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Altered Expression of Epidermal Growth Factor Receptor (EGFR) in Glioma

M. Pooladi[a,b], M. Rezaei-Tavirani[a], M. Hashemi[c], S. Hesami-Tackallou[d], S. Khaghani Razi Abad[a,b], L. Firozi Dalvand[b] and A. Alaei[b]

[a]*Proteomics Research Center, Faculty of Paramedical Sciences, Shahid Beheshti University of Medical Sciences, Tehran-Iran*
[b]*Department of Biology, School of Basic Sciences, Science and Research Branch, Islamic Azad University, Tehran, Iran*
[c]*Department Of Molecular Genetics, Tehran Medical Branch, Islamic Azad University Tehran, Iran*
[d]*Department of Biology, Garmsar Branch, Islamic Azad University, Garmsar, Iran*

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ABSTRACT

EGFR is a key molecule in cancer cells. EGFR signaling was shown to promote tumor cell proliferation and survival, invasion and angiogenesis and mediate resistance to treatment, including ionizing radiation in preclinical models. We extracted proteins from astrocytoma (III and IV) oligodendroglioma(IV) tumors and normal brain tissues and then evaluated the protein purity by Bradford test and spectrophotometry method. In this study, we separated proteins by the two-dimensional gel (2DG) electrophoresis method, and the spots were analyzed and compared using statistical data and Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF-TOF MS). EGFR position showed in the diagram cluster for oligodendroglioma tumors (t-test and one-way ANOVA; $P < 0.05$). EGFR proteins were definitely with isoelectric pH, molecular weight and protein databank detected which has an up-regulation. Proteomics analysis revealed an unexpected alteration in the expression of certain protein networks in EGFR gliomas. The EGFR dependent signaling pathways are most frequently reported in high grade oligodendroglioma and affect all histological closes. Thus EGFR could be a candidate biomarker in glioma tumors.

Keywords: EGFR, Glioma, Proteomics and 2DG Electrophoresis

INTRODUCTION

Glioma is the most common type of primary brain tumors, and is grouped into four grade according to the World Health Organization (WHO) criteria [1,2]. Gliomas are a classification of nervous system tumors arising from glial cells, the most common of which are astrocyromas and oligodendrogliomas, arising from astrocytes and oligodendroglial, respectively [3-5].

Epidermal growth factor receptor (EGFR) is a tyrosine kinase that binds to extracellular EGF and dimerizes, thus transducing signal across the cell membrane. Testing for EGFR amplification serves mainly as a refinement of diagnosis because its presence in brain [6]. EGFR signaling was shown to promote tumor cell proliferation and survival, invasion and angiogenesis [7,8] and mediate resistance to treatment, including ionizing radiation in preclinical models [9,10]. Mutations in egfr kinase domain have been associated with responsiveness [10,11]. It is not known, however, whether such mutations affect the responsiveness of other types of cancer to EGFR kinase inhibitors. EGFR has biological importance in cancer [12,13] and its gene is located on the same chromosome (chromosome 7 for glioma). EGFR itself is a transmembrane spanning protein, with an extracellular ligand binding and a cytoplasmatic tyrosine kinase domain [14 ,15], and its main ligand EGF is a polypeptide consisting of 53 amino acids that binds to domain I and II of the extracellular part of the receptor. Upon binding of EGF to EGFR, the receptor undergoes conformational changes [16,17]. EGFR is a key molecule in cancer cells. It has been proposed that trastuzumab in hibits the downstream signaling pathways, including phosphoinositide 3 kinase (P13K)/Akt and mitogen-

*Corresponding author. E-mail: Mehdi.pooladi7@gmail.com

activated protein kinase (MAPK) pathways and in deuces receptor-mediated cytotoxity through immune response [18-20].

In the present study, we investigated the EGFR expression change in human brain astrocytoma (III and IV) and oligodendroglioma(III) tumors. To get an understanding of data and specific software for molecular diagnosis of glioma, we extracted proteins of tumoral and normal brain tissues and evaluated the protein quantities. We separated proteins by two-dimensional gel electrophoresis and identified alternation in spot characteristics using statistical data, specific software (Progenesis Same Spots) and MALDI TOF-TOF.

MATERIALS AND METHODS

Patient Samples

Tissues were obtained, with informed consent and institutional review board approval, from patients undergoing tumor resectioning. For this study, all individuals filled a written informed consent form. Astrocytoma (III and IV) and oligodendroglial(III) tumors were surgically removed at Shohada Tajrish Hospital. The tumors were classified by a team of neuropathologists according to the guidelines of the WHO classification of tumors of the central nervous system. Ten tumors [4 astrocytoma(III), 3 astrocytoma(IV) and 3 oligodendroglioma(III)] from surgery operated patients with malignant glioma have been separated. Non-tumoral brain tissues were obtained from normal areas (either grey or white matter) of brain tissues removed from patient undergoing non-tumor epileptic surgery. In all phases of research, ethical issues have been considered. Also, informed consent from patients or their relatives to participate in this research were taken.

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 -80 ºC until used for proteomic analysis. To obtain tissue extracts, the samples were broken into suitable pieces and were homogenized in lysis buffer II consisting of lysis buffer I {7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl) dimethylammonio]-1-propane-sulfonate (CHAPS), 0.2% 100 × Bio-Lyte 3/10}, dithiothreitol (DTT), and 1 mM ampholyte and protease inhibitor (AEBSF 2 mM, Sigma) on ice. Cell lysis was completed by subsequent sonication (4 × 30 pulses). Then samples were centrifuged 20000 g at 4 °C for 30 min to remove insoluble debris. Supernatants were combined with acetone 100% and centrifuged at 15000 g, and then the supernatants were decanted and removed (3 times). Acetone 100% was added to the protein precipitant and kept at -20 °C (overnight). Samples were then centrifuged again at 15000 g and the precipitant incubated for 1 hour at room temperature. The protein samples were dissolved in rehydration buffer [8 M urea, 1% CHAPS, DTT, ampholyte pH 4 and protease inhibitor]. Protein concentrations were determined using the Bradford test and spectrophotometry method, and the protein extracts were then separated and used for 2D gel electrophoresis

Two-Dimensional Gel Electrophoresis

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

Image Analysis

Analytical gels were scanned by a Densitometer GS-800 (BioRad) scanner at 600 dpi in tagged image file format (TIFF). Image Master^{TM} 2D platinum v6.0 software was then used to extract and digitize data from graphical images of scanned gels through detecting, normalizing, matching and comparing protein spots according to their volume percent. The gel images were analyzed (automatically and) by Progenesis Same Spots software to identify spots

differentially expressed between astrocytoma tumors (III, IV) and control samples, oligodendroglial tumor(III) and normal tissue and astrocytoma(III) and astrocytoma(IV) based on difference were defined as altered. The spots were carefully matched individually and only spots that showed a definite difference were defined as altered. Spots were detected by isoelectric pH, Molecular Weights, databanks and Comparison with previous research.

MS Analysis

The identify of differentially expressed proteins ($P < 0.05$ and fold > 2) was established using MALDI TOF TOF Mass Spectrometery. In gel digestion was done as mentioned in a MS analysis was performed described previously.

RESULTS

Using 2D-PAGE proteomic analysis, we compared protein expression patterns between asterocytoma (gread III & IV) and oligodendroglioma (gread III) samples relative to control tissue (Fig. 1). The 2D-gel electrophoresis revealed consistent protein profiles for each group. Simple statistical the test was used to establish a putative hierarchy in which the change in protein level were ranked according a cutoff point with $P < 0.05$. The 2D gel showed totally 800 spots for astrocytoma(III). A total of 343 spots showed statistically significant differences (student's *t*-test; $P < 0.05$) in gel, of which 164 spots exhibited up-regulation in expression level, whereas the remaining 179 spots were decreased in astrocytoma tumor relative to normal tissue. Among them the statistically significant protein spots ($p < 0.05$) EGFR proteins were definitely with (first spot: as shown in Fig. 1) isoelectric pH mean 6.27 and molecular weight mean 12.3 kDa detected which has an up-regulation about 1.6 (fold = 1.6) (Fig. 2), and showed totally 876 spots for astrocytoma(IV). A total of 420 spots showed statistically significant differences (student's *t*-test; $p < 0.05$) in gel, of which 188 spots exhibited up regulation in expression level, whereas the remaining 232 spots were decreased in astrocytoma tumor relative to normal tissue. Among them the statistically significant protein spots ($p < 0.05$) EGFR proteins were definitely with (first spot: as shown in Fig. 1) isoelectric pH mean 6.32 and molecular weight mean 14 kDa detected which has an up-regulation about 2.8 (fold = 2.8) (Fig. 3).

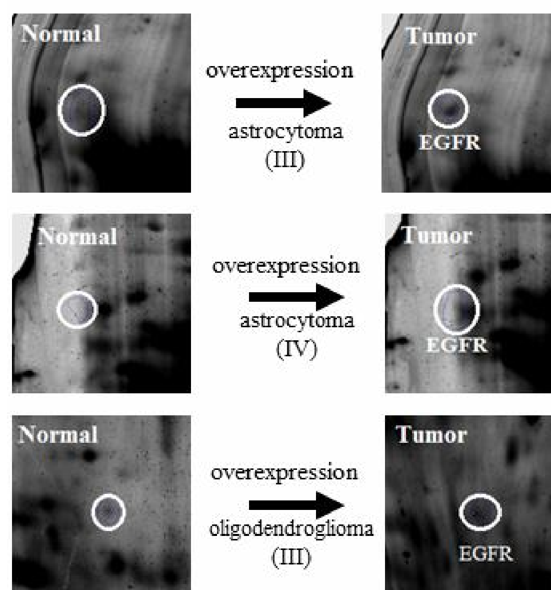

Fig. 1. Comparison of expression changes Spots (EGFR) in astrocytoma(III), astrocytoma(IV) and oligodendroglioma(III) with normal brain tissue.

Also, showed totally 1328 spots for oligodendroglioma(III), A total of 433 spots showed statistically significant differences (student's *t*-test; $p < 0.05$) in gel, of which 157 spots exhibited up regulation in expression level, whereas the remaining 276 spots were decreased in astrocytoma tumor relative to normal tissue. Among them the statistically significant protein spots ($P < 0.05$) EGFR proteins were definitely with (first spot: as shown in Fig. 1) isoelectric pH mean 6.25 and molecular weight mean 13.3 kDa detected which has an up-regulation about 1.5 (fold = 1.5) (Fig. 4). As can be seen in the picture, astrocytoma shows more expression than oligodendroglia changes, and also, gread III astrocytoma showed more expression than gerad IV astrocytoma changes.

In order to continue to further understanding of rates of change, each of the tumors (oligodendroglioma III, astrocytoma III, and astrocytoma IV) has been compared with their controls. Molecular weight and isoelectric pH values are recorded in Table 1, and the trend lines are shown in diagram 1. Afterwards, Statistical analysis we

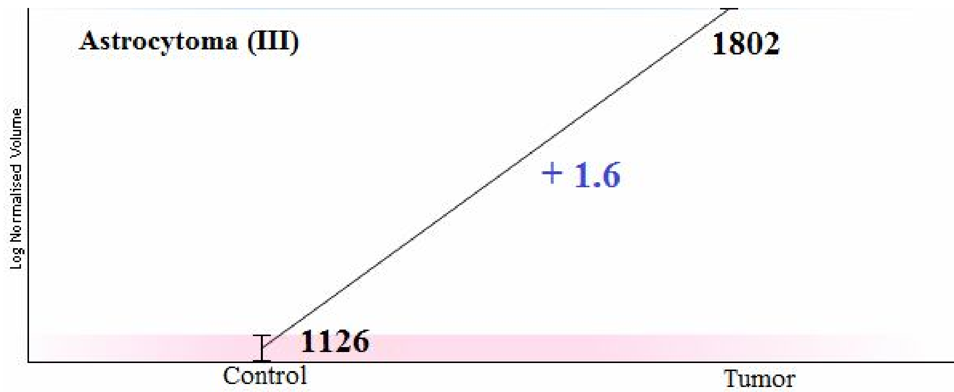

Fig. 2. EGFR protein has an up-regulation about 1.6 (fold = 1.6) in asterocytoma(III) brain tumors with normal brain tissue.

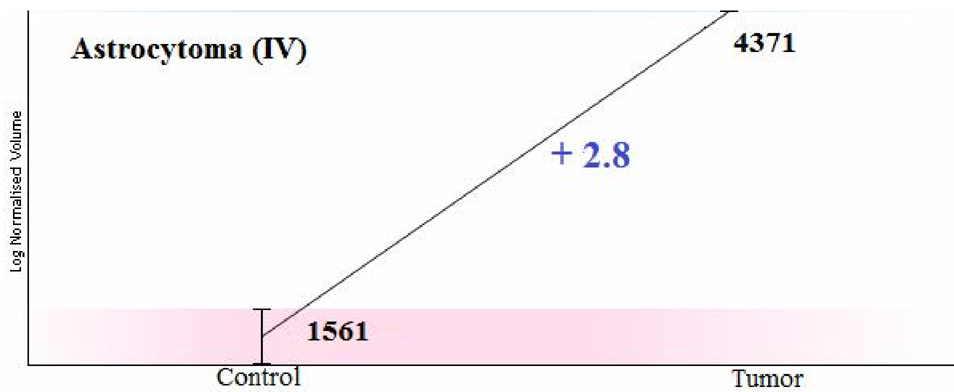

Fig. 3. EGFR protein has an up-regulation about 2.8 (fold = 2.8) in asterocytoma(IV) brain tumors with normal brain tissue.

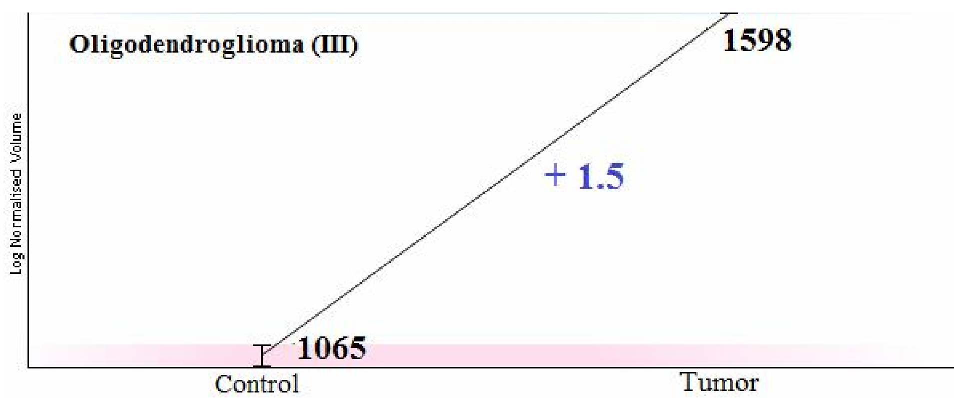

Fig. 4. EGFR protein has an up-regulation about 1.5 (fold = 1.5) in oligodendrogloma(III) brain tumors with normal brain tissue.

Table 1. The Molecular Weight and Isoelectric pH of Each of the Tumors (Oligodendroglioma III, Astrocytoma III, and Astrocytoma IV) Compared to Control Independently

	Tumor	Grade	Sex	Age	EGFR		
					$P < 0.05$	PI	MW
Case 1	Oligodendroglioma	III	Man	48	6.542e-009	6.43	14
Case 2	Oligodendroglioma	III	Man	69	4.683e-009	6.18	12
Case 3	Oligodendroglioma	III	Woman	63	1.318e-007	6.13	14
Case 4	Astrocytoma	III	Man	39	3.878e-007	6.27	13
Case 5	Astrocytoma	III	Man	60	2.078e-007	6.36	13
Case 6	Astrocytoma	III	Woman	51	5.345e-006	6.19	11
Case 7	Astrocytoma	III	Man	62	2.106e-007	6.23	12
Case 8	Astrocytoma	IV	Woman	49	5.868e-006	6.41	14
Case 9	Astrocytoma	IV	Man	55	1.115e-006	6.29	15
Case 10	Astrocytoma	IV	Man	56	1.080e-006	6.34	15

Table 2. Statistical Analysis of Molecular Weight and Isoelectric pH for EGFR

	Valid	Missing	Mean	Median	Error of mean	Variance	Min	Max	Rande
PI	10	0	6.28	6.28	0.09	0.0081	6.13	6.43	0.3
MW	10	0	13.3	13	1.1	1.2	11	15	4

Table 3. EGFR Protein Matching the Same set of Peptides by Databank

	Expressed proteins change	Fold change	Number of peptides	Score	Matches	Sequences coverage
Oligodendroglioma (III)	Up-Regulated	1.5	16	4	37(41)	41
Astrocytoma (III)	Up-Regulated	1.6	17	6	39(40)	40
Astrocytoma (IV)	Up-Regulated	2.8	19	6	41(44)	44

have examined more closely, and it has been presented in Table 2.

Proteins identification was performed by MALDI TOF TOF, in this experiment; we changed EGFR expression (Up-regulated) in oligodendroglioma(III), astrocytoma(III) and astrocytoma(IV) tumors than control identified by MALDI TOF TOF. Levels of EGFR spots were markedly higher in tumor than non-tumor. We analyzed data from the MALDI TOF TOF, were showed in Table 3.

DISCUSSIONS

Cancer biomarkers for the early detection of malignancies and selection of therapeutic strategies have

been requested in the clinical field. Proteomics has produced an enormous number of biomarker candidates for cancer, but this effort has not been accompanied by an increase in validated biomarkers [21-23].

The selective expression of EGFR by glioma of higher degree and the potential of sparing normal brain tissue, also suggest the possibility of successful radio sensitization in this setting [24]. In contrast, molecular abnormalities in these engraft models affected the EGFR associated with (TTEN) less. Recent works attempted to identify molecular subtypes in glioblastomas and diffuse glioma, were similary described in oligodendroglioma. Abnormalities on the EGFR and the EGFR dependent signaling pathways are most frequently reported in high grade oligodendroglioma and effect all histological closes [25-28].

Most of the investigate, focused on the acstrocytic too tumors. We investigated so far about EGFR expression change in glioma tumor. Ekstrand *et al.* [29] EGFR gene amplification was found in WHO grade II oligodendroglioma and anaplastic oligodenoglioma (WHO grade III and IV). None of the tumors had EGFR gene amplification. Diedrich *et al.* [30] investigated two anaplastic oligodendroglioma. Both showed strong immunoreactivity for protein and one had EGFR gene amplification [31]. In essays, to investigate the passible potential applicability of this approach in future clinical sample analysis, EGFR-characterized tumor samples from cancer patients were studied. The analyzed samples were grouped based on a clinically relevant cut off level for EGFR, and it was possible to discriminate samples with expression levels below or above cut off using the described assay setup [32]. EGFRs with mutations in the tyrosine kinase domain selectively activate antiapoptotic signals through the PI3 K-Akt signaling pathway [33]. Akt, a kinase involved in cellular proliferation and apoptosis, is activated by signals generated by pI3 k [12]. Currently, activating mutations of EGFR are the only validated biomarkers of response to EGFR tyrosine kinase inhibitors in Non-Small Cell Lung Cancer (NSCLC) [34].

In gliomas, the EGFR gene is amplified in approximately 40% of glioblastomas and less than 10% of anaplastic astrocytoma. Amplification of the EGFR gene in malignant gliomas consistently results in overexpression of EGFR mRNA and protein [35,36]. We showed expression changed of EGFR in gliomas, however, has focused on the protein. The EGFR pathway is commonly altered in gliomas, such as protein insertion in to membrane, positive regulation of epithelial cell proliferation, negative regulation of apoptosis, translation, and signal transduction [37].

We have also shown in this study that changes in EGFR expression in malignant astrocytoma (III and IV) and malignant oligodendroglioma(III) tumors are indeed observed. Thus EGFR could be a candidate biomarker in glioma tumors. Due to the wide variation in pI and molecular weight of EGFR, in a variety of malignant gliomas, we propose this biomacromolecular as being a candidate biomarker for the diagnosis and prediction of glioma tumors. Hopefully in the future, by examining the types of glioma biomarker candidates, we will be able to achieve a more unified and functional approach.

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