Search for Differentially Expressed Proteins Involved in the Treatment of Human Nasopharyngeal Carcinoma Cells with NGX6 Using Two-dimensional Electrophoresis and Mass Spectrometry*

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Abstract The current study was designed to reveal the preliminary function of NGX6 to NPC cells. In search of mechanisms of NGX6 2-D PAGE was used to identify proteins that were overexpressed in HNE cells that were transfected with NGX6. After staining and image analysis, spots of interest were isolated and subjected to mass spectrometry. Seven proteins such as Fas, ZNF and MHC-Ⅱ Ag were identified. The possible significance of these findings is discussed. NGX6 may exert its effect on NPC cells by many ways.

Key words two-dimensional gel electrophoresis, cell transfection, matrix-assisted laser desorption, ionization mass spectrometry

Nasopharyngeal carcinoma (NPC) is one of aggressive malignant head and neck squamous cancers (HNSCC) with a high incidence in southern China and southeast Asia[1]. Epstein Barr virus (EBV) infection, genetic factors, dietary and certain environmental factors were found to be closely associated with this disease. The fact that 5% ~ 10% NPC patients have family history suggests that genetic susceptibility might play an important role in the pathogenesis of NPC[2~4].

We performed loss of heterozygosity analysis and cloned a novel putative tumor suppressor gene (TSG), named NGX6, with significantly down-regulated expression in NPC from the minimal common deletion region D9S161-D9S1853[5]. A recent study by our group showed that NPC cells transfected with a construct carrying NGX6 displayed a reduced growth rate in culture conditions (article revised), but the mechanism is not clear.

Two-dimensional (2-D) gel electrophoresis is a powerful tool since the expression of a large number of proteins can be evaluated in single experiments[6,7]. We have employed 2-D analysis of human tumor cell extracts to examine protein expression patterns between HNE1 cells and cells transfected by NGX6, and identified several proteins, which were overexpressed. These *in vitro* studies may be helpful in studying mechanisms that may lead to the function of NGX6.

1 Materials and methods

1.1 Chemicals and materials

The IPGphor isoelectric focusing system, peristaltic pump1, linear immobilize dry strips pH3-10, dithiothreitol (DTT), β-mercaptoethanol, carrier ampholyte mixture, and low molecular weight calibration kit were from Amersham Pharmacia-Biotech (Uppsaia, Sweden). PDQuest software was from Bio-Rad (Richmond, CA, USA), as well as urea, piperzine diacrylyl (PDA), zinc-imidazole staining kit and p*I* calibration markers. Agarose was from Gibco BRL (Grand Island, NY, USA).

1.2 Cell lines and cell culture

HNE-1 cell line was constructed by our laboratory[8]. Cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS). The cells were collected during the exponential growth phase.

1.3 Construction of vectors

The NGX6 cDNA 2.1kb fragment that was obtained from the plasmid pBluescript Ⅱ SK including NGX6 was cloned into the *Eco*R Ⅰ/*Xho*Ⅰ sites of

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the pcDNA3.1 (+) vector (Invitrogen, Carlsbad, Calif). The directional cloning of NGX6 cDNA insert was confirmed by restriction mapping.

1.4 Transfection

HNE-1 cells were transfected with the NGX6 vector, or with the pcDNA3.1 (+) vector using Lipofectin (Life Technologies) according to the supplier's instructions. Cells grown to 80% confluency were harvested for experiment.

1.5 Protein extraction

Cells were centrifuged at 2 000 r/min, washed in ice-cold PBS 4 × 10 min, resuspended in PBS, and then counted. The number of cells was adjusted to 3×10^8 cells/ml. Soluble protein were extracted with buffer containing 50 mmol/L Tris-HCl, pH 7.4, 10 mmol/L EDTA, 65 mmol/L DTT, 1.5 mmol/L phenylmethylsulfonyl fluoride (PMSF), and one tablet of anti-proteases for 10 ml of buffer as previously described. After centrifugation, the supernatant containing soluble proteins was supplemented with 7 mol/L urea, 2 mol/L thiourea and 4% CHAPS. Aliquots were stored at − 20 ℃ until used.

1.6 2-D electrophoresis with immobilized pH gradient strips

2-D electrophoresis was performed as described[9], using precast immobilized pH gradient (IPG) strips (pH 3~ 10, linear, Pharmacia, Uppsala, Sweden) in the first dimension (IEF). Samples were applied via rehydration of IPG strips in sample solution overnight. Before application, samples were diluted to a total volume of 350 μl with 8 mol/L urea, 2% CHAPS, 2% IPG buffer (pH 3~ 10, linear), 0.3% DTT and a trace of bromophenol blue. Typically, 500 μg protein were loaded on each IPG strip and focusing was carried out for 45 500 Vh. After IEF separation, the strips were immediately equilibrated 2 × 15 min with 50 mmol/L Tris-HCl, pH 6.8, 6 mol/L urea, 30% glycerol and 2% SDS. In the first equilibration solution, DTT (2%) was included, and 2.5% iodoacetamide was added in the second equilibration step to alkylate thiols. SDS-PAGE was performed using 0.75 mm thick, 10%~ 13% SDS-polyacrylamide gradient gels with piperizine diacrylamide as cross-linker. The strips were held in place with 0.5% agarose dissolved in SDS-Tris running buffer and electrophoresis was carried out at constant current (40 mA/gel) and temperature (20 ℃). After electrophoresis, gels were stained with silver nitrate.

1.7 Image analysis and spot identification

Image analysis was performed using the PDQuest system according to the protocol provided by manufacturer. To account for experimental variations, three gels were prepared for each cell line. The gel spot pattern of each gel was summarized in a standard after spot matching. Thus, we obtained one standard gel for each cell line. These standards were then matched to yield information about new spots related to the gene transfection (up- or down-regulation of spots).

1.8 In-gel protein digestion

The stained protein spots were excised from preparative gels using biospy punches. Proteins were in-gel digested as previously described[10]. Briefly, the spots were washed several times with 50% acetonitrile, which was then removed. Gel pieces were dried in a vacuum centrifuge. The cysteine reduction and alkylation steps consisted of incubation first in 10 mmol/L DTT, 100 mmol/L NH_4HCO_3 for 45 min in the dark at room temperature. The gel pieces were then dried again and rehydrated in 30 μl of 50 mmol/L NH_4HCO_3 containing trypsin for 45 min in ice. The concentration of trypsin used was 0.1 mg/L. The excess liquid was removed and the pieces of gel were immersed overnight in 50 mmol/L NH_4HCO_3 at 37 ℃. The resulting peptide mixture was extracted from the gel by centrifugation. Desalting of peptides was performed using Ziptips following the manufacture's instructions.

1.9 MALDI-TOF-MS analysis

Mass spectra were recorded in positive mode of a MALDI-TOF-MS equipped with a delayed extraction device. DBA was used as matrix. A volume of 0.5 μl was mixed with the same volume of the sample. The TOF was measured using the following parameters: 21 kV accelerating voltage, 74% grid voltage, 0% guide wire voltage, 200 ns delay and low mass gate of 500. External calibration was preformed using desArg1-bradykinine ($M+H^+$, 904.46) and ACTH (clip 18~ 39 $M+H^+$, 2465.20) in the same series as the samples to be measured. Internal calibration was also performed using autodigestion peaks of trypsin ($M+H^+$, 906.50 and 2163.06)[10].

2 Results and discussion

2.1 Two-dimensional polyacrylamide gels of HNE1 cells and NGX6-transfected cells

Pairs of samples collected at the same time from

normal and treated cells were loaded onto first-dimension gels for isoelectric focusing using pH 3~ 10 immobilized pH gradient strips. Horizontal SDS-PAGE was performed immediately following IEF; pairs of gels were run simultaneously from the same power supply. A representative gel is shown in Fig. 1. The image analysis software typically detected approximately 500 spots on each gel following silver staining. This approach to sample preparation and 2D PAGE favors the detection of relatively abundant cytosolic proteins. Basic proteins as well as very large or poorly soluble proteins are discriminated against. Three pairs of gels from different batches of control and transfected cells were analyzed for the purpose of quantitative spot comparisons with the image analysis software. Several preparation of cells were pooled, subsequently, to amass enough protein so that spots of interest could be digested and analyzed using mass spectrometry and computer database searches.

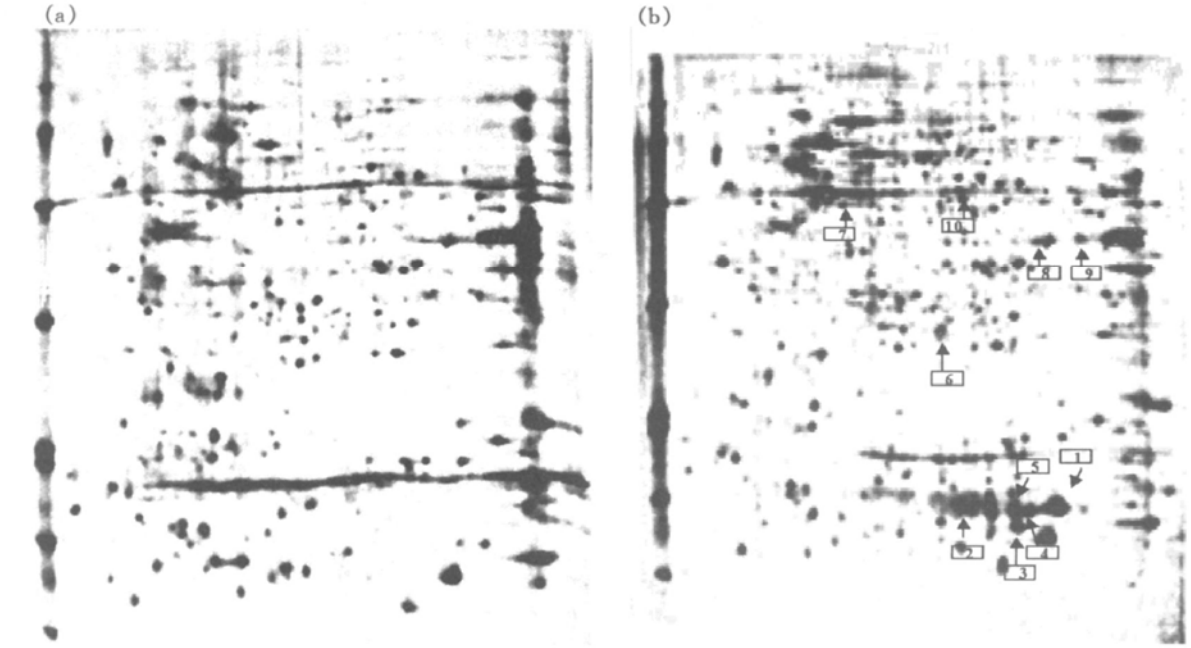

Fig. 1 2-D electrophoresis pattern of cell extracts from HNE cell line (a) and NGX6-transfected (b) cells
150 μg protein was loaded, IEF at pH 3~ 10 linear, 10%~ 13% SDS-PAGE and silver staining. Spots that were found to be overexpressed have been annotated.

2.2 Image Analysis

Silver-stained gels were digitized using a flatbed color scanner and saved as grayscale images. Images were studied with the aid of PDQuest analysis software. Gels were matched in pairs and differences in abundance were calculated for each spot; spots that increased or decreased consistently could then be identified, even if relative abundance of particular spots varied with the cell preparation. If the abundance of a given protein is not related to transfection, then its spot will increase in intensity on some pairs of gels, and decrease on others, whereas proteins up- or down-regulated in treated cells will consistently go up or down in abundance. This analysis revealed that 10 spots showed higher expression in treated cells ($P < 0.05$) (Fig. 1). The spots that clearly overexpressed in the treated cells are marked with an arrow.

2.3 Mass spectrometry and protein identification

In view of the large number of proteins to be identified (Fig. 1), MALDI-TOF-MS peptide was adopted. Ten spots distributed on different places of the gels were excised and in-gel digested. Fig. 2 showed the spectrum of the trypsin digest of spot 8. Seven proteins were successfully identified by MALDI-TOF-MS. One protein could not be identified by mass fingerprinting, and two digests produced no spectrum. Table 1 lists the identified protein. These proteins were not yet positioned on 2-DE maps in SWISS-2D-PAGE.

ZNF255 is probable a nuclear protein and may function as a transcription factor, which belongs to the krueppel family of c2h2-type zinc-finger proteins[11]. The zinc finger domain is a very ubiquitous structural element whose hallmark is the coordination

of a zinc atom by several amino acid residues (cysteines and histidines, and occasionally aspartate and glutamate). These structural elements are associated with protein-nucleic acid recognition as well as protein protein interactions. It could lead to differential expression of some proteins known to be involved in the proliferation of cells. NGX6 may exert its function by up-regulating the expression of ZNF255.

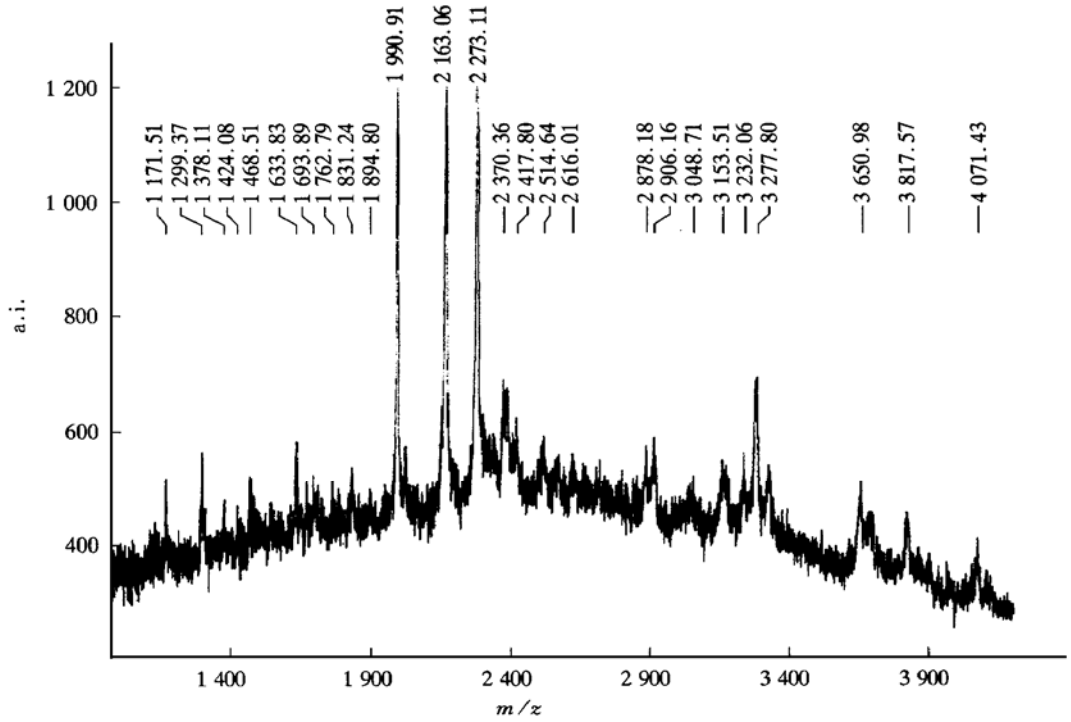

Fig. 2 Peptides mass fingerprint of spot 8

The spot was in-gel digested with trypsin. After desalting, the peptide mixture was analyzed by MALDI-TOF-MS.

Table 1 Protein identified by mass spectrometry

Spot (cf. Fig. 1)	SWISS-PROT (Accession No.)	Detected peptides	Score	Coverage seq. /%	Protein identified
1	Q16652	14	0.43	43.9	Fas soluble protein
2	Q9UFW2	15	0.27	39.8	Hypothetical protein
3	Q9TP94	23	0.17	44.9	MHC class II antigen
4	P01824	19	0.22	51.9	IG heavy chain V-II region
5	(-)	(-)	(-)	(-)	(-)
6	(-)	(-)	(-)	(-)	(-)
7	P35237	16	0.44	37.8	Protease inhibitor 6
8	Q9UID9	20	0.50	49.2	ZNF255
9	(-)	(-)	(-)	(-)	(-)
10	Q16654	19	0.26	16.3	PDK4

Fas (CD95/APO-1) is a cell surface "death receptor" that mediates apoptosis upon engagement by its ligand, FasL[12]. The Fas-mediated pathway could contribute to the early steps of drug-induced apoptosis while sensitization to the cytokine TRAIL could be used to amplify the response to cytotoxic drugs[13]. So the expression of NGX6 may stimulate Fas signaling and induce the apoptosis of tumor cells.

Major histocompatibility complex (MHC) is a cluster of genes encoding the major histocompatibility antigens (cell-surface glycoproteins involved in antigen recognition in immune responses), some complement proteins and other surface proteins of immune system cells. One limitation of cancer immunotherapy is that natural tumor antigens elicit relatively weak T cell responses, in part because high-affinity T cells are rendered tolerant to these antigens. The higher expression of MHC improved immunity to tumor cells.

Immunol globulin (Ig) is also involved in immunology response. Expression of NGX6 may improve the immunity to tumor cells.

The relation of the two proteins (protease inhibitor 6 and PDK4) to function of NGX6 is unclear. PDK4 inhibits the mitochondrial pyruvate dehydrogenase complex by phosphorylation of the E1 alpha subunit, thus contributing to the regulation of glucose metabolism[14]. Protease inhibitor 6 is a cytoplasmic protein, which belongs to the serpin family[15].

3 Conclusion

Although we cannot present the exact mechanisms through which these proteins lead to the function of NGX6, Fas protein, ZNF and MHC are promising objects for further investigation on how they can play a part in NGX6 function.

Advances in 2D electrophoresis, mass spectrometry, and bioinformatics along with progress in genomic sequence analysis now make possible the large-scale examination of protein expression patterns. These improved methods were used to identify seven proteins found to be up regulated in expression level in NGX6-transfected HNE cells. These informations can help us understand the mechanism of function of NGX6 well. Further automation of these integrated techniques should enable the high throughout analysis of protein expression in different cell types, stages of development, and disease states.

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用双向电泳和质谱技术检测 NGX6 转染后人鼻咽癌细胞表达差异的蛋白质*

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摘要 NGX6 是克隆的鼻咽癌相关基因，它的功能与作用机制目前尚不十分清楚．通过脂质体转染把 NGX6 导入鼻咽癌细胞株中，采用双向凝胶电泳分离细胞内所有蛋白质，通过软件分析，找到与未处理细胞表达差异的蛋白质，通过质谱分析和生物信息学资料处理．鉴定出七种表达上调的蛋白质，其中包括 Fas 蛋白，锌指蛋白(ZNF)，主要组织相容性抗原Ⅱ（MHCⅡ）等．Fas 蛋白参与细胞凋亡的信号传导途径，它的上调可以促进细胞凋亡；ZNF 蛋白参与基因的转录调控，它的上调也可影响细胞异常增殖的信号传导通路；MHCⅡ可以促进机体对肿瘤细胞的免疫应答．这些结果说明 NGX6 可能通过多种途径抑制鼻咽癌细胞的生长，为研究 NGX6 的作用机制提供了很好的实验资料，对鼻咽癌的基因治疗奠定了一定的研究基础，也为研究其他基因的作用机制提供了新思路．

关键词 双向电泳，细胞转染，基质辅助性激光解吸飞行时间质谱
学科分类号 R73

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书讯：《蛋白质纯化与鉴定实验指南》

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