/z 842.51, and m/z 2 211.10). Monoisotopic peptide masses were assigned and used for database search.

Database search

The MALDI-TOF-MS data were searched against a NCBI nonredundant protein sequence database using Mascot tool from Matrix Science. All proteins present in the NCBI database were taken into account without any pl or Mr restrictions. Search parameters included a maximum allowed peptide mass error of 100 ppm with a consideration of one incomplete cleavage per peptide. Accepted modifications included carbamidomethylation of cysteine residues (from iodoacetamide exposure) and methionine oxidation, a common modification occurring during SDS-PAGE. Protein identifications were assigned when three criteria were met:
(1) Statistical significance (P<0.05) of the match when tested by Mascot (matrixscience.com); (2) >20% sequence coverage by the tryptic peptides; (3) Concordance (±15%) with the molecular weight and pI of the parent 2-D PAGE protein spot; and (4) Protein identifications not fulfilling criterion 2 were still assigned, if criteria 1 and 3 were fulfilled and no other Homo sapiens proteins with peptide mass-matched P values <0.05 were identified by Mascot; identified protein was inferred.

RESULTS
Changes in nuclear matrix proteins upon addition of 5 mmol/L HMBA

Samples of nuclear matrix proteins extracted from MGe80-3 cells treated with or without 5 mmol/L HMBA were subjected to 2-D gel electrophoresis for at least three repeats per experimental condition. Analysis of proteins was based on evaluation of at least two gels. Typical 2-DE map of nuclear matrix proteins from MGe80-3 cells in the presence or absence of HMBA is shown in Figure 1. There were 22 protein spots that were changed remarkably, whereas most of the spots were similar in the expression patterns of nuclear matrix proteins from differentiated MGe80-3 cells compared to that from control. Among the changed spots, 12 spots were downregulated, one spot disappeared in the differentiated MGe80-3 cells, whereas 8 spots were upregulated, and 1 spot emerged as a new protein spot that appeared in the differentiated cells (Figures 1 and 2). Relative expression levels of the changed proteins were shown using Melanie software (Figure 3), relative volume (%vol) of spot was employed to make the data independent of uninteresting variation between gels, such as differences in protein loading or staining (Table 1).

Identification of the altered proteins
In the 22 differentially expressed protein spots, 15 were identified as 11 proteins by peptide mass fingerprinting according to the criteria, while 8 spots failed to be identified by peptide mass fingerprinting. The Spot C33 that disappeared in the HMBA-treated MGe80-3 cells was identified as hnRNP A2/B1. Nine of the twelve spots downregulated in the HMBA-treated cells were actin, vimentin, prohibitin, porin 31HL, hnRNP A2/B1, ATP synthase and heat shock protein 60. The new spot present after HMBA treatment was oxygen-regulated protein. In the eight upregulated spots, four spots were matched with heat shock protein gp96, heat shock protein 90-beta, ubiquitin thiolesterase (EC 3.1.2.15) and valosin-containing protein individually.

Table 1 Nuclear matrix proteins from the 2-DE map of MGe80-3 cells identified by peptide mass fingerprinting

| Accession number | Protein name | Sequence coverage (%) | Theoretical values | Experimental values | Number of matched peptides |
|------------------|--------------|-----------------------|-------------------|---------------------|---------------------------|
|                  |              |                       | M (Da)            | pI                  |                          |
|                  |              |                       |                   |                     |                           |
|                  |              |                       |                   |                     |                           |
| Disappeared proteins:                                                                                             |
| C33 gi|4504447 Heterogeneous nuclear ribonucleoprotein A2/B1 | 26 | 36 041 | 8.67 | 37 100 | 8.47 | 7 |
| Downregulated proteins:                                                                                           |
| C19 gi|15277503 ACTB protein | 37 | 40 536 | 5.55 | 29 000 | 5.51 | 10 |
| C20 gi|4505773 Prohibitin | 40 | 29 843 | 5.57 | 31 500 | 5.58 | 8 |
| C29 gi|238427 Porin 31HL | 34 | 30 737 | 8.63 | 34 500 | 8.78 | 7 |
| C30 gi|4504447 Heterogeneous nuclear ribonucleoprotein A2/B1 | 43 | 37 429 | 8.97 | 36 600 | 8.85 | 11 |
| Newly emerged proteins:                                                                                           |
| C38 gi|15277503 ACTB protein | 35 | 40 536 | 5.55 | 48 400 | 5.13 | 10 |
| C39 gi|5030431 Vimentin | 34 | 41 651 | 4.82 | 46 800 | 4.82 | 11 |
| C40 gi|2119204 Vimentin | 20 | 53 676 | 5.06 | 52 800 | 4.98 | 9 |
| C41 gi|32189394 ATP synthase | 29 | 56 525 | 5.26 | 55 200 | 5.05 | 15 |
| C70 P10809 Heat shock protein 60 | 17 | 61 187 | 5.70 | 59 400 | 5.51 | 6 |
| Unidentified proteins                                                                                              |
| S78 gi|5453632 oxygen regulated protein | 14 | 111 494 | 5.16 | 92 300 | 5.50 | 12 |
| Upregulated proteins:                                                                                              |
| S72 gi|15010550 heat shock protein gp96 precursor | 14 | 90 309 | 4.73 | 98 000 | 4.62 | 12 |
| S74 gi|72222 heat shock protein 90-beta | 27 | 83 584 | 4.97 | 79 700 | 5.16 | 19 |
| S75 gi|2134982 ubiquitin thiolesterase (EC 3.1.2.15) | 12 | 96 596 | 4.90 | 82 000 | 5.14 | 9 |
| S75 gi|6005942 valosin-containing protein | 34 | 89 950 | 5.14 | 82 000 | 5.50 | 20 |

Downregulated: C23, C27, C28; Upregulated: S41, S44, S53, S66, S54.
In the present study, nuclear matrix proteins extracted from differentiated MGc80-3 cells induced by HMBA were compared with those from MGc80-3 cells using 2-D gel electrophoresis. 2-D gel image analysis software was used to confirm the identification of specific nuclear matrix proteins associated with the differentiation of carcinoma cells combined with the visual observation. The protein patterns were highly reproducible. There were 22 spots that were changed remarkably during the differentiation process. Most of interested proteins differed qualitatively between control and differentiated MGc80-3 cells, except for two proteins, in which one was absent, while the other was only present in the HMBA-treated cells. The differentially expressed proteins confirm that specific nuclear matrix proteins are accompanied with the differentiation of MGc80-3 cells.

Fifteen of the twenty-two changed proteins were identified. The identified proteins that associated with proliferation and/or differentiation of MGc80-3 cells were grouped into six classes: (1) common nuclear matrix proteins:

**Figure 1** 2-D PAGE gels of nuclear matrix proteins from MGc80-3 cells, Coomassie blue-stained: A: MGc80-3 cells; B: MGc80-3 cells exposed to 5 mmol/L HMBA for 7 d. Arrows pointed to changed proteins. Disappeared protein: C33; downregulated proteins: C19, C20, C23, C27, C28, C29, C30, C38, C39, C40, C41, C70; new protein: S78; upregulated proteins: S4, S44, S53, S54, S72, S74, S75.

**Figure 2** Enlarged maps of changed nuclear matrix proteins from MGc80-3 cells, Coomassie blue-stained: A–H: nuclear matrix from control cells; I–P: nuclear matrix from HMBA-treated cells. Arrows pointed to changed proteins. C33 disappeared, while C19, C20, C23, C27, C28, C29, C30, C38, C39, C40, C41, C70 were downregulated in the HMBA-treated cells; S78 was a new protein, whereas S4, S44, S53, S54, S72, S74, S75 were upregulated after HMBA treatment.
vimentin and actin; (2) heterogenous nuclear ribonucleoprotein family: hnRNP A2/B1; (3) heat shock proteins; heat shock protein 60, heat shock protein gp 96, heat shock protein 90-beta, oxygen-regulated protein; (4) enzymes: ATP synthase, ubiquitin thiolesterase, and valosin-containing protein; (5) tumor suppressor protein: prohibitin; and (6) chloride channel protein: porin 31HL [12].

Previously, several nuclear matrix proteins identified in this study, such as hnRNP A2/B1 [13], vimentin, actin [14], prohibitin [15], heat shock protein 60 (Hsp60), heat shock protein gp96 [17], and heat shock protein 90-beta [18], were reported previously to be associated with cell growth and/or differentiation, although the roles they played in cell differentiation remain unclear.

In the downregulated proteins, vimentin and hnRNP A2/B1 have drawn intense attention to their relationship with cellular differentiation [12,13]. In accordance to the result in this study, vimentin and hnRNP A2/B1 were highly expressed in cancer cell lines, but not detected or expressed in a lower level in well differentiated cells. Low expression of vimentin was reported to be correlated with the decreased in a lower level in well differentiated cells. Low expression expressed in cancer cell lines, but not detected or expressed in this study, vimentin and hnRNP A2/B1 were highly expressed in the differentiated MGc80-3 cells. The deep involvement of heat shock protein family in the differentiation of MGc80-3 was consistent with the reports that heat shock proteins played an important role in the gastric tumors.

The proteins found in this study may be directly or indirectly involved in altered gene expression associated with the differentiation of carcinoma cells.

The findings described here will facilitate the understanding of the signal pathways and the mechanism of HMBA-induced differentiation. Further characterization of these proteins is now underway. These specific proteins are potential markers for tumor development or therapeutic target.

In this study, proteomic methods were used to systematically analyze the altered protein profiles in differentiated cancer cells. Specific nuclear matrix proteins associated with the differentiation of carcinoma cells were found and identified. It is of significance for revealing the signal pathway and mechanism of cell growth and differentiation, as well as carcinogenesis. Further characterization of those proteins will show the mechanism of cellular proliferation and differentiation, as well as cancer differentiation.

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