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A proteomics analysis for certain signature proteins of rabbit lacrimal passages after ^{125}I seeds brachytherapy

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Abstract To search for certain signature proteins and the expression profiles in lacrimal passage stenosis, rabbit models of lacrimal passage stenosis were treated by ^{125}I seed brachytherapy. All the signature proteins were separated by two-dimensional electrophoresis, and identified by mass spectrometry. The results show that the up-regulated proteins are peptidyl-prolyl cis-trans isomerase A (PPIase A), and epidermal fatty acid-binding protein (E-FABP), while the down-regulated proteins are myosin light chain 1(isomer of skeletal muscle), myosin light polypeptide 6 (isomer 1 of smooth muscle and non-muscle), myosin light chain 1(isomer of slow-twitch muscle A), isomer 2 of ERC protein 2, and α-crystallin family protein. The proteins may play a role in healing the wound and regulating synaptic active zone of neurons due to correlation to cell apoptosis, proliferation and migration of smooth muscle cell. These provide molecular mechanism for preventing stenosis and restenosis of lacrimal passage.

Key words Lacrimal passage stenosis, Radioactive nuclide, Probing of lacrimal passage, Differential expression protein, Proteome.

1 Introduction

Lacrimal passage stenosis is a common ophthalmic disease in elder people, and probing lacrimal passage is considered as a strategy of therapeutic efficacy. The therapeutic outcomes, however, were not desirable[1], and for most patients of the probing treatment, *in situ* restenosis occurred within several months. It was reported that radioactive brachytherapy with ^{125}I seeds could affect short-term outcomes on treating lacrimal passage stenosis and preventing restenosis[2]. The ^{125}I seeds were applied in rabbit models of lacrimal passage stenosis. The tissue growth was inhibited and the proliferation capacity of fibroblasts in lacrimal passage was reduced significantly.

In this study, the signature proteins for ^{125}I brachytherapy of rabbit models of lacrimal passage stenosis were separated and identified by 2-DE (2-D electrophoresis) and mass spectrometry. The results provided a molecular basis for preventing lacrimal passage stenosis and restenosis.

2 Materials and methods

2.1 Experimental animals

Twenty white rabbits of New Zealand (2.5±0.5 kg, 8–12 weeks old) were provided by the Animal Center of Jilin University. Their unobstructed lacrimal passages at normal anatomical locations were demonstrated by digital subtraction angiography (76% diatrizoate).

2.2 Reagent and instruments

The IPG prefabricated gel strips (pH 3–10, 7 cm and 17 cm), IPG buffer (pH 3–10), covering solution, and staining kit were from Phamacia, Sweden. Acrylamide, bisacrylamide, uera, glycine, Tris, CHAPS, and SDS were purchased from Amresco Inc., USA. Mixed bed resin, thiourea, TBP, DTT, and PMSF (Bio-Rad, USA), trypsin, CCA, TFA, and iodoacetamide (Sigma-Aldrich Corp.), and the standard protein (2-DE Mr)

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(Pierce Biotechnology Inc.) were purchased overseas. $Na_2S_2SO_3$, NH_4HCO_3, and $CaCl_2$ (AR) were purchased in China. The ^{125}I seeds were from China Institute of Atomic Energy. Isoelectric focusing instrument (IPGphor), vertical electrophoresis tank (EttanDALT), ultra-centrifuge (Beckman L7) and image scanner were from Amersham Biosciences, Plc. Electro-phoresis apparatus (PRO-TEAN II xiceII), and image analysis software (PD-Quest) were from Bio-Rad.

2.3 The rabbit lacrimal passage stenosis model

The rabbit model of bilaterally lacrimal passage stenosis[3] was prepared by hooked probe scratch method. All the rabbits were fed regularly until the epiphora symptom occurred. The digital subtraction angiography analysis revealed that lacrimal passage stenosis was located at 4-mm lacrimal punctum. The ^{125}I seeds and medical thermoplast tube were prepared according to Ref.[4]. Ten rabbits received the ^{125}I seed treatment at 7–8 Gy, while the control group had 10 rabbits received physical probing of lacrimal passage.

2.3 Preparation of tissue protein

Forty specimens of bilateral lacrimal passages were collected on the 30th day, 20 specimens each from the treatment and control groups. The tissue samples were rinsed by pre-cooled normal saline, drained and cryo-preserved in liquid nitrogen (LN_2). The 240–260 mg tissue was grinded in a mortar under LN_2. After nitrogen volatilizing, 3 mL sample was slowly added into a mixture of 0.047 g DTT, 0.12 g CHAPS, 0.46 g thiourea, 1.44 g uera, 0.015 g Tris, and 30 μL 100 mmol·L^{-1} PMSF, and lysed at 4°C for 14 h. Centrifuged at 12000 rpm for 30 min, the supernatant fluid was dialyzed in 0.101 mol·L^{-1} PBS for 24 h, 10 times diluted by pre-cooled acetone, kept at –20°C for 2 h, and centrifuged again at 10000 r·min^{-1} for 10 min. The sediment obtained by volatilizing acetone at 4°C was put in a 5-times lysate buffer (7 mol·L^{-1} urea, 0.5% ampholyte with pH 4–7, 2 mol·L^{-1} thiourea 4% CHAPS, 100 mmol·L^{-1} DTT, 40 mmol·L^{-1} Tris) for 2 h, and centrifuged at 10000 r·min^{-1} for 10 min to obtain the supernatant fluid.

2.4 2-D electrophoresis

2-D electrophoresis of the samples from the treatment and control groups were conducted in parallel. The first-dimensional isoelectric electrophoresis was performed by the IPGphor isoelectric system using Gorg method[5]. The gel equilibrium was achieved after focusing, and the IPG prefabricated gel strips were moved to the upper end of the 0.75 mm thick spacer gel. The 2-DE was performed by injecting the SDS standard protein, and the resultant proteins were obtained by silver staining as described in Ref.[6].

2.5 Image analysis

The 2-DE gel images were obtained by fluoroscopic scanner using a 300 DPI resolution at a 1:1 scale of image and gel. The intensity, background and point of detection were emendated by the PD-Quest software. The three 2-DE images were matched into an averaged gel, using the 2-DE images of control group as reference to compare with treatment group. Same protein spots in different gels can be automatically matched by the software using their correlated position, molecular mass and appearance. If not, the spots were marked as a specific protein.

2.6 LCQ-DECA XP^{plus} analysis and database scanning

Marked protein spots were retrieved from 2-DE gel, and conducted by in-gel digestion using 2 mg·L^{-1} trypsinase. The peptide mass fingerprint (PMF) was analyzed by the LCQ-DECA XP^{plus} analysis (liquid chromatography-electrospray ionization mass spectrometry, Proteomics Research Center, Chinese Academy of Medical Sciences), and matched by the extracted peptides in ITI database using SEQUEST software.

3 Results and Discussion

3.1 2-DE analysis of control/^{125}I seeds groups

The 2-DE images of the treatment and control groups are shown in Fig.1. The protein spots were calibrated by molecular weight and isoelectric points, and evaluated by statistical analysis using student′s t-test under normalization and gel matching. The results show that the two groups differ in five spots.

3.2 LCQ-DECA XP^{plus} analysis of 5 protein spots

The 5 protein spots were analyzed by LCQ-DECA XP^{plus} mass spectrometry (Figs.2–5, the results will be discussed in Section 3.3). The corresponding amino acid sequences were acquired to identify seven proteins using database alignment (Table 1). The ^{125}I probing of lacrimal passage showed that the proteins of up-regulated expression were PPIase A and E-FABP, and the down-regulated expression profiles, accordingly, included the myosin light chain 1 (isomer of skeletal muscle), myosin light polypeptide 6 (isomer 1 of smooth muscle and non- muscle), myosin light chain 1 (isomer of slow-twitch muscle A), isomer 2 of ERC protein 2, and α-crystallin family protein.

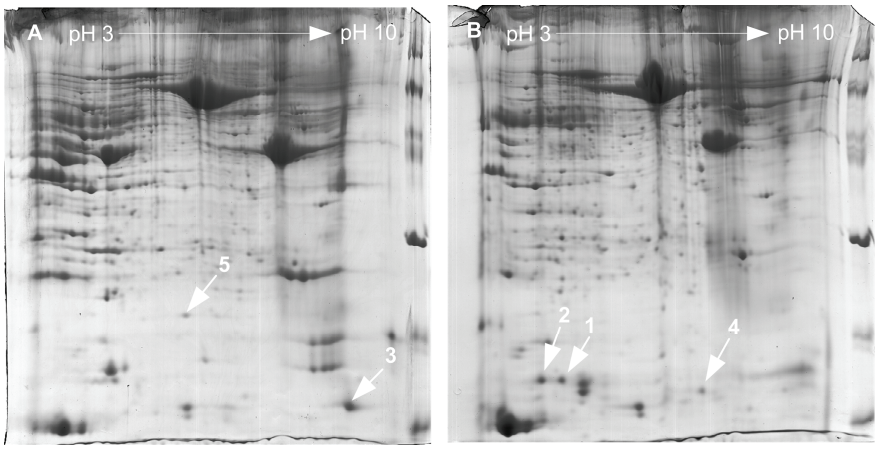

Fig.1 2-D electrophoresis gel images stained by coomassie for expressing proteins in rabbit lacrimal passage, (A) the control group, and (B) ^{125}I brachytherapy group. The differential protein spots were marked with numbers of 1–5.

Table 1 Identification results of the tissue proteins differently expressed between the control and treatment groups.

Spots[a]	Accession[b]	Proteins	Average mass	pI	Sequence coverage%
1 and 2	IPI00419585.8	Peptidyl-prolyl cis-trans isomerase A (PPIase A)	17882.2	7.82	7.32
3	IPI00216070.2	Myosin light chain 1 (isomer of skeletal muscle)	21014.8	4.97	21.24
	IPI00335168.8	Myosin light polypeptide 6 (isomer 1 of smooth muscle and non-muscle)	16931.0	4.56	10.60
	IPI00027255.1	Myosin light chain 1 (isomer of slow-twitch muscle A)	22764.8	5.56	6.25
	IPI00456707.1	Isomer 2 of ERC protein 2	111608.0	6.46	1.04
4	IPI00007797.2	Epidermal fatty acid-binding protein (E-FABP)	15034.2	6.82	8.21
5	IPI00022433.5	α-crystallin family protein	18784.4	7.91	5.71

[a] The spot number is according to spot position in 2-DE gel indicated as Fig. 1; [b] ITI accession number.

3.3 Discussion

Lacrimal passage obstruction leads usually to constant lacrimal pains, but some patients may suffer chronic dacryocystitis, seriously suppurative keratitis, corneal perforation, and endo-ophthalmitis, and even blindness. So far, the clinical treatment strategies have failed to achieve long-term cure for post-treatment cicatricial stenosis due to the hyperplasia of fibrous tissue. In clinical practice, β-rays have been widely used to treat benign disorders of fibrotic hyperplasia, e.g. post-operation cicatricial hyperplasia and cutaneous hemangioma, and achieved remarkable positive effects. In this study, the lacrimal passage restenosis[2] could be effectively inhibited by the ^{125}I seeds emitting low -energy γ-rays, due to the pathological hyperplasia related to epithelial cells and the suppressed phorocyte, and to the irradiation-caused apoptosis of epithelial cells and phorocyte under membranes of basement tissue.

In the Post-Genomic Era, proteomic analysis popularly facilitates protein expression profile, protein modification and interaction after translation, and provides the informative messages for exploring gene functions and epigenetic appearances. In this study, it was used to evaluate the changes of protein expression for lacrimal passage stenosis treated by ^{125}I irradiation, using 2-DE protein separation and mass spectrometry.

The seven proteins identified with the mass spectrometry played certain roles in the ^{125}I-treated lacrimal passage stenosis via possible pathways, such as apoptosis induction, effect of histiocytic phenotype changing on proliferation, migration and diffusion, promoting wound healing, and joining in construction and interactions in neuron synaptic activating sites.

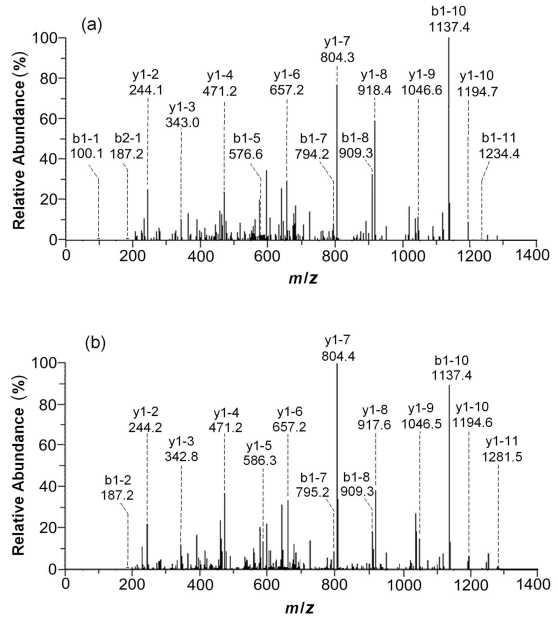

Fig.2 Mass spectra of protein Spot 1 (a) and Spot 2(b) (PPIase A).

Fig.2 shows the mass spectra of Spots 1 and 2, which belong to PPIase A (molecular chaperone family) that can change the protein backbone and nature if peptide bond of cis-trans isomerism are catalyzed between terminal carboxyl/amino groups of proline with polypeptide chain. In many physiological processes[7], such as signal transmission, programmed cell death and protein folding, the PPIase A could lead to apoptosis of neurocyte due to reaction of caspase cascade[8]. Leverson *et al*[9] found that the PPIase A could take part in regulation of transcription factor activity. In our experiment, the up-regulated PPIase A treated by the ^{125}I seeds might be involved in induction of apoptosis, such as, changing the protein nature and influencing the signal transmission, and inhibiting the development of lacrimal passage restenosis.

The myosin light chain corresponding to the mass spectrum of Spot 3 were considered to modulate ATPase activity in smooth muscle contraction (SMC), as shown in Fig.3(a)–(c). The SMC can be divided into synthetic or contractile phenotype. The former is less intracellular myofilaments contents due to its poor differentiation or un-differentiation, and can secrete multiple cytokines via proliferation, migration and matrix synthesis, while the latter highly dominate differentiation due to poor or no proliferation and migration. Fareh[10] proved that β-rays *in vitro* could turn the SMC between synthetic/contractile phenotype. So, the myosin light chain in smooth muscle was major markers for synthetic SMC[11].

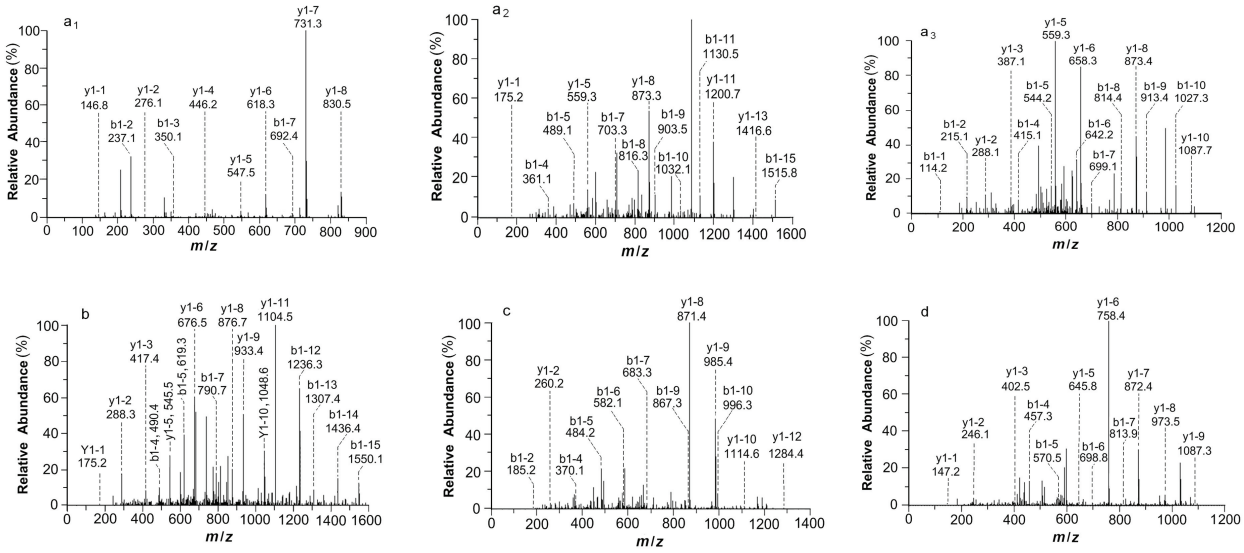

Fig.3 Mass spectra of protein Spot 3. (a) Myosin light chain 1 (isomer of skeletal muscle), (b) Myosin light polypeptide 6 (isomer 1 of smooth muscle and non-muscle), (c) Myosin light chain 1 (isomer of slow-twitch muscle A), (d) Isomer 2 of ERC protein 2.

In our study, SMC expression was down-regulated after the ^{125}I γ-ray irradiation. This indicates that the γ-ray irradiation changes the SMC phenotype and inhibites the hyperplasia in lacrimal passage tissue due to decreasing cell proliferation and migration.

The ERC2 protein in neuronal, known in the ERC (ELKS-Rab6-interacting protein-CAST) protein family, is a specific protein in the active site and presynaptic localization of RIM1[12–15], which is an active protein[16,17] released by regulating neuro-transmitter. Fig.3(d) shows that the ERC2 protein indexed from ITI database increased after radioactive probing, and could bind the RIM and various known isomers of liprin-α in culture neurocyte[18] to increase Synapsis concentration. This indicates that the interaction between ERC-liprin-α1 is related to presynaptic localization, active site composition and transportation. The ERC2 protein might be involved in construction and activity of neuron synapsis. However, whether the radionuclide inhibiting lacrimal passage restenosis is related to neuronal activities need be further clarified.

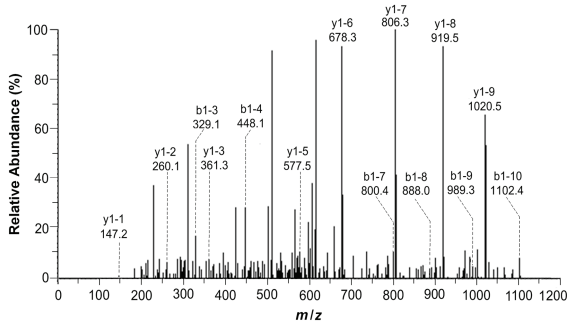

Fig.4 Mass spectrum of protein Spot 4 (E-FABP).

Fig.4 shows mass spectrum of Spot 4 corresponding to E-FABP in cytoplasm of epidermal cells, which is linked with life activities, such as transport and metabolism of fatty acid, intracellular signal transduction, and gene expression, and a critical to wound healing. The E-FABP genetic deletion can inhibit migration of keratinocyte, and adversely affect the wound healing process[19]. In addition, the E-FABP has anti-oxidation function due to the ability of clearing lipid peroxidative products form poisonous reagents[20,21]. The ^{125}I γ-rays produce free radicals and oxidative multiple molecules to worsen the tissue damages, the E-FABP high expression after ^{125}I γ-ray radiotherapy may reduce the oxidative damages of lacrimal passage tissues and accelerate tissue repairing process via promoting migration of keratinocyte, and eventually contributes to wound healing.

The mass spectrum of α-crystallin oligomer in the crystalline lens is shown in Fig.5. This belongs to a heat shock protein family and can be expressed in multiple tissues of non-crystalline lens as a micro-molecular chaperone due to the anti-oxidation and anti-apoptosis as dioptric media[22–24]. Besides, it indirectly modified counterparts influenced functional proteins. The αA and αB are subunits of the crystalline, and can inhibit activation of caspase-3[25,26] that mediates and reduces the apoptosis of myoblast[25]. *In vitro* experiment showed that the α-crystallin was localized in epithelial cells of porcine lens migrating, and could significantly aggregate in the front of cell membrane edge and lamellipodium cells migrating and potentiate the cell migration[27]. In this paper, the α-crystallin expression in the lacrimal passage tissue was down-regulated by the ^{125}I irradiation. This might reduce corresponding anti-oxidative and anti-apoptotic capacities in local area, and cause apoptosis of epithelial cells, fibroblasts and SMCs. In addition, the α-crystallin played a role in inhibiting lacrimal passage stenosis via inhibiting migration of the epithelial cells.

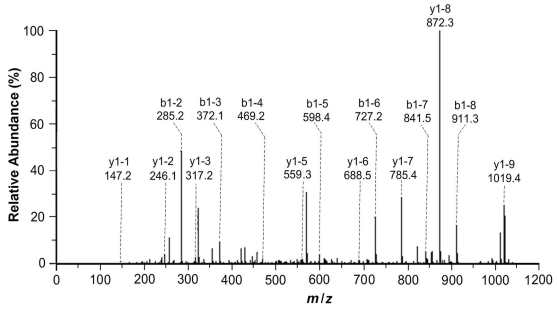

Fig.5 Mass spectrum of protein Spot 5 (α-crystallin family protein).

4 Conclusions

The seven novel proteins related to lacrimal passage stenosis treated by ^{125}I γ-rays were identified by using proteomics analysis, and could be used to partially explain mechanisms for tissue hyperplasia of lacrimal passage cicatrice. The ^{125}I seeds might provide a clue for curing lacrimal passage obstruction in clinical practice.

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