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• ESOPHAGEAL CANCER •

Separation and identification of differentially expressed nuclear matrix proteins between human esophageal immortalized and carcinomatous cell lines

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Abstract

AIM: To separate and identify differentially expressed nuclear matrix proteins (NMPs) between the immortalized human esophageal epithelial cell line (SHEE) and the malignantly transformed esophageal carcinoma cell line (SHEEC), and to provide new ways for finding specific markers and the pathogenesis of esophageal carcinoma.

METHODS: SHEE and SHEEC cell lines were used to extract NMPs. The quality of NMPs was monitored by Western blot analysis including DNA topoisomerase IIα, proliferation cell nuclear antigen (PCNA) and histone. NMPs of SHEE and SHEEC were analyzed by two-dimensional electrophoresis (2-DE), silver staining and PDQuest6.2 image analysis software. Three spots in which the differentially expressed NMPs were more obvious, were selected and analyzed with matrix-assisted laser desorption/ionization time of flying mass spectrometry (MALDI- TOF-MS) and database search.

RESULTS: Western blot analysis revealed that DNA topoisomerase IIα and PCNA were detected, and the majority of histones were deleted in NMPs of SHEE and SHEEC. After 2-DE image analysis by PDQuest6.2 software, the 2-DE maps were detected with an average of 106±7.1 spots in SHEE and 132±5.0 spots in SHEEC. Most of them were matched one another (r=0.72), only 16 protein spots were found differing in intensity. Three NMPs including cytoskeletal tropomyosin, FK506-binding protein 6, similar to retinoblastoma binding protein 8 were preliminarily identified by MALDI- TOF-MS.

CONCLUSION: These differentially expressed NMPs may play an important role during malignant transformation from SHEE to SHEEC. Their separation and identification will contribute to searching for specific markers and probing into the pathogenesis of esophageal carcinoma.

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INTRODUCTION

Nuclear matrix (NM) represents the insoluble structural framework of nucleus which removes membrane lipid, soluble protein and chromatin. According to many investigations, nuclear matrix has been shown to play an important role not only in maintaining the structure of nucleus, but also in chromatin/chromosome construction, DNA replication, gene expression and regulation (RNA synthesis, RNA splicing and RNA transportation)[1]. Recently changes of the composition, structure and function of NMPs in the generation and development of tumors have been more and more concerned. Separation and identification of tumor associated NMPs have been a new way to search for tumor specific markers and to study tumor pathogenesis. Nowadays several tumor specific NMPs have been separated and identified from hepatocellular carcinoma[2], colon cancer[3] and prostate cancer[4], etc. Some of them (such as NMP22) have been applied to clinical diagnosis and therapy[5], but studies of the separation and identification of esophageal carcinoma specific NMPs have not been carried out.

Esophageal carcinoma is one of the most common malignant tumors in China[6-27]. In recent years, it has been increasingly concerned about the roles of human papilloma virus (HPV) in esophageal carcinogenesis[28-32]. In our previous work, we transfected human embryonic esophageal mucosal cells with HPV18 E6E7 genes, and established an immortalized epithelial cell line SHEE[33,34]. The SHEE cells were further exposed to the tumor promoter (12-O-tetradecanoyl-phorbol-13-acetate, TPA) to induce malignant transformation from which a human embryonic esophageal epithelial carcinoma cell line SHEEC was then established[35,36]. These studies not only provided the evidence for the close relationship between HPV and esophageal carcinogenesis, but also established a reliable model for studying the molecular mechanisms of esophageal carcinogenesis. In the present study, the differentially expressed NMPs between SHEE and SHEEC were investigated by Western blot, 2-DE and MALDI-TOF-MS, and three esophageal carcinoma associated NMPs were preliminarily identified. The separation and identification of these proteins may contribute to searching for specific markers and studying the pathogenesis of esophageal carcinoma.

MATERIALS AND METHODS

Cell culture

SHEE and SHEEC were cultured in MEM medium (Gibco) supplemented with 100 ml/L fetal bovine serum (100 u/ml penicillin, 100 u/ml streptomycin) and incubated at 37 ℃ in humidified atmosphere of 50 ml/L CO_2. Cells were harvested when they grew into a full monolayer and kept at -70 ℃ until use.

Extraction of NMPs

The method used was modified from Fey *et al*[37]. Cultured cells were extracted by cytoskeleton (CSK) buffer (100 mM KCl, 3 mM $MgCl_2$, 1 mM EGTA, 10 mM PIPES pH6.8, 300 mM sucrose, 0.5 % triton X-100, 1.2 mM PMSF) for 5 min at 4 ℃. After centrifugation at 650 g for 5 min, the supernatants contained cytoplasmic proteins. The pellets were resuspended in digestion buffer (same as CSK buffer except with 50 mM NaCl instead of KCl) containing 400 μg/ml DNase I and 100 μg/ml RNase A. Enzyme digestion was carried out for 20 min at room temperature and terminated by adding cold ammonium sulfate to a final concentration of 0.25 M. After centrifugation at 1 000 g for 10 min, the pellets were then solubilized in disassembly buffer (8 M urea, 20 mM Mes pH6.6, 0.1 mM $MgCl_2$, 1 mM EGTA, 1 % 2-mercaptoethanol, 1 mM PMSF) and dialyzed overnight at 4 ℃ against 1 000 volumes of assembly buffer (150 mM KCl, 5 mM $MgCl_2$, 0.125 mM EGTA, 25 mM imidazole hydrochloride pH7.1, 2 mM dithiothreitol, 0.2 mM PMSF). The samples were centrifuged at 200 000 g for 100 min. The protein concentration of supernatants containing NMPs was determined by Bradford method and then precipitated in 5 volumes of absolute ethanol. The dried pellets were resuspended in electrophoresis sample buffer. The sample aliquots were stored at -70 ℃ until use.

Western blot

The experimental procedures were referred to *Molecular cloning*[38]. Briefly, the proteins were separated by SDS-PAGE and then transferred onto nitrocellulose membranes. Nonspecific reactivity was blocked by incubation overnight at 4 ℃ in buffer (10 mM Tris-HCl pH7.5, 150 mM NaCl, 2 % Tween-20, 4 % bovine sreum albumin). The membrane was then incubated with primary antibody including mouse anti-human histone (Roche), mouse anti-human DNA topoisomerase IIα. (Roche) and mouse anti-human PCNA (Dako). The secondary antibody (Zymed) was used to detect bound primary antibody. Reactive protein was detected by Western blot luminol reagent (Santa Cruz) and exposed to X-films (Kodak). The X-films were scanned and analyzed with Kodak 1-D 3.5 software (Kodak).

Two-dimensional electrophoresis

To separate NMPs, the 2-DE procedures were referred to Xiong *et al*[39]. Briefly, 2-DE was carried out by using the Mini-PROTEAN II 2-D apparatus (Bio-Rad). 90 μg of the NMPs was mixed with the rehydration solution to a total volume of 125 μl. After rehydration, the isoelectric focusing (IEF) and equilibration, and the IPG strips (pH4-7, 7 cm) were placed on a 1.0 mm thick, 10 % SDS-PAGE gel. On electrophoresis, the SDS-PAGE gels were stained with PlusOne™ silver staining kit (Phamacia).

Image acquisition and analysis

Image scanning for the silver-stained 2-D gels was performed with EDAS290 digital camera system (Kodak) and image analysis with the PDQuest 6.2 software (Bio-Rad). To obtain reliable results, three gels were employed for each cell line. After the background subtraction, spot detection and match, one standard gel for each cell line was obtained. These standard gels were then matched to yield information about the spots of differentially expressed NMPs.

Protein identification by MALDI-TOF-MS

Three spots in which the differentially expressed protein was more obvious in each cell line were cut out from the gel. The gel pieces were treated by a series of steps including silver-removal, reduction, alkylation and in-gel digestion with trypsin. The peptide mass maps were generated by Applied Biosystems Voyager System 6192 MALDI-TOF-mass spectrometry (ABI, USA). Peptide masses were analyzed using the MS-Fit search program (http:// prospector.ucsf.edu/ucsfhtml4.0u/ msfit.htm).

RESULTS

Evaluation of NMPs quality

The NMPs extracted from SHEE and SHEEC were evaluated by Western blot, and the aim of evaluation was to monitor whether some NMPs were lost and whether other non-NMPs components were mixed. In recent years, many studies proved that DNA topoisomerase IIα and PCNA were the major components of NMPs[40,41], the existence of histone in nucleosome was the major soluble protein of nucleus. In our study, 170 kD DNA topoisomerase IIα was detected in the nuclear protein fraction of SHEE and SHEEC at the same position of NMPs fraction, but the density in NMPs fraction was weaker than that in the nuclear protein fraction (Figure 1). This agreed with other reports[40]. PCNA (36kD) was detected in the NMPs fraction of SHEE and SHEEC, and this showed PCNA was the major protein of NMPs (Figure 2). Moreover, histone was seen at 23 kD and 12-15 kD in the nuclear protein fraction (Mw of histone: H1:23 kD, H2A:14.5 kD, H2B: 13.8 kD, H3:15 kD, H4:11.8 kD) and almost no histone was detected at the same position of NMPs (Figure 3). This showed majority of histone was removed during extraction and there was almost no histone in the NMPs fraction of SHEE and SHEEC. According to these results, the NMPs fractions extracted from SHEE and SHEEC almost deleted the histone and retained the major NMPs components including DNA topoisomerase IIα and PCNA, so the NMPs were pure in high quality.

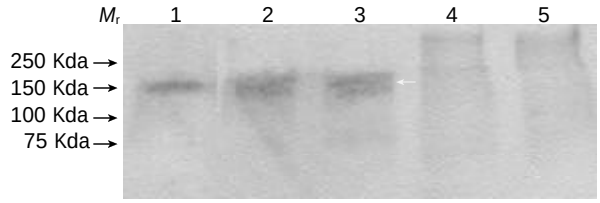

Figure 1 Western blot analysis of DNA topoisomerase IIα. Lane 1, nuclear matrix fraction of SHEE. Lane 2, total crude nuclear protein fraction of SHEEC. Lane 3, total crude nuclear protein fraction of SHEE. Lane 4, cytoplasmic protein fraction of SHEEC. Lane 5, cytoplasmic protein fraction of SHEE. M_r, molecular weight standard.

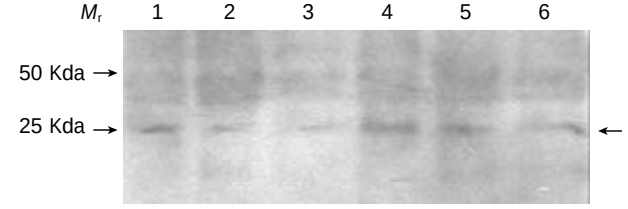

Figure 2 Western blot analysis of PCNA. Lane 1 to Lane 3, nuclear matrix fraction of SHEEC. Lane 4 to Lane 6, nuclear matrix protein of SHEE. M_r, molecular weight standard.

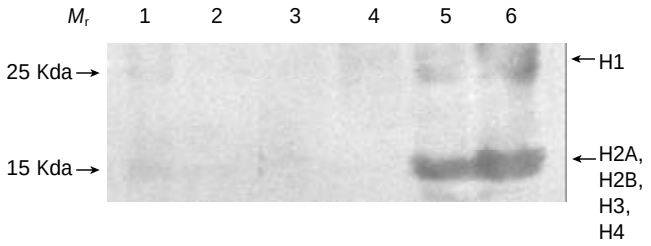

Figure 3 Western blot analysis of histone. Lane 1, nuclear matrix fraction of SHEEC. Lane 2, nuclear matrix protein of SHEE. Lane 3, cytoplasmic protein fraction of SHEEC. Lane 4, cytoplasmic protein fraction of SHEE. Lane 5, total crude nuclear protein fraction of SHEEC. Lane 6, total crude nuclear protein fraction of SHEE. *M*r, molecular weight standard.

2-D map and image analysis

To obtain a higher electrophoretic resolution, IPG strips (pH4-7) were selected to separate the NMPs of SHEE and SHEEC in our study because the p*I* range of NMPs was mainly in acid pH. Three pairs of gels from different batches of SHEE and SHEEC were analyzed by using the software PDQuest6.2. There were 106±7.1 and 132±5.0 protein spots observed in SHEE and SHEEC respectively. Most of them were matched one another (r=0.72), only 16 protein spots were found differing in intensity. These 16 protein spots belonged to 4 types. Namely A: one protein spot was detected in SHEE (No.2). B: Seven protein spots were detected only in SHEEC (No.3, 4, 9, 11, 12, 14, 16), C: One protein spot was expressed higher in SHEE (No.1), D: Seven protein spots were expressed higher in SHEEC (No.5, 6, 7, 8, 10, 13, 15). According to the standard molecular weight and pH gradient of IPG strips, the positions of these 16 protein spots were estimated with the software PDQuest6.2 (Table 1). Three protein spots (No.4, 14, 16) which belonged to type B and had a higher intensity were selected and analyzed with MALDI-TOF-MS (Figure 4).

MALDI-TOF-MS analysis and protein identification

These three protein spots (No.4, 14, 16) were cut out from the gels and analyzed with MALDI-TOF-MS. Peptide mass fingerprint (PMF) of each protein spot was then generated (Figure 5). By searching the NCBInr protein database with the MS-Fit search program, we identified these three proteins combined with the searching results. The characteristics of the protein, the number and intensity of peptide matching peak, the sequence coverage of matching peptide, as well as the theoretical and approximate values of M_r and p*I*, the identified protein names, accession numbers, as well as the sequence coverages, the theoretical M_r and p*I* values for each protein spot are listed in Table 2.

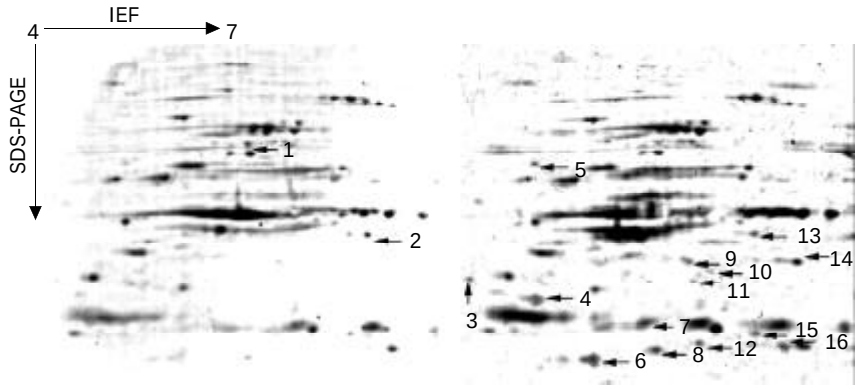

Figure 4 Differentially expressed NMP spots observed in SHEE (left) and SHEEC (right) two-dimensional gels (IPG dry strips: pH 4-7, 7 cm). The arrows show differentially expressed protein spots. Three protein spots (No.4, 14, 16) were selected and analyzed with MALDI-TOF-MS.

Table 1 Differentially expressed protein spots between SHEE and SHEEC

Spot No.	Type	Experimental M_r	Experimental p*I*	Spot No.	Type	Experimental M_r	Experimental p*I*
1	C	73.0	5.55	9	B	38.2	5.79
2	A	45.1	6.60	10	D	35.8	5.96
3	B	34.0	4.10	11	B	33.5	5.83
4	B	30.0	4.60	12	B	23.1	5.88
5	D	62.5	4.50	13	D	44.6	6.32
6	D	21.0	5.03	14	B	37.6	6.66
7	D	26.2	5.45	15	D	24.6	6.33
8	D	22.2	5.53	16	B	22.7	6.54

Table 2 Proteins identified by MALDI-TOF-MS

Spot No.	Accession No.(gi)	Theoretical M_r	Theoretical p*I*	Intensity matched	Length (aa)	Name of protein
4	37424	27975	4.8	44 %	239	Cytoskeletal tropomyosin
14	17149849	37227	6.9	29 %	327	FK506-binding protein 6
16	13647876	26775	5.7	54 %	230	Similar to retinoblastoma binding protein 8

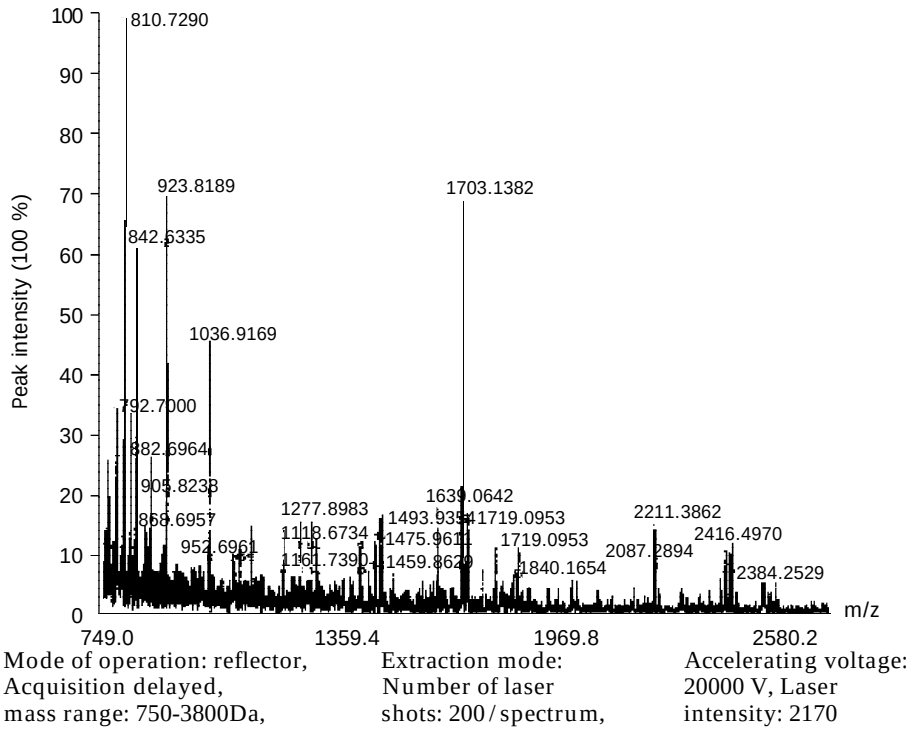

Figure 5 MALDI-TOF mass spectrum map of protein spot 16.

DISCUSSION

In the present study, we studied the differentially expressed NMPs extracted from SHEE and SHEEC by Western blot, 2-DE and MALDI-TOF-MS, and preliminarily identified three differentially expressed NMPs. According to their characteristics, these three proteins were connected with diseases such as colon carcinoma and myofibroblastic tumor, etc, but they were not related to esophageal carcinoma.

Cytoskeletal tropomyosin (tropomyosin 3, cytoskeletal (TPM3)) belongs to the tropomyosin family. There were at least 5 isoforms in tropomyosins including TPM1, TPM2, TPM3, TPM4, TPM5 which were produced by a tissue-specific alternative mRNA splicing mechanism[42]. The tropomyosins were a group of actin-binding proteins which served to mediate the effect of Ca^{2+} on the actin-myosin interaction[43]. Actin is one of the major components of NMPs. Owing to active cell division during the course of malignant transformation, cytoskeletal tropomyosin connected with actin in the nucleus becomes overexpressed. Zeng and his associates[44] found that tropomyosin was localized in the nuclear matrix and chromosome scaffold, and dispersively distributed in the interphase nuclei and metaphase chromosomes. Many studies have proved that cytoskeletal tropomyosin is related to the generation and development of tumors. Martin-Zanca and his associates[45] found the oncogene-Onc D (named later "trk" (tropomyosin(t)-receptor(r) -kinase(k)) in colon carcinoma. Sequence analysis for the oncogene Onc D found that it was generated by a somatic rearrangement of two genes, one of which coded for cytoskeletal tropomyosin (TPM3) and the other for a tyrosine-specific protein kinase. The amino end of TPM3 has been found to be fused with tyrosine protein kinase domain and has become a new oncogenic protein. These results indicate that cytoskeletal tropomyosin is related to the generation and development of colon carcinoma. Furthermore, Lawrence and his associates[46] reported that TPM3-ALK (anaplastic lymphoma kinase, ALK) and TPM4-ALK fusion genes occurred in inflammatory myofibroblastic tumors. In our study, cytoskeletal tropomyosin (TPM3) showed overexpression in the NMPs fraction of SHEEC cells, indicating that cytoskeletal tropomyosin as a oncoprotein might be related to esophageal carcinoma. But its characteristics and functions in esophageal carcinoma remain to be further studied.

FK506-binding protein 6 (FKBP6) is one member of FKBP family. The FKBP family proteins were those directly binding to the immunosuppressant drug FK506[47]. So far there are several FKBP family members, namely FKBP6, FKBP12, FKBP13, FKBP25 and FKBP52. These FKBP family members differ in their subcellular localization. FKBP12 and FKBP52 are localized in the cytosol, FKBP13 in the endoplasmic reticulum, and FKBP25 in the nucleus. Like other FKBP members, FKBP6 had some peptidyl-prolyl cis-trans isomerase activity and a drug binding site[48]. Owing to its possession of cis-trans isomerase activity, FKBPs are thought to be essential for protein folding during protein synthesis. FKBP6 gene is localized in chromosome 11 and consists of nine exons. Meng and his associates[49] found FKBP6 gene was deleted in Williams syndrome (WS), and this might contribute to certain defects such as hypercalcemia and growth delay in WS. In our study, FKBP6 was overexpressed in the NMPs fraction of SHEEC. This indicated that FKBP6 as an active NMP might play a role during malignant transformation of the immortalized human esophageal epithelial cells.

Similar to retinoblastoma binding protein 8 (STRBP8), it contains 230 amino acid residues. Its sequences are the same as residues 668-897 of retinoblastoma binding protein 8 (RBP8), and so it is named STRBP8. So far there are not any reports about the function of STRBP8. Moreover, RBP8 is localized in the nucleus. It might interact with some tumor suppressive factors including CtBP1, Rb1 and BRCA1, and was involved in transcription regulation and tumor suppression[50,51]. The interaction with CtBP1 requires a short amino acid motif PLDLS (residues 490-494) of RBP8, whereas the Rb1 tumor suppressor binding to a LXCXE motif is located within the N-terminal region of RBP8 (residues 153-157). Unlike RBP8, STRBP8 has no Rb1 binding motif (sequence LXCXE) and CtBP1 binding motif (sequence PLDLS). The results indicate that STRBP8 possibly loses the function of tumor suppression and on the contrary possesses carcinogenetic effect. The fact that STRBP8 was overexpressed in SHEEC in our study, showed that STRBP8 as a new oncoprotein might

be involved in the malignant transformation of SHEE.

Although three NMPs have been found to be associated with esophageal carcinoma in the present study, but whether they would become specific markers of esophageal carcinoma needs to be further studied. In addition, the generation and development of tumors are the extraordinary pathological complex phenomena, and the process of coordinated interaction and programed development of many proteins. It will help elucidate the process of tumor generation and development to study the network relationship of these tumor associated proteins. Therefore, the network functional relationship of these differentially expressed NMPs needs to be further studied.

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