Original Article

The Study of "Dihydropyrimidinase Related Proteins (DRPs)" Expression Changes Influence in Malignant Astrocytoma Brain Tumor

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Abstract

Background: Dihydropyrimidinase Related Proteins (DRPs) have known homologous to the Collapsing Response Mediator Proteins (CRMPs). The DRP gene family has comprised four members, DRP 1, 2, 3, and 4, all out of which have considered to be involved in axonal outgrowth and path-finding.

Methods: The protein has extracted from tumor, normal brain tissues, and then the protein purity has evaluated by Bradford test and spectrophotometric methods. In this study, proteins has separated by Two-Dimensional Gel (2DG) electrophoresis method and then spots have analyzed and compared using statistical data and specific software (Progenesis Same Spots).Spots have identified by pH isoelectric, molecular weights and data banks.

Results: The 2D gel has shown 800 spots totally. Two spots have reported for DRP2, and one spot has reported for DRP3 in the human brain proteome, that have differed in pH isoelectric, and Molecular Weights values.

Conclusion: This protein family has involved in neuronal differentiation and axonal guidance, and abundantly influenced in the developing brain, but their expression persisted into adulthood. DRP2 has regulated by phosphorylation, Glycogen synthase kinase 3, regulate phosphorylation of DRP2 an inactive from, and induced neuronal polarity.

Keywords: Asterocytoma; Proteomics; DRP and 2DG Electrophoresis

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Introduction

The aim of some medical studies is to identify Dihydropyrimidinase Related Proteins (DRP) has known homologous to the Collapsing Response Mediator Proteins (CRMPs). Four DRPs (DRP 1-4) have identified, all out of which have expressed in the developing neurons [1-3]. Nonhuman counterparts of the human DRPs have identified and named in relation to the history of their discovery, chicken (CRMP)-62, rat turned on after Division (TOAD)-64, and mouse unc-33 like phosphoprotein [4]. The DRP gene family has comprised four members, DRP 1, 2, 3, and 4, all out of which have thought to be involved in axonal outgrowth and path findings [5, 6], One of the underlying mechanisms which required to build the conventions network, between neurons during brain development, would be the guidance of axons to their correct target. Function impairment of the proteins which have involved in this mechanism, might lead to abnormalities in guidance and outgrowth of the axons in many neurons. Hence, dysfunction of DRP family might result in neurodevelopmental abnormalities [2]. Based on the comparison of primary structures, those genes have classified into four groups [7]. Amino acid sequences of DRP proteins have shown 64-79% identity to each other (94-99% interspecies conservation including chicken) [1, 7]. DRP2 which also has independently identified as CRMP2, TOAD-64, ULIP2, UNC-33 or DPYSL-2, is a homolog of human dihydropyrimidinase [8]. CRMP2 protein has

recently reported to be involved in neural development in venous and bovine [7, 9, 10]. DRP family members and response mediates protein the intracellular response to collapsing a repulsive extracellular guidance cue for axonal outgrowth, the human DRP2 gene has located on chromosome 8p21 [11, 12]. The mentioned DRP2 gene might be considered as a functional and positional candidate gene for Bipolar Disorder (BPD) [1, 11]. DRP3 cDNA has isolated from fetal human brain as a homologue of dihydropyrimidinase along with the other homologues [13, 14]. The DRP3 gene has expressed in testis as a shorter mRNA than the brain form. As a first step in understanding the extra neuronal function of DRP3, the structure and expression of testis DRP3 have examined [10, 13].

Gliomas were the most common human primary brain tumors, [15-18] they were highly invasive and heterogeneous, and have responded poorly to treatment [15,19]. More than80% of gliomas have appeared to arise from astrocytomas, the most abundant type of glial cells, and have known as astrocytomas [16,19]. Patients with high-grade (grade IV) astrocytomas would have a life expectancy of <1 year even after surgery, chemotherapy, and radiation therapy [20]. The grade IV glioma, commonly known as Glioblastoma Multiforme (GBM), would be the most aggressive and lethal type with an average life expectancy of one year or less, from the disease diagnosis time [21-23]. Proteomics have increasingly employed in both neurological and oncological research to provide a guide view into the molecular basis of disease but rarely has a coherent, novel pathophysiological insight emerged [24, 25]. Proteomics analysis has now applied widely for any area of neuroscience research including brain cancer [26, 27].

In this paper, we have studied "the Dihydropyrimidinase related proteins changes" influence in human brain astrocytoma tumor. To obtain a brief understanding of data and specific software molecular diagnosis of astrocytomas, we have extracted proteins of tumoral and normal brain tissues, and evaluated the protein purity. We have separated proteins by two-dimensional gel electrophoresis method, and then we have identified spot alteration using statistical data and specific software (Progenesis Same Spots). Using different proteomics approaches, we have identified multiple differentially expressed astrocytoma proteins, few out of which could further investigate as potential surrogate marker for astrocytoma.

Materials and Methods

Patient samples

Tissues have obtained, with informed consent and institutional review board approval, from the patients which undergone tumor resection. For this study, all individuals have filled a written informed consent form. Astrocytoma tumors have surgically removed in Shohadaye Tajrish Hospital. The tumors have classified by a team of neuropathologists, according to the WHO classification guidelines of the CNS tumors. Eight tumor surgeries that have operated patients with malignant astrocytoma have separated as below: four tumors have selected for protein extraction, and two-dimensional electrophoresis. Non-tumoral brain tissues have obtained from normal areas (either grey or white matter) of brain tissues removed from patient undergone non-tumor epileptic surgery.

Tissue and samples preparation

Tissue samples of both tumoral and normal brain tissue were snap-frozen immediately after operation in liquid nitrogen and stored at -80ºC until used for proteomic analysis. To obtain tissue extracts, the samples have broken into suitable pieces and have homogenized in lysis buffer II consisting of lysis buffer I {7 M urea, 2Mthiourea, 4% 3-[(3-Cholamidopropyl) Dimethylammonio]-1-Propanesulfonate (CHAPS), 0.2% 100×Bio-Lyte 3/10}, Dithiothreitol (DTT), and 1mM ampholyte and protease inhibitor on ice. Cell lysis has completed by subsequent sonication (4×30 pulses). Then samples have centrifuged 20,000 g at 4°C for 30 minutes to remove insoluble debris. Supernatants have combined with acetone 100% and centrifuged at 15,000 g, and then the supernatants have decanted and removed (3 times). Acetone 100% has added to the protein precipitant and kept at -20°C (overnight). Samples then have centrifuged again at 15,000 g and the precipitant incubated 1 hour at room temperature. The protein samples have dissolved in rehydration buffer [8 M urea, 1% CHAPS, DTT, ampholyte pH (4) and protease inhibitor]. Protein concentrations have determined using the Bradford test and Spectrophotometry method, and the protein extracts have then separated and used for 2D gel electrophoresis.

Two-dimensional gel electrophoresis

The isoelectric focusing for first-dimensional electrophoresis has performed using 18 cm; pH 3–10 Immobilized pH Gradient (IPG) strips (BIO-RAD, Protean IEF cell). The samples have diluted in a solution containing rehydration buffer, IPG buffer, and DTT to reach a final protein amount of 500µg per strip. The strips have subsequently subjected to voltage gradient as described in the instructions of the manufacturer. Once focused, the IPG strips have equilibrated twice for 15 minutes in equilibration buffer I [50mM Tris-HCl (pH: 8.8), 6M urea, 30% glycerol, 2% Sodium Dodecyl Sulfate (SDS), and DTT] and equilibration buffer II. The second-dimension SDS-PAGE has carried out using 12% PAGEs. Following SDS-PAGE, the gels have stained using the Coomassie Blue method (overnight).

Image analysis

All gel images have analyzed by Progenesis Same Spots software to identify spots differentially expressed between tumor and control samples based on differences which have defined as altered. The spots have carefully matched individually and only spots that have shown a definite difference have defined as altered. Spots have detected by pH isoelectric, Molecular Weights, databanks and Comparison with previous research.

Results

Using 2D-PAGE proteomic analysis, we have compared protein expression patterns between astrocytoma samples relative to control tissue. The 2D-DIGE (DIGE: Difference gel electrophoresis) has revealed consistent protein profiles for each group. Simple statistical test have used to establish a putative hierarchy in which the change in protein level have ranked according to a cutoff point with $p<0.05$. The 2D gel has shown totally 800 spots. A total of 343 spots have shown statistically significant differences (student's *t*-test; $p<0.05$) in the gel, out of which 164 spots exhibited up-regulation in expression level, whereas the remaining 179 spots have decreased in astrocytoma tumor relative to normal tissue. Among them the statistically significant protein spots ($p<0.05$) DRP2 proteins have seen definitely with (first spot) with 5.82 isoelectric pH, and 63kDa molecular weight has detected with an up-regulation about 2.8 (fold=2.8) (Figure 1), and (second spot) with 5.35 isoelectric pH and 53kDa molecular weight have detected with

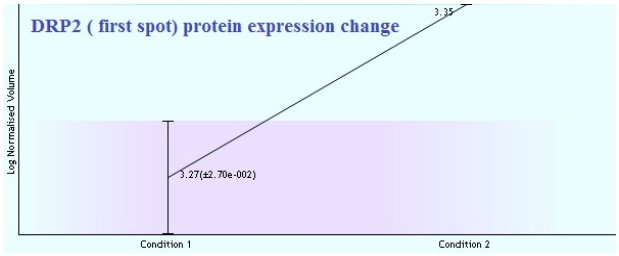


Figure 1. DRP2 (first spot) protein has an up-regulation about 2.5 (fold=2.5) in astrocytoma brain tumors than normal brain tissue.

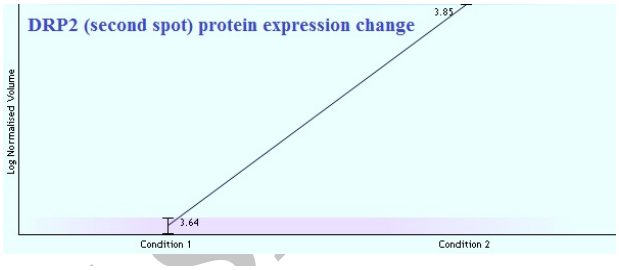


Figure 2. DRP2 (second spot) protein had an up-regulation about 1.6 (fold=1.6) in astrocytoma brain tumors, rather than normal brain tissue.

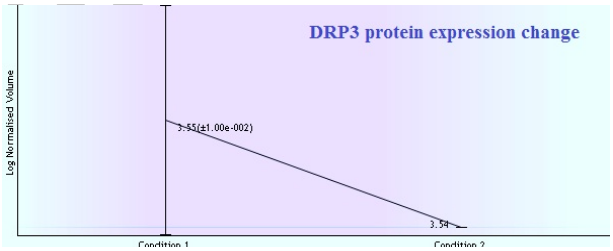


Figure 3. DRP3 protein has a down-regulation about 4.3 (fold=4.3) in astrocytoma brain tumors than normal brain tissue.

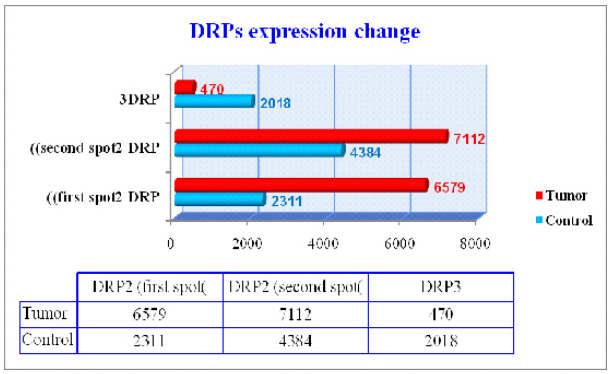
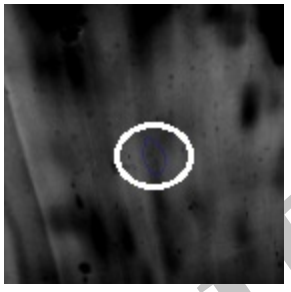
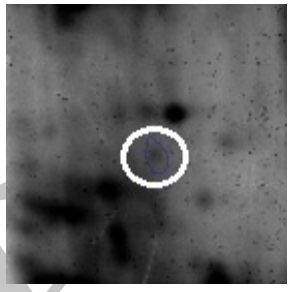
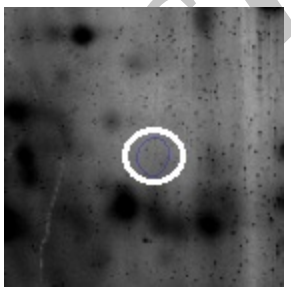
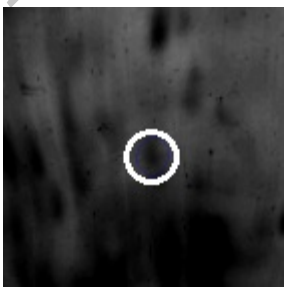
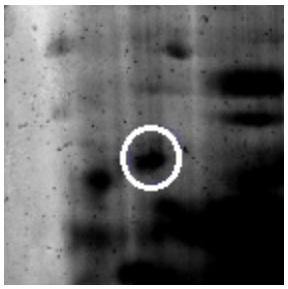
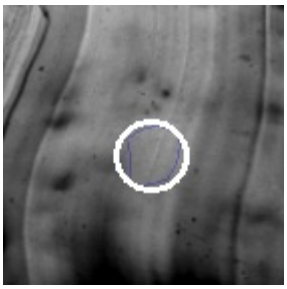


Figure 4. DRP spots expression chart: showing differential expression between the control (blue) and astrocytoma (red).

Among them the statistically significant protein spots ($p<0.05$) DRP3 protein was definitely with

Table 1. Comparison of spots (DRP2 and DRP3), between normal brain tissue and astrocytoma tumor, differentially expressed protein in astrocytoma tumor identified by 2D gel analysis.

Name protein	Fold change	expressed proteins Change	Control	Tumor
DRP2 (First spot)	2.8	Up-regulation		
DRP2 (Second spot)	1.6	Up-regulation		
DRP3	4.3	Down-Regulation		

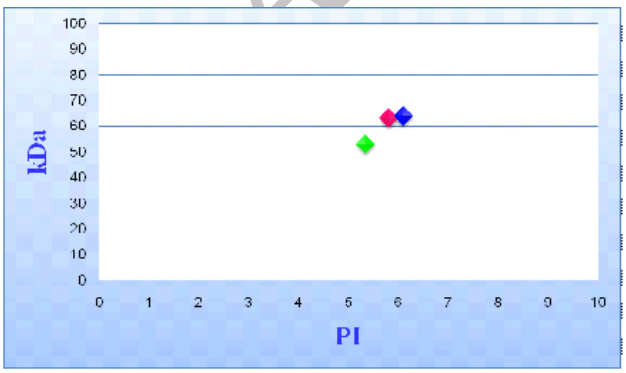


Figure 5. DRP proteins position in Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), red: DRP2 (first spot), green: DRP2 (second spot) and blue: DRP3.

6.11 isoelectric pH, and 64kDa molecular weight has detected with a down-regulation about 4.3 (fold=4.3) in astrocytoma brain tumors, rather than normal brain tissue (Figure 3, Table1 and Figure 4). Each of the tumors has compared separately with the control, and the results have shown in table 2.

Discussion

DRP genes have been studied mainly in the Central Nervous System (CNS) of the developing embryo. A Little has known about DRP involvement in the Enteric Nervous System (ENS) during development and adulthood. Enteric neurons received a minority of their innervation from the

Table 2. Each of the tumors has compared with controls separately.

Asterocytoma	Grade	DRP 2 (First spot)			DRP 2 (Second spot)			DRP 3		
		Anova (p)	MW	pI	Anova (p)	MW	pI	Anova (p)	MW	pI
Case1	**IV**	2.464e-011	65	5.66	1.405e-009	52	5.55	2.880e-011	65	6.02
Case 2	**IV**	2.316e-010	63	5.85	1.361e-008	53	5.49	2.403e-009	67	6.15
Case 3	**IV**	4.229e-011	63	5.75	_	_	_	3.119e-009	66	6.46
Case 4	**IV**	1.023e-009	63	5.89	1.683e-009	55	5.25	2.586e-009	67	6.10

CNS, have supported glia instead of Schwann cells, and collagen has excluded from the interior of enteric ganglia [4, 7]. This protein family has involved in neuronal differentiation and axonal guidance [28], and then abundantly expressed in the developing brain, but their expression persists into adulthood. Veyrac et al. (2005) studied the differential expression of DRP (CRMP-1 and 5) in the mature and immature neurons in the adult olfactory system and suggested that these proteins have still associated with neurogenesis in the adult brain [29, 30].

DRP2 has remained expressed in adult brain, suggesting that the process of axonal outgrowth was important as mechanism of repair and regeneration of adult neurons. Additionally, evidence that CRMP2 has involved in regulating the dynamics of microtubules has been recently reported [31], DRP2 has expressed early in neuronal differentiation, which correlated to the highly active period of neuronal development including axonogenesis and synaptic connectivity, and would be significantly down-regulated in the well-integrated neurons [32, 33]. DRP2 (CRMP2/ulip2) and DRP3 (CRMP4/ulip1) were members of CRMP, which involved in regulation of neuritis guidance and synapse formation: they have highly expressed during brain development and rarely in adult brains [34, 35]. Except DRP2 which could be detected until adulthood in several types of specialized neurons in the hippocampus, cerebellum and dorsal root ganglion [34].

Nonetheless, some reports respectively out lined that DRP2 down expression and DRP3 up-regulation in astrocytoma. Our comparison between astrocytoma and healthy subjects revealed differentially expressed and statically significant ($p<0.05$) DRPs in brain tumor.DRP3 which has identified in 2D gel in only a single spot. Our findings have revealed that DRPs2 has over expressed and DRP3 down expressed in astrocytoma [Figure 4]. We have identified two spots for DRP2 (first spot: 63kDa and 5.82 for molecular weight and pH isoelectric respectively and second spot: 53kDa and 5.35 for MW and pI respectively) and have identified one spot for DRP3 (64kDa and 6.11 for molecular weight and pH isoelectric respectively) (Figure 5).

In contrast, Franzen et al. (2003) reported 3 spots for DRP2 in the mouse brain proteome that has differed in MW and pI values, and then we have suggested two spots that differed in MW and pI values only. The DRP family has known to be highly phosphorylated [36-38].

A protein spot has identified as DRP2, has increased in cadmium treated cells. DRP2 has also known as CRMP2, and would be a member of the TOAD/Ulip/CRMP family of cytosolic phosphoproteins [39, 40]. DRP2 has regulated by phosphorylation, Glycogen synthase kinase 3 β phosphorylates DRP2 an inactive from, and induced neuronal polarity [41]. Phosphorylation of DRP2 by Rho kinase has deleted its ability to bind to tubulin dimers and microtubules and thus inactivates its ability to promote microtubule assembly during growth cone collapse [11, 31]. In contrast neurotrophin-3 has increased non phosphorylated DRP2, an active from, probably via activation, of

pI3 kinase and Akt, and induces axon outgrowth [41, 42]. DRP2 would be also a brain specific substrate of Glycogen Synthase Kinase3 (GSK3) and Cyclin Dependent Kinase 5 (CDK 5). Mutation of the GSK3 phosphorylation sites on the DRP2 to Ala residues has been shown to reduce DRP2 induced axon elongation. Some studies have shown that DRP2 would be important for modulating synaptic vulnerability [39].

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Authors' Contribution

Mehdi Pooladi, Mostafa Rezaei-Tavirani, Mehrdad Hashemi, Saeed Hesami-Tackallou and Solmaz Khaghani Razi Abad, have designed the study, have analyzed the data and written the paper. Masoumea Mousavi, Leila Firozi Dalvand and Roghaye Omidi have contributed to study design. Afshin Moradi, Ali Reza Zali, Azadeh Rakhshan and Mehdi Pooladi have contributed to samples collection and indentation.

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