Journal of
Proteomics & Bioinformatics

Kanamoto et al. J Proteomics Bioinform 2011, 4:3
DOI: 10.4172/jpb.1000167

Research Article Open Access

Proteomic Analyses of Proteins Differentially Expressed in Recurrent and Primary Pterygia

Takashi Kanamoto[1]*, Nazariy Souchelnytskyi[2], Ryotaro Toda[1], Ulfah Rimayanti[1] and Yoshiaki Kiuch[1]

[1]*Department of Ophthalmology and Visual Sciences, Graduate School of Biomedical Sciences, Hiroshima University*

[2]*Karolinska Biomics Centre, Department of Oncology Pathology, Karolinska Institute, Stockholm, Sweden*

Abstract

We describe a proteomic approach to identify proteins which may be involved in the recurrence of pterygia. Tissues from a recurrent pterygium and from a primary pterygium were surgically resected and were analyzed by proteomics to identify proteins that were significantly up- or down-regulated. The proteins showing significant differences in the two tissues were identified by mass spectrometry. Eleven proteins were differentially expressed; seven proteins were up-regulated and four proteins were down-regulated in the recurrent pterygium. The identified proteins are known to regulate cell cycle, cell organization, extracellular matrix, cholesterol metabolism, and cell signaling. Up- and down-regulation of proteins for cellular signaling are most likely involved in the recurrence of pterygia.

Keywords: Pterygia; Recurrency; Proteomic approach

Introduction

Pterygia are growths on the ocular surface that invade the bulbar conjunctiva at the margin of the cornea and can become inflamed by neovascularization [1]. Studies on pterygia show that their pathogenesis is complex, and several factors are associated with the development of pterygia. Ultraviolet-B (UV-B) light is a major cause of pterygia, and chronic UV-B exposure induces oxidative stress that leads to an up-regulation of several mediators of the growth of pterygia [2]. UV-B irradiation stabilizes p53, a major cell cycle regulator, and the increased p53 is confined to the basal epithelial cells of pterygia [3]. These findings suggested that cell cycle-related molecules affect the growth of pterygia.

UV-B exposure also induces inflammatory mediators, such as interleukin-1 (IL-1), IL-6, IL-8, and tumor necrosis factor-alpha [4]. These mediators can increase the expression of metalloproteinase to modulate the expression of extracellular matrix (ECM), and E-cadherin and beta-catenin are abundantly expressed in pterygial tissue [5], and they play a role in the pathogenesis of pterygia and epithelial-mesenchymal transition [6].

Growth factors controlling cell signaling pathways are required for the development of pterygia [7]. Vascular endothelial growth factor (VEGF) is particularly elevated in pterygia [8] and is co-expressed with nitric oxide synthetase, and they contribute to angiogenesis and inflammation. In addition, cholesterol metabolism is involved in the development of pterygia, and a modification of cholesterol metabolism stimulates the proliferation of fibroblasts and pioglitazone (PIO) and everolimus (EVE), inhibitors of cholesterol ester cycle, which decrease the proliferation of fibroblasts in pterygia [9].

The postoperative recurrence rate of pterygia is 18%, [10] and the pathogenesis of a recurrent pterygium has still not been determined. A mutation of *Ki-ras* gene, a cell cycle regulator, has been associated with the postoperative recurrence of pterygia [11]. The expression of VEGF is higher in a recurrent pterygium than in a primary pterygium [12]. TIMP-2 has also been found in recurrent pterygia [13]. The number of CD34 positive cells was higher in a recurrent pterygium than in a primary one [14]. Human papilloma virus and herpes simplex virus are present in a recurrent pterygium [15]. These findings suggest significant differences between primary and recurrent pterygia and may be related to the pathogenesis of pterygia. However, little is known on the differences between primary and recurrent pterygia.

Thus, the purpose of this study was to identify proteins that are differentially expressed in recurrent and primary pterigia by proteomic profiling. We report eleven proteins whose expression was significantly different in primary and recurrent pterygia.

Experimental Section

Sample preparation

The procedures used in this experiment conformed to the tenets of the Declaration of Helsinki, and a signed informed consent was obtained from two patients to resect their pterygia and to use the tissue for research. The protocol was approved by the Institutional Review Board of Hiroshima University.

A sample of a recurrent pterygium was derived from a male patient, who was 75-years–of-age. He had undergone a surgical resection by the cut-and-suture method for the primary pterygium on the left eye 45-years earlier. Although the pterygium recurred when he was 69-years-old, he did not use any medication and the recurrent pterygium grew progressively. Finally, the recurrent pterygium was surgically resected (Figure 1A).

A primary pterygum was resected from a 54-year-old female patient who found the pterygium on her left eye at 51-years-of-age but did not seek treatment (Figure 2A).

The samples of the pterygia were washed in phosphate-buffered

***Corresponding author:** Takashi Kanamoto, Department of Ophthalmology and Visual Sciences, Graduate School of Biomedical Sciences, Hiroshima University, 1-2-3, Kasumi, Minami-ku, Hiroshima, Japan-734-8551, Tel: +81-82-257-5247; Fax: +81-82-257-5249; E-mail: tkana@hiroshima-u.ac.jp

Received March 06, 2011; **Accepted** March 24, 2011; **Published** March 26, 2011

Citation: Kanamoto T, Souchelnytskyi N, Toda R, Rimayanti U, Kiuch Y (2011) Proteomic Analyses of Proteins Differentially Expressed in Recurrent and Primary Pterygia. J Proteomics Bioinform 4: 058-061. doi:10.4172/jpb.1000167

saline (PBS) two times and solubilized in sample buffer (8 M urea, 4% CHAPS, 0.5% dithiothreitol (DTT), IPG buffer, pH 3-10). Aliquots of the lysates were stored at –70°C. The protein concentration in the lysates was measured by the Bradford assay.

Two-dimensional electrophoresis

Two-dimensional electrophoresis and protein identification were performed as described early [16]. Isoelectrofocusing was performed on the strips with an immobilized pH gradient (pH 3-10 non-linear gradient, 18 cm: GE Healthcare). First-dimension isoelectrophoresis was performed in IPGphor (GE Healthcare) according to manufacturer's instructions. After the isoelctrofocusing, the strips were placed in equilibration buffer-1 (50 mM Tris-HCl, pH 8.8, 6.0 M urea, 2.0% SDS, 30% glycerol, 1% DTT) and then in equilibration buffer-2 (50 mM Tris-HCl, pH 8.8, 6.0 M urea, 2.0% SDS, 30% glycerol, 4% iodoacetamide). The equilibrated strips were loaded onto SDS-containing 12% polyacrylamide gel, and SDS-polyacrylamide gel electrophoresis (PAGE) was performed. After the electrophoresis, the gels were fixed in 7.5% acetic acid and 20% methanol, and sensitized in 25% ethanol, 0.2% sodium thiosulfate, and 3.4% sodium acetate. The gels were then stained with 0.25% silver nitrate and developed with 2.5% sodium carbonate and 0.04% formaldehyde.

Gel analyses

Silver-stained gels were scanned by an image scanner (EPSON) and analyzed with calculations of the volumes of the spots with the PD-Quest software (BioRad) following the manufacturer's instructions. Three gels from each type of chicken were prepared and a master gel was generated for each type of mouse. The values of the volume of each matched spot were compared. Spots with differences in expression were then identified by mass spectrometry.

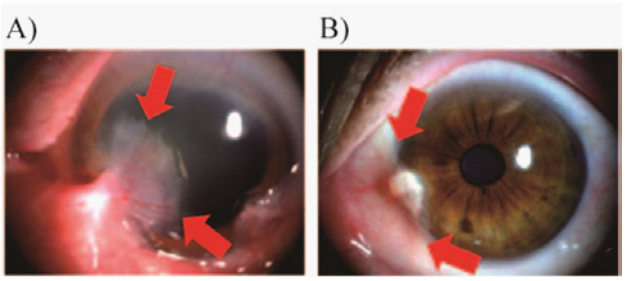

Figure 1: Slit-lamp examination of subjects.
A. Recurrent pterygium can be seen to have expanded onto the nasal cornea and is adherent to the lower palpebral conjunctiva.
B. primary pterygium with hyperemia can be seen on the nasal bulbar conjunctiva. Arrows indicate the resected pterygum.

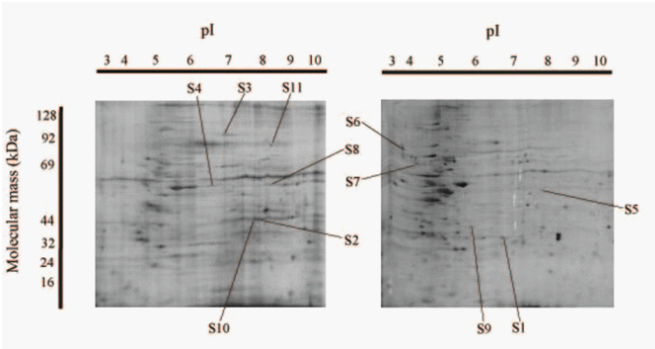

Figure 2: Images of two-dimensional electrophoresis gels with annotation of the spots of the identified proteins. The image on the left shows a silver-stained gel of a recurrent pterygium. The right gel shows the results of the tissue from primary pterygium. Spots S1 through S11 represent the annotated spots. The pI gradient of the first dimension electrophoresis is shown at the top of the gels, and the migration of the molecular mass markers for SDS-PAGE in the second dimension is shown at the side of the gel.

Protein identification

The excited protein-containing spots were de-stained with 30 mM potassium ferricyanide and 100 mM sodium thiosulfate. Then, the gel pieces were dipped in 0.1 M sodium hydrocarbonate and washed with acetnitril. After the gel pieces were dried, in-gel digestion was performed with trypsin. Then, 10% trifluoroacetic acid (TFA) and acetonitrile were used to extract the peptides, and the extract was desalted on a nano-column. After washing the column with 0.1% TFA, the matrix was eluted with acetnitril containing alpha-cyano-4-hydroxycinnamic acid directly onto the MALDI target. Spectra were generated on a MALDI-TOF-MS (Bruker Daltonics). The spectra were internally calibrated using known internal tryptic peptides from trypsin and searches were made in the NCBI sequences using ProFound. No restrictions on species and pI were applied, and tolerance was set on less than 0.5 Dalton. The search results were evaluated by considering the probability, the Z-value, peptide coverage, and correspondence to experimental pI and molecular mass.

Results

Two-dimensional proteomic maps of pterygia

To identify the proteins associated with the recurrent pterygium, we compared the proteome of the recurrent pterygium tissue to that from the primary pterygium. The total lysates of the tissue were resolved by two-dimensional gel electrophoresis. We detected an average of 80 protein spots on the two-dimensional gels after silver staining (Figure 2).

The volume of all of the protein spots was quantitatively analyzed, and the maximum volume of a single spot was 162,668 arbitary units (au). We selected 33 protein spots, that were expressed either only in the panel of the recurrent pterygium (11 spots) or only in that of the primary pterygium (22 spots). Small and weak spots whose protein volume was less than 10,000 units were deleted from the analyses because their weak spots tend to fail technologically to be identified by mass spectrometry in the next experimental step. Seven protein spots in the recurrent pterygium and 9 in the primary pterygium were left.

MALDI TOF mass spectrometry was used to identify the proteins and 11 spots were successfully identified with a high quality spectra and sufficient reliability.

Clustering of identified proteins

Analyses of the identified proteins showed that the recurrent pterygium required proteins that were involved in different cellular functions. We found that the expression of 7 of the 11 (64 %) proteins, S2, S3, S4, S5, S8, S10, and S11, were specifically identified in the recurrent pterygium and the others, S1, S6, S7, and S9, were not expressed in the recurrent pterygium (Table 1).

Discussion

Earlier studies using the DNA microarray method showed the expression of extracellular matrix [17], increased expression of insulin-like growth factor binding protein-2 (IGFBP-2) [18] and decreased of IGFBP-3 in recurrent pterygia [19]. But the identified proteins did not

Spot	Protein	Sequence coverage(%)	ncbi ID	Theoretical value		Experimental value		Changes		
				pH	Mr (kDa)	pH	Mr (kDa)	RP	:	PP
S1	Hypothetical protein	36	NP_065189.1	6.2	22.1	7	36	(-)	:	(+)
S2	Glyceraldehyde-3-phosphate dehydrogenase	34	CAA25833.1	8.4	36.21	8	45	(+)	:	(-)
S3	Single recognition particule (54kDa)	17	NP_003127.1	9	55.97	7	150	(+)	:	(-)
S4	ACTB protein	25	AAH12854.1	5.6	40.54	6	60	(+)	:	(-)
S5	Similar to human albumin	9	AAA64922.1	5.7	53.43	7.5	60	(+)	:	(-)
S6	STAG1 protein	10	AAG64699.1	5.3	141.04	3.5	90	(-)	:	(+)
S7	Sarcolectin	22	CAB41416.1	5.6	51.45	4	80	(-)	:	(+)
S8	Arginiosuccinate synthetase 1	30	NP_446464.1	8.3	46.8	9	60	(+)	:	(-)
S9	Proteasome activator hPA28 subunit beta	52	BAB08205.1	5.4	27.5	5.5	40	(-)	:	(+)
S10	Hypothetical protein	10	CAH10535.1	5.8	55.73	8	45	(+)	:	(-)
S11	Transkeratolase	28	NP_001055.1	7.8	68.54	8.5	100	(+)	:	(-)

Table 1: Differentially expressed proteins identified by proteomics from recurrent pterygium tissue obtained. S1 to S11 represent ID number of spots and sequence coverage, and the theoretical value of pI and Mr were obtained from the Pro Found search. The calculations of the experimental pI and Mr were based on the migration of the protein on a 2D gel. (RP : recurrent pterygium, PP: primary pterygium).

include genes indicating that the identified proteins were differentially modified post-translationally.

Our results showed that STAG1 (S6) was down-regulated in the recurrent pterygium. STAG1 is one of the transcriptional factors for p53 and mediates p53-dependent apoptosis [20]. These properties indicate that STAG1 may play a role in a recurrent pterygium through regulation of cell cycles.

PA28 (S9), which is an interferon gamma-inducible proteasome activator, was down-regulated in the recurrent pterygium. PA28 is associated with the processing of p53 [21], and is also homologous to a Ki-antigen that contains the "KEKE" motif [22]. In addition, sarcolectin (S7), which is co-expressed with p53 and co-localized with Ki-67 in leiomyoma [23], was also down-regulated. These findings suggest that PA28 and sarcolectin are associated with p53 and Ki-ras-dependent signaling pathway for cell cycle regulation, and they may also have an effect on the recurrency of a pterygium.

ACTB protein (S4) is a neural-related gene expressed in the heart, and is one of mediators of the production of cholesterol [24]. In addition, transkeratilase-1 (S-11) is inactivated by thiamine therapy, and is involved with lipogenesis [25]. Both ACTB and transkeratilase-1 were up-regulated in the recurrent pterygium indicating that cholesterol metabolism was modified in the recurrent pterygium.

Conclusions

We have identified eleven proteins whose expressions were significantly different in a recurrent pterygium from that in a primary pterygium. Identified proteins included those that are involved in cell cycle, cholesterol metabolism, and various cellular functions.

References

1. DiGirolamo N, Chui J, Coroneo MT, Wakefield D et al. (2004) Pathogenesis of pterygia: role of cytokines, growth factors, and matrix metalloproteinases. Prog Retin Eye Res 23: 195-228.
2. Kau HC, Tsai CC, Lee CF, Kao SC, Hsu WM et al. (2006) Increased oxidative DNA damage, 8-hydroxydeoxyguanosine, in human pterygium. Eye 20: 826-831.
3. Tan DT, Lim AS, Goh HS, Smith DR (1997) Abnormal expression of the p53 tumor suppressor gene in the conjunctiva of patients with pterygium. Am J Ophthalmol 123: 404-405.
4. Di Girolamo N, Kumar RK, Coroneo MT, Wakefield D et al. (2002) UVB-mediated induction of interleukin-6 and -8 in pterygia and cultured human pterygium epithelial cells. Invest Ophthalmol Vis Sci 43: 3430-3437.
5. Kase S, Osaki M, Sato I, Takahashi S, Nakanishi K et al. (2007) Immunolocalization of E-cadherin and beta-catenin in human pterygium. Br J Ophthalmol 9: 1209-1212.
6. Kato N, Shimmura S, Kawakita T, Miyashita H, Ogawa Y et al. (2007) Beta-catenin activation and epithelial-mesenchymal transition in the pathogenesis of pterygium. Invest Ophthalmol Vis Sci 48: 1511-1517.
7. Kria L, Ohira A, Amemiya T (1998) Growth factors in cultured pterygium fibroblasts: immunohistochemical and ELISA analysis. Graefes Arch Clin Exp Ophthalmol 236: 702-708.
8. Aspiotis M, Tsanou E, Gorezis S, Ioachim E, Skyrlas A (2007) Angiogenesis in pterygium: study of microvessel density, vascular endothelial growth factor, and thrombospondin-1. Eye 21: 1095-1101.
9. Peiretti E, Dessi S, Mulas C, Abete C, Norfo C et al. (2007) Modulation of cholesterol homeostasis by antiproliferative drugs in human pterygium fibroblasts. Invest Ophthalmol Vis Sci 48: 3450-3458.
10. Fernandes M, Sangwan VS, Bansal AK, Gangopadhyay N, Sridhar MS et al. (2005) Outcome of pterygium surgery: analysis over 14years. Eye 19: 1182-1190.
11. Detorakis ET, Zafiropoulos A, Arvanitis DA, Spandidos DA (2005) Detection of point mutations at codon 12 of KI-ras in ophthalmic pterygia. Eye 19: 210-214.
12. Di Girolamo N, Wakefield D, Coroneo MT (2006) UVB-mediated induction of cytokines and growth factors in pterygium epithelial cells involves cell surface receptors and intracellular signaling. Invest Ophthalmol Vis Sci 47: 2430-2437.
13. Kuo CH, Miyazaki D, Nawata N, Tominaga T, Yamasaki A et al. (2007) Prognosis-determinant candidate genes identified by whole genome scanning in eyes with pterygia. Invest Ophthalmol Vis Sci 48: 3566-3575.
14. Nolan TM, Di Girolamo N, Coroneo MT, Wakefield D (2004) Proliferative effects of heparin-binding epidermal growth factor-like growth factor on pterygium epithelial cells and fibroblasts. Invest Ophthalmol Vis Sci 45: 110-113.
15. Detorakis ET, Sourvinos G, Spandidos DA (2001) Detection of herpes simplex virus and human papilloma virus in ophthalmic pterygium. Cornea 20: 164-167.
16. Kanamoto T, Hellman U, Heldin CH, Souchelnytskyi S (2002) Functional proteomics of transforming growth factor-beta-1stimulated Mv1Lu epithelial cells: Rad51 as a target of TGF beta1-dependent regulation of DNA repair. EMBO J 21: 1219-1230.
17. Molykutty J-A, Nicholas D, Cynthia JJ, Cox CA, Schultz G et al. (2006) Microarray and protein analysis of human pterygium. Mol Vis 12: 55-64.
18. Solomon A, Grueterich M, Li D-Q, Meller D, Lee SB, et al. (2003) Overexpression of insulin-like growth factor-binding protein-2 in pterygium body fibroblasts. Invest Ophthalmol Vis Sci 44: 573-580.
19. Wong YW, Chew J, Yang H, Tan DT, Beuerman R (2006) Expression of insulin-like growth factor binding protein-3 in pterygium tissue. Br J Ophthalmol 90: 769-772.
20. Anazawa Y, Arakawa H, Nakagawa H, Nakamura Y (2004) Identification of STAG1 as a key mediator of a p53-dependent apoptotic pathway. Oncogene 23: 7621-7627.
21. Kuckelkorn U, Ferreira EA, Drung I, Liewer U, Kloetzel PM et al. (2002) The effect of the interferon-gamma-inducible processing machinery on the generation of a naturally tumor-associated human cytotoxic T lymphocyte epitope within a wild-type and mutant p53 sequence context. Eur J Immunol 32: 1368-1375

22. Ahn JY, Tanahashi N, Akiyama K, Hisamatsu H, Noda C et al. (1995) Primary structures of two homologous subunits of PA28, a γ-IF-inducible protein activator of the 20s proteasome. FEBS Letters 366: 37-42.

23. Choufani G, Mahillon V, Decaestecker C, Lequeux T, Danguy A et al. (1999) Determination of the levels of expression of sarcolectin and calcyclin and of the percentages of apoptotic but not proliferating cells to enable distinction between recurrent and nonrecurrent cholesteatomas. Laryngoscope 109: 1825-1831.

24. Schweitzer NB, Alessio HM, Berry SD, Roeske K, Hagerman AE (2006) Exercise-induced changes in cardiac gene expression and its relation to spatial maze performance. Neurochem Int 48: 9-16.

25. Babaei JR, Karachalias N, Kupich C, Ahmed N, Thornalley PJ (2004) High-dose thiamine therapy counters dyslipidaemia in streptozotocin-induced diabetic rats. Diabetologia 47: 2235-2246.