# Introducing aldolase C as a differentiation biomarker: A proteomics approach

**Mostafa Rezaei-Tavirani[1,2,*], Hakimeh Zali[1], Ferdous Rastgar Jazii[3], Mohammad Hossain Heidari[4], Behrooz Hoseinzadeh-Salavati[5], Fatemeh Daneshi-Mehr[5], Kambiz Gilany[6]**

[1]Clinical Proteomics Research Center, Faculty of Paramedical Sciences, Shahid Beheshti University of medical sciences, Tehran, Iran.
[2] Asre-Novin Institute of Research and Industrial Services, Tehran, Iran.
[3] National Institutes of Genetic Engineering and Biotechnology, Tehran, Iran.
[4] Faculty of Paramedical Sciences, Shahid Beheshti University of medical sciences, Tehran, Iran.
[5] Departments of Molecular Cell Biology, Khatam University, Tehran, Iran.
[6] University of Antwerpe-campus Drie Eiken, Department of Biomedical Sciences, Universiteitsplein 1, B-2610 Antwerp, Belgium.

*Corresponding author: e-mail address: rezaei.tavirani@ibb.ut.ac.ir (M. Rezaei-Tavirani)

## ABSTRACT

Aldolase C as a glycolytic enzyme is associated with cellular structure at developmental stages of all cells, and this is particularly evident during the early stages of morphogenesis. It seems that expression of aldolase C can be regulated by the rate of differentiation that depends on the level of transcription or mRNA stability. There are several techniques to detect gene expression, here proteomics was used for determining expression of aldolase C (as a differentiation factor) in several cell types and basal cell carcinoma (BCC) tissue. The human astrocytes were differentiated from mesanchymal stem cells, fibroblast cells were cultured as primary cell culture and BCC tissue was taken from the patient. The fibroblast cells divided into two groups including sham and exposed groups. The exposed cells are them that were exposed to continue Extremely Low-Frequency Electromagnetic Fields (ELF-EMF). The analysis of 2DE gels, showed different expression of aldolase C in mentioned cells. The findings indicate that the amount of aldolase C expression decreases as differentiation process develops.

**Keywords:** Aldolase C; Proteomics; Mesanchymal stem cells; Fibroblast; Basal cell carcinoma

## INTRODUCTION

The glycolysis pathway is undoubtedly one of the most centrally situated pathways in metabolism. Glycolysis enzymes are associated with cellular structure at all developmental stages, and this is particularly evident during the early stages of morphogenesis. Certain enzymes bind more readily to cellular structure (such as phosphofructokinase, aldolase, glyceraldehyde phosphate dehydrogenase, pyruvate kinase and dehydrogenase) than the other glycolytic enzymes and much of this binding is associated with actin-containing filament [1]. Aldolase C, found on human chromosome 17, has kinetic properties that are intermediate between those of A and B isoforms [2]. Findings have indicated that Zebrin II which is an $3\times10^3$ Mr antigen, is aldolase C and its expression is regulated at the level of transcription or mRNA staibility [3]. It seems that aldolase C gene is almost transcribed in all cell types. Studies using isozyme-specific antibodies report its location in gray matter astrocytes and cells of the pia matter [2, 4]. Aldolase C mRNA expression is highest in the cerebellum [5]. Aldolase C increases during the development of mammalian fetal brain, and is Expressed at high levels in adult brain [6,7]. Although very low level of aldolase C has been found in some non nervous tissues, its gene is transcribed mostly in neurons.

Astrocyte is an absolute differentiated cell. Stem cell is mesenchymal cell with high capacity to differentiate into any mature cell types. Fibroblast seems to be the least specialized cells in the connective-tissue family. One of the underlying principles of specific types of cells in multicellular organisms is that the functions and properties of each cell type are determined by the proteins it contains. The procedure that is used to analyze change in expression of many proteins in a cell is proteomics. In the proteomics, the proteins are separated and can then be identified by mass spectrometry. Today, this technique is used in many

researches in order to identify gene expression in specific types of cells in multicultural organism [9, 10]. Here, the amount of expression of aldolase C in the normal human stem cell, fibroblast, exposed fibroblast cell (the cell that is exposed to continues Extremely Low-Frequency Electromagnetic Fields (ELF-EMF)) and astrocytes were investigated by proteomics. For getting more illustration, these results were compared to an abnormal condition like as Basal cell carcinoma (BCC) tissue.

## MATERIALS AND METHODS

### Sample preparation

Human skin fibroblasts were prepared from foreskin tissue and grown to 80% confluence in Dulbecco's modified Eagle medium (DMEM; 4.5 g/liter glucose) supplemented with 10% fetal bovine serum, 0.58 g/liter L-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin at 37 °C under isobaric conditions (5% CO2, 95% air) in humidified atmosphere.

To compare the effect of environmental stress condition on gene expression, one group of fibroblast cells were exposed to continues Extremely Low-Frequency Electromagnetic Fields (ELF-EMF) (3Hz, sinusoidal, 3h and 4mT). Then media was removed, cells were washed three times with phosphate-buffered saline and lysed by adding lysis buffer (7 M urea, 2 M thiourea, and 1% Triton X-100) and prepared for 2DE process [11].

It is important to compare between normal and cancer cell to find out the differential rate of cellular protein contain so, used Basal cell carcinoma (BCC). BCC tissues sample was taken from patient. Fresh tissue samples of skin were snap frozen and kept in liquid nitrogen until use. Tissue samples were powdered by microdismembrator at 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 betamercapto ethanol, 0.5% CHAPS and 10% glycerol. After 30 minutes 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[12].

### Isolation of mesenchymal stem cells (MSCs)

Briefly, each aspirate was diluted 1:1 with DMEM and layered over 1:1 Ficoll (Ficoll-Paque Plus; GE Healthcare Bio-Sciences, Baie-d'Urfé, QC). After centrifugation at 900×g for 30 min, the mononuclear cell layer was removed from the interface, washed with DMEM, and resuspended in DMEM supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 mm l-glutamine. The cells were plated in 20 ml of medium in a 176-cm2-culture dish and incubated at 37 °C in a 5% CO2 humidified atmosphere. After 72 h, non-adherent cells were discarded and the adherent cells were thoroughly washed twice with DMEM. Thereafter, the cells were expanded [13].

### Immunophenotyping of MSCs by flow cytometry

Expanded MSCs were detached from the culture flask by the use of PBS (pH 7.4) containing trypsin (0.05%) and EDTA (0.02%), washed once with DMEM and once with filtrated PBS. Cells were next suspended at a concentration of 1 × 106 cells in 50 µl PBS and incubated for 45 min at 4 °C in the dark with FITC or PE-conjugated antibodies as follows: anti-CD29-PE, anti-CD54-PE, anti-CD44-FITC, anti-CD45-FITC, anti-CD73-FITC, anti-CD166-FITC, anti-CD105-PE, anti-CD34-FITC, and anti-CD31-FITC. In parallel, cells were incubated with an irrelevant antibody (anti-Aspergillus niger glucose oxidase, Dako) as a negative isotype control to exclude non-specifically labelled cells from the calculation. Upon completion of the incubation time, cells were washed twice with PBS supplemented with 2% BSA and fixed with 1% paraformaldehyde solution in PBS. Analysis was next performed using a flow cytometer (FACsort, BD, USA). Before each test, the percentage of viability that was more than 95% was measured with trypan-blue staining and dead cells were counted with a neobar slide.

**Differentiation of MSCs into Neural like cells:** The adult human mesenchymal stem cells used, were frozen twice and passaged a total of seven times. $2 \times 10^5$ mesenchymal stem cells were thawed and suspended in the neurosphere-inducing media in 35 mm dishes. The neurosphere media consisted of Neurobasal A media (GIBCO BRL), B27 proliferation/cell expansion supplement, 1% antibiotic/antimycotic, 20 ng/ml epidermal growth factor (EGF) and 20 ng/ml basic fibroblastic growth factor (bFGF) (Sigma).

The floating neurospheres that formed received additional EGF and bFGF every 3–5 days for 15 days. Ten μM of forskolin and 0.1 mM of isobutylmethylxanothine (IBMX), a phosphodiesterase inhibitor, were added to the preliminary neurosphere media to increase cyclic AMP in later experiments. An increase in intracellular cAMP has been shown to promote neuronal signaling mechanisms [14].

**Immunocytochemistry**

Immunocytochemical staining was performed to examine the NF expression in MSCs at 24 h post neuron induction. The cultures were washed with D-PBS and fixed with 4% paraformaldehyde at 37°C for 10 min. The cells were then permeabilized with 1% Triton at room temperature for 10 min. Following 2 rinses with 0.5 mg/ml sodium borohydride, the cells were incubated with 5% normal goat serum and then 6% BSA, each at room temperature for 30 min, to block non-specific antibody binding. The cells were subsequently incubated with a rabbit anti-human NSE antibody at 37°C for 1 h, and a goat anti-rabbit IgG antibody conjugated with Alexa Fluor 488 at 37°C for 40 min. In other experiments, cells were incubated for 60 min at room temperature with monoclonal antibodies against neuron specific enolase (NSE; 1:20, Cymbus Biotechnology). Subsequently, slides were rinsed three times in PBS-Triton, incubated for 30 min with biotinylated antimouse IgG (1:200, Vector) and rinsed and incubated with horseradish peroxidase conjugated avidin D (1:800, Vector), followed by 10 min incubation with DAB tablet sets (SIGMA FAST). Negative controls were routinely performed for each experiment, incubating the samples with non-immune serum and with biotinylated anti-mouse IgG. Six different experiments were performed for each marker.

**Two dimensional SDS-PAGE**

Briefly, human astrocyte on 25mm$^2$ dishes was washed three times in PBS. 300μl lysis buffer (7M urea, 2M thiourea, 4%CHAPS, 0.2-0.3%DTT,1-2% ampholine 3-10) was added to cell culture and shaking was done at room temperature for 1 h. The lysate was centrifuged at 10000 g for 10 min at room temperature. The supernatant was used for protein assay according to Bradford protein assay (15) and subsequently was kept at -20˚C. 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). Sample (400μg) was applied during rehydration. The first dimension of 2D electrophoresis was performed on the PROTEAN IEF Cell system (Bio-Rad). Next, gels were equilibrated for 15 min in 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 second dimension gel electrophoresis. Equilibrated IPG strips were placed on the surface of second dimension gels and were then sealed with 0.5% agarose in SDS electrophoresis buffer (25mM Tris base, 192mM glycine, 0.1%SDS) and were run vertically [16].

**2DE gel staining**

After electrophoresis, the gels were stained with silver stain [17] Coomassie Brilliant Blue staining [18].

**Bioinformatics analysis**

2DE gels were scanned and gels were analyzed by Bionumeric and fliker soft wares to compare the spots in one statement in gels and get the density of same spot in each of gel. Then, the spots were compared to data banks to detect the spots in one statement in every experiment gel and data bank references gels.

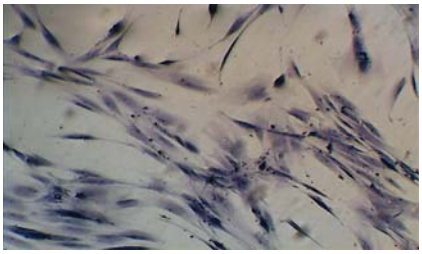
**Mass spectrometry**

Stained protein spot containing the interested protein was destained thoroughly with 1% $H_2O_2$ (typically 1 min) and lyophilized to dryness [19]. stain removal using $H_2O_2$ was performed to enhance peptide adsorption by matrix-assisted laser desorption/ionization time-of- flight mass spectrometry (MALDI) [20]. The dehydrated gel bands were hydrated with 15 μg/l (Promega, Madison, WI) of porcine trypsin in 25 mmol/L NH4HCO3, pH8.2 on ice for 45 min. Excess trypsin was removed; gel bands were covered with 25 mmol/l NH4HCO3, pH8.2 and incubated at 37°C overnight. Tryptic peptides were extracted from the gel bands with 70% acetonitrile and 0.1% trifl uoroacetic acid. Sample was desalted with C18 Zip Tips (Millipore, Bedford, MA) as permanufacturer's protocols. 0.5 μl of 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,

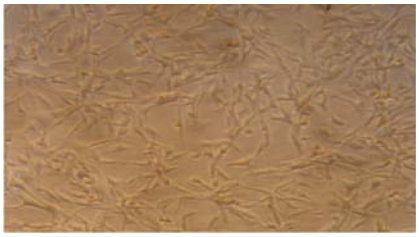
Applied Biosystems, Foster City, CA). MALDI-TOF/TOF spectra were internally calibrated (< 20 ppm) using trypsin autolysis products. Post-acquisition baseline correction and smoothing was carried out using software provided with the TOF/TOF instrument. Spectra were submitted to Mascot (http://matrixscience.com)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

## RESULTS

Since fibroblast cell is a least differentiated cell and is near to stem cell and astrocyte is a differentiated cell, the comparison of their proteome will show pathway of differentiation. Figure1 shows the human mesenchymal stem cells (MSCs), human fibroblast and astrocyte cell NSE staining (neuron specific enolase), respectively.



a)

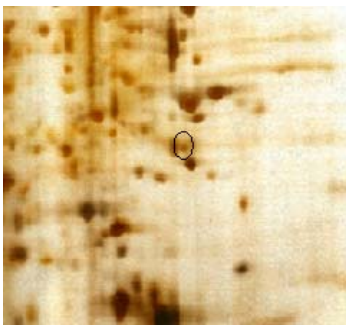


b)

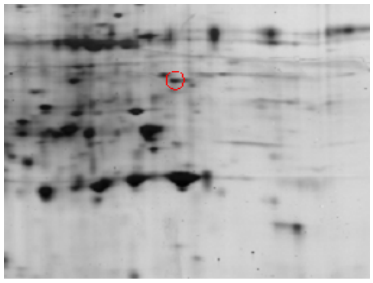
c)

**Figure 1.** a) mesenchymal stem cells-NSE staining (neuron specific enolase), b) human fibroblast and c) astrocyte.

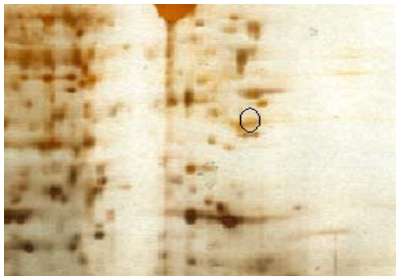
2DE in combination with mass spectrometry and bioinformatics analysis were used for protein identification. The first step in proteomics study is protein extraction and the second is protein separation. The proteomes of stem cell, fibroblast, astrocyte, exposed fibroblast and BCC tissue were provided and analyzed by bionameric and flicker software. As it is shown in figure 2, the similar part of gel that contains aldolase C is represented.



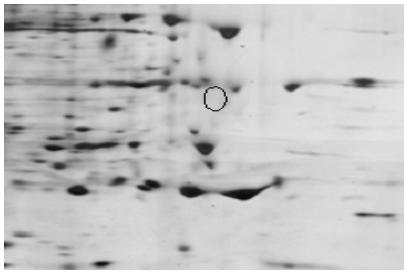
a)



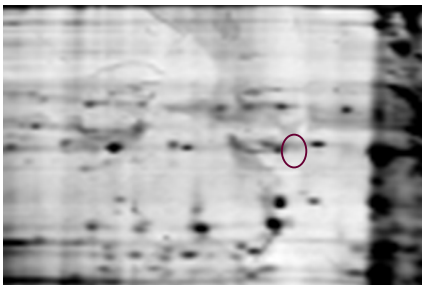
b)



c)



d)



e)

**Figure 2.** Two dimensional images of a) mesenchymal stem cells, b) human fibroblast, c) astrocyte, d) exposed fibroblast and e) BCC tissue. The selected spot of every gel refers to aldolase c protein.

Bionumerics software is a suitable tool for detection of density of the spots compared to the similar ones on the other gels. By using Bionumerics software, density of the mentioned spot in all gels was determined and were tabulated in table1. Flicker analysis indicated that the mentioned spot correspond to the aldolase C. For confirming the flicker analysis, mass spectrometry (MALDI/TOF/TOF / MASCOT) was used. The finding of mass spectrometry (Mascot Search Results) indicates that the desired protein is aldolase C.

**Table 1.** Aldolase C expression of different cells was determined by using Bionamerics software

| Type of cell | **max volume** | **volume** |
|---|---|---|
| Stem cell | 4.5±78 | 18.7±2089 |
| Fibroblast | 5.6± 32 | 13.6±1177 |
| Astrocyte | 15 ± 0.09 | 10.4±985 |
| Exposed fibroblast | 0 | 0 |
| BCC | 0 | 0 |

## DISCUSSION

Class I aldolases include three tissue-specific isoenzymes in vertebrate tissues: aldolase A predominates in skeletal muscle and red blood cells, aldolase B in liver, kidney, and small intestine, and aldolase C in neuronal tissues and smooth muscle [21]. It was also identified aldolase A in human skin fibroblasts [29]. As it is depicted in the figures 1 stem cell, fibroblast and astrocyte have different morphology. Astrocyte is an absolute differentiated cell while stem cell is undifferentiated cell with high capacity for differentiation. Fibroblast seems to be the least specialized cells in the connective-tissue family [9, 30]. The actions and properties of each cell type in multicellular organisms are determined by the proteins it contains. Many of the proteins within cells are enzymes, other proteins allow cells to move and do work, maintain internal cell rigidity, and transport molecules across membranes. In the figure 2, the similar and desired part of 2DE gel correspond to the stem cell, fibroblast cells, astrocyte, exposed fibroblast and basal cell carcinoma tissue are shown. By using Bionamerics software, the amounts of aldolase C for all cell lines and also BCC tissue that are shown in table 1 indicate that a) aldolase C expression was suppressed completely in exposed fibroblast and also BCC tissue, b) amount of aldolase C expression reduced from stem cell, fibroblast and astrocyte, respectively and c) it is interesting that radiation as an environmental stress (differentiated motor factor) suppresses aldolase C expression. However, the radiation type is none ionized.

Aldolase serves as scaffolding protein linking insulin-responsive glucose transporter (GluT4) and hence GluT vesicle to the actin cytoskeleton [31]. Glycolytic enzyme can modulate cellular life span. Early passage MEFs have high glycolytic activity, which declines during replicative senescence and is reestablished during spontaneous immortalization. Inhibition of glycolytic enzymes can induce senescence. Like neurofilament, aldolase A and C are expressed early in embryonic development, are up regulated during postnatal life [7]. The molecular mechanisms of the brain-specific expression of this gene are still poorly understood. A complete set of the regulating elements required for CNS-specific expression of the aldolase C gene is present between -6 Kb and +7 Kb of the gene [32].

Finally, it can be concluded that amount of aldolase C expression is a suitable biomarker for differentiation progress in the cell lines, so its expression decreases as cell differentiation develops.

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