Proteomic Analysis of Gene Expression in Basal Cell Carcinoma

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Background: Basal Cell Carcinoma (BCC) is a type of non-melanoma skin cancer. Alteration in gene expression is the important event that happens in cancer cell. Detection of this event is possible by proteomics techniques.

Methods: Normal and tumor tissues were taken from BCC patient. Total proteins were purified by standard methods, and proteins were separated by two-dimensional electrophoresis (2DE). Mass spectrometry (MS-MALDI method) was used as a powerful instrument for protein identification.

Results: Eighty seven spots of protein were detected in 2DE gels out of which, 76 spots had different expression in normal and cancer gel images. Comparison with the data base showed 11 proteins. Nine spots had no association with BCC, but expression of ceruloplasmin and C3b were decreased and increased respectively in cancer tissues, compared to normal cell. The mass spectrometry results were aldolase C, Prx-cis and VDAC.

Conclusion: These recognized proteins, either with increased or decreased expression in cancer tissue, might be considered as new biomarkers in BCC patients.

Keywords: proteomics, basal cell carcinoma, biomarker, ceroluplasmin, prothrombin, C3b

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INTRODUCTION

Skin cancer is divided into two major groups: non-melanoma and melanoma. Basal cell carcinoma (BCC) is a type of non-melanoma skin cancer; it is the most common form of cancer [1] and can be destructive and disfiguring. The risk of developing BCC is increased by the following: family history, high cumulative exposure to UV light via sunlight [2] and carcinogenic chemicals, especially arsenic. Surgery, topical chemotherapy, x-ray, cryosurgery and photodynamic therapy are introduced for its treatment. It is rarely life-threatening but if left untreated, it can be disfiguring [3]. Genetic studies have shown that loss-of-function mutations in the tumor-suppressor genes or gain-of-function mutations in the smoothened gene lead to the formation of sporadic basal cell tumors [3,4]. A significant negative correlation between p53 and bcl-2 expression has been found in the basal cell carcinomas. A disturbance in either p53 or bcl-2 suffices to enhance skin tumor formation by suppressing apoptosis; bcl-2 appears to reduce the rate of spontaneous apoptosis, but an aberrant p53 expression does not, and this factor may solely affect the apoptosis from exogenous genotoxicity [5]. Frequencies of pathogenic genetic mechanisms are contained truncating mutation, missense mutation, splice site mutation and intragenic or large scale deletions or rearrangements. Ptc and smo mRNA expressions might be associated with BCC tumor progression and divide the BCC histological types into two subtypes, superficial and nodular [6]. The positive rate of the expression of p-STAT3 well

correlates with the depth of tumor invasion and with metastasis, but there is no correlation between the positive rate and tumor size [7]. By the way, the procedure that is used to analyze changes in expression of proteins in a cell is proteomics. In proteomic methods, by using two dimensional electrophoresis and mass spectrometry, gene expression can be analyzed. Here, we measured the amount of the expression of proteins in BCC by proteomic techniques and compared them with normal tissue.

PATIENTS AND METHODS

Sampling

Normal and BCC tissues samples were taken from patients. Every section of the sample was confirmed as normal or BCC tissue by a pathologist.

Protein purification [8]

Fresh tissue samples of skin were snap frozen and kept in liquid nitrogen until use. Tissue samples were powdered by micro dismembrator at the maximum speed for 60 seconds under liquid nitrogen conditions. Each powdered tissue sample was added to an appropriate amount of lysis buffer containing 10 mM Tris-HCl pH=7.5, 1 mM MgCl2, 1 mM EGTA, 0.1 mM Phenylmethylsulfonyl fluoride (PMSF), 5 mM beta mercapto ethanol, 0.5% CHAPS and 10% glycerol. After 30 minutes of incubation on ice, the lysate was centrifuged at 16000g for 30 minutes at 4°C. Protein concentration of all samples was estimated using a Bradford based microassay.

Two dimensional SDS-PAGES [9]

Briefly, normal and cancer cells on 25 mm^2 dishes were washed three times in PBS. About 300µl lysis buffer (7M urea, 2M thiourea, 4%CHAPS, 0.2-0.3%DTT, 1-2% ampholin 3-10) was added to cell culture and shaking was done in room temperature for about 1 hour. The lysate was centrifuged at 10000 g for 10 min at room temperature. The supernatant was saved at -20°C until used. Linear pH 3-10 Immobilized Dry Strip (17 cm) were rehydrated overnight at 20°C in rehydration buffer (8.5M urea, 2% CHAPS, 40mM DTT, 0.1% ampholin, 0.001% bromophenol blue). The sample (400µg) was applied during rehydration. The first dimension of the 2D electrophoresis was performed on the PROTEAN IEF Cell system (Bio-Rad). Next, gels were equilibrated for 15 min in the equilibration buffer I (6M urea, 2% SDS, 0.375 M Tris Hcl pH8.8, 20% glycerol, 130mM DTT). A 12% SDS-Polyacrylamide slab gel was used for the second dimension gel electrophoresis. Equilibrated IPG strips were placed on the surface of the second dimension gels and then sealed with 0.5% agarose in SDS electrophoresis buffer (25mM Tris base, 192mM glycine, 0.1%SDS) and were run vertically.

2DE gel staining

After electrophoresis, the gels were stained with Coomassie Brilliant Blue staining [10].

Bioinformatics analysis

2DE gel was scanned and both BCC and normal tissue gels were analyzed by flicker software to compare the spots in one statement in both gels and to get the density of the same spot in each gel.

Mass spectrometry

Silver stained protein spot containing the interested protein was distained thoroughly with 1% H2O2 (typically 1 min) and lyophilized to dryness. Silver stain removal using H2O2 was performed to enhance peptide adsorption by matrix-assisted laser desorption/ionization time-of- flight mass spectrometry (MALDI) [11]. The dehydrated gel bands were hydrated with 15 µg/L (Promega, Madison, WI) of porcine trypsin in 25 mmol/L NH4HCO3, pH =8.2 on ice for 45 min. Excess trypsin was removed; gel bands were covered with 25 mmol/L NH4HCO3, pH=8.2 and incubated at 37°C overnight. Tryptic peptides were extracted from the gel bands with 70% acetonitrile and 0.1% trifluoroacetic acid. The sample was desalted with C18 Zip Tips (Millipore, Bedford, MA) according to the manufacturer's protocols. About 0.5 µL of the sample was co-crystallized with 0.5 µL of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile, 1% trifluoroacetic acid and spotted directly on a stainless steel MALDI target plate. Mass spectra were acquired using a MALDI-TOF/TOF mass spectrometer (Voyager 4700, Applied Biosystems, Foster City, CA). MALDI-TOF/TOF

spectra were internally calibrated (< 20 ppm) using trypsin autolysis products. Post-acquisition baseline correction and smoothing was carried out using software provided with the TOF/TOF instrument. Spectra were submitted to Mascot (http://matrixscience.com) for peptide mass fingerprinting.

Bioinformatics detection of proteins

Scanned images of 2DE BCC and normal tissue gels were compared to the data banks by flicker software to detect the spots in one statement in every experiment gel and data bank reference gels.

RESULTS

The spots correspond to the 1) transthyretin (TTR), 2) aldolase C, 3) Apo poss, 4) FGG, 5) ceruloplasmin, 6) unknown protein, 7) cis peroxide reductase (Prx-cis), 8) protrombin, 9) C3b, 10) voltage dependent anion channel (VDAC) and 11) LRG in the two dimensional gels of electrophoresis for the normal skin and BCC skin are shown in the Figures 1 and 2, respectively. Figures 3a and 3b show the spots which correspond to the ceruloplasmin and C3b protein in the two dimensional gels of electrophoresis for the normal skin and BCC skin respectively after analysis by flicker software. The mass spectrometry findings for aldolase C, Prx-cis and VDAC sequencing are illustrated in Figure 4. The amounts of the volume of the labeled spots are tabulated in Table 1.

DISCUSSION

Proteomics is a powerful science for analyzing different proteins in a cell or tissue. The regulation of gene expression varies extensively among tissues, individuals, strains, populations and species [12-17]. As it is depicted in the Figures 1 and 2, the two dimensional images of normal and BCC cells have different patterns. The variation in gene expression is the hallmark of cancer in these tissues. One of the best ways for detection of the gene expression is proteomics. Previous studies have demonstrated that mutation in genes p53, p14ARF, p16 and PTCH can alter gene expression, resulting in initiating and promoting of BCC [5,18,19]. Comparison of 2DE gels showed alterations of gene expression in every labeled spot in figures 1 and 2. About 87 spots were recognized in both gels out of which 76 spots had different gene expression. Some of them were up-regulated and the rest were down-regulated (not published). Flicker is a suitable software for determining desired spots. Here, by using flicker, 11 pointed spots were detected which referred to transthyretin, aldolase C, Apo poss, FGG, ceruloplasmin, unknown protein, Prx-cis, protrombin, C3b, VDAC and LRG. Among 11 pointed spots, C3b had a higher expression in BCC than normal cells. Transthyretin, Apo poss and ceruloplasmin, had a lower expression in BCC than normal cells and aldolase C, FGG, unknown protein, Prx-cis, protrombin, VDAC and LRG had no expression in BCC. Mass spectrometry (MS-MALDI method) is a powerful instrument for

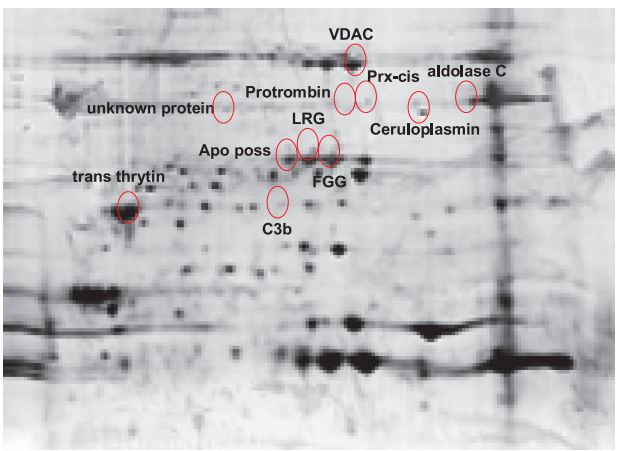

Figure 1. The spots corresponding to transthyretin, aldolase C, Apo poss, FGG, Ceruloplasmin, unknown protein, Prx-cis, Protrombin, C3b, VDAC and LRG in the two dimensional gels of electrophoresis for the normal skin are shown.

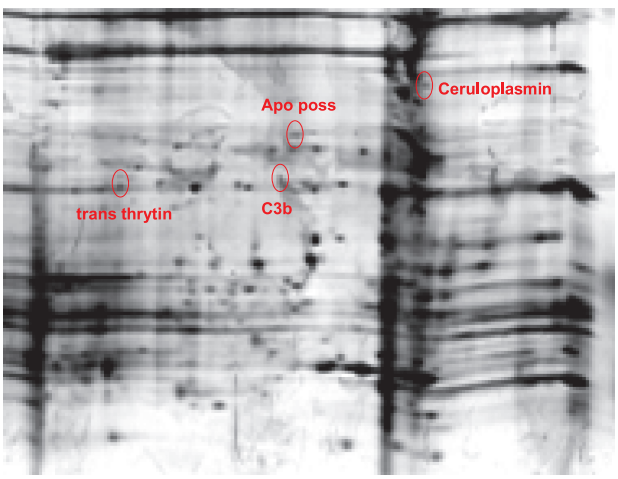

Figure 2. The spots that correspond to the transthyretin, Apo poss, Ceruloplasmin and C3b in the two dimensional gels of electrophoresis for the BCC skin are shown.

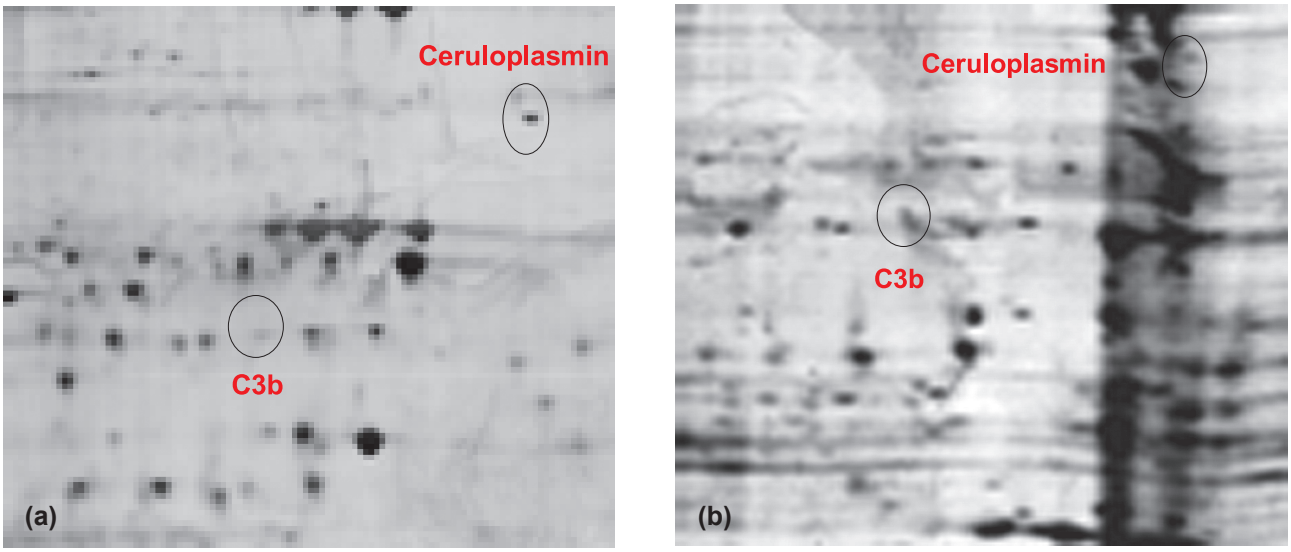

Figure 3. Two dimensional images of **a)** normal skin and **b)** BCC skin, the selected spots that are labeled by arrow refer to the ceruloplasmin and C3b protein.

protein identification. The mass results (Figure 4) confirmed that the Flicker findings were real and the yielded proteins were aldolase C, Prx-cis and VDAC. Aldolase C is one of the glycolytic enzymes. The glycolysis pathway is undoubtedly one of the most centrally situated pathways in metabolism and of the most ancient [20]. Glycolytic enzymes are associated with cellular structures at all developmental stages, and this is particularly evident during the early stages of morphogenesis [21]. Prx-cis is an antioxidant

a)

1 MPHSYPALSA EQKKELSDIA LRIVTPGKGI LAADESVGSM AKRLSQIGVE
51 NTEENRRLYR QVLFSADDRV KKCIGGVIFF HETLYQKDDN GVPFVRTIQD
101 KGILVGIKVD KGVVPLAGTD GETTTQGLDG LLERCAQYKK DGADFAKWRC
151 VLKISDRTPS ALAILENANV LARYASICQQ NGIVPIVEPE ILPDGDHDLK
201 RCQYVTEKVL AAVYKALSDH HVYLEGTLLK PNMVTPGHAC PIKYSPEEIA
251 MATVTALRRT VPPAVPGVTF LSGGQSEEEA SLNLNAINRC PLPRPWALTF
301 SYGRALQASA LNAWRGQRDN AGAATEEFIK RAEMNGLAAQ GRYEGSGDGG
351 AAAQSLYIAN HAY

b)

1 MPGGLLLGDE APNFEANTTI GRIRFHDFLG DSWGILFSHP RDFTPVCTTE
51 LGRAAKLAPE FAKRNVKLIA LSIDSVEDHL AWSKEAKQCF PKGVFTKELP
101 SGKKYLRYTP QP

c)

1 MATHGQTCAR PMCIPPSYAD LGKAARDIFN KGFGFGLVKL DVKTKSCSGV
51 EFSTSGSSNT DTGKVTGTLE TKYKWCEYGL TFTEKWNTDN TLGTEIAIED
101 QICQGLKLTF DTTFSPNTGK KSGKIKSSYK RECINLGCDV DFDFAGPAIH
151 GSAVFGYEGW LAGYQMTFDS AKSKLTRNNF AVGYRTGDFQ LHTNVNDGTE
201 FGGSIYQKVC EDLDTSVNLA WTSGTNCTRF GIAAKYQLDP TASISAKVNN
251 SSLIGVGYTQ TLRPGVKLTL SALVDGKSIN AG

Figure 4. Mascot search results for **a)** aldolase C, **b)** Prx-cis and **c)** VDAC respectively that the Matched peptides of aldolase C (6 items), Prx-cis(3 items) and VDAC (5 items) are shown in **Bold Red**.

Table 1. The amounts of the volume of the labeled spots are tabulated

ID	Normal	BCC	fliker	Mass Spectrometry
1	10778 ± 35.2	9611 ± 32.6	TTR	
2	8046 ± 25.3		Aldolase c	Aldolase c
3	6695 ± 12	6277 ± 16.9	Apo poss	
4	9426 ± 16		FGG	
5	5574 ± 23.6	4779 ± 18.6	Ceruloplasmin	
6	3244 ± 12.8		Unknown	
7	4659 ± 21.6		Prx-cis	Prx-cis
8	3274 ± 19		Protrombin	
9	3697 ± 12.5	4736 ± 21.5	C3b	
10	8416 ± 25.7		VDAC	VDAC
11	4308 ± 18.4		LRG	

and VDAC, a small abundant protein found in all eukaryotic kingdoms, is a class of protein ion channel that is located on the outer mitochondrial membrane and forms a voltage-gated pore when incorporated into planar lipid bilayers. VDAC is also the binding site of the metabolic enzymes hexokinase and glycerol kinase to the mitochondrion in what may be a significant metabolic regulatory interaction [22]. VDAC regulates the activity of BAK and provides a connection between mitochondrial physiology and the core apoptotic pathway [23-25]. Mitochondrial transmembrane carrier deficiencies are a recently discovered group of disorders, which belong to the so-called mitochondriocytopathies. Expression in brain, placenta, lung, and kidney is lower than other tissues. Patient studies have shown that cultured skin fibroblasts may not be a reliable alternative for skeletal muscle in screening for human mitochondrial carrier defects [26]. As shown in figure 3, C3b had a higher expression in cancer cell than normal. Our findings indicated the association of C3b in BCC patient. C3b binds to the cell surface for opsonization and activation of the alternate pathway [27]. Sayama K et al, performed immunohistochemical studies to establish the distribution of membrane cofactor protein (MCP; CD46), decay-accelerating (DAF; CD55) and homologous restriction factor (HRF20; CD59), in normal skin appendages, and in benign and malignant skin neoplasms. At least two of these regulators were detected on normal eccrine glands, apocrine glands and sebaceous glands. They were also found in basal cell carcinoma. Although there were slight differences in their distribution, these regulators were found in all examined cells, indicating that they were essential factors in human skin as well as other organs and neoplasms for preventing autologous complement attack [28]. Ceruloplasmin, a plasma antioxidant that has been proposed to function in copper transport, oxidation of organic amines, iron(II) oxidation and the regulation of cellular iron levels, catechols, radical scavenging and other antioxidant processes [29] significantly decreases in the BCC group compared to controls [30]. Our finding suggested that BCC altered the expression of C3b and ceruloplasmin as indicated. Such biological variances and differences in gene expression are used to describe cancers and other diseases [31,32]. However, it can be concluded that alteration of gene expression induced by different pathways in cancer cells due to the alteration in the expression of other factors leads to BCC. Gene expression alteration in BCC can be monitor by proteomics. Mapping of the new biomarkers can lead to identification of the mechanism and pathway of the disease. This study also provided feasibility for determining some diagnostic biomarkers and also drug targets in patients. To summarize, not only C3b with up-regulation can be assumed as a biomarker, but also aldolase C, FGG, unknown protein, Prx-cis, Protrombin, VDAC and LRG proteins with no expression can be introduced as new biomarkers in BCC patients. However, it is necessary to conduct further experiments. To be more accurate, it is suggested that these biomarkers should be analyzed in the mucous secretions and blood.

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