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Glioblastoma cell secretome: Analysis of three glioblastoma cell lines reveal 148 non-redundant proteins

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ABSTRACT

Glioblastoma multiforme (GBM) is the most aggressive among human gliomas with poor prognosis. Study of tumor cell secretome – proteins secreted by cancer cell lines, is a powerful approach to discover potential diagnostic or prognostic biomarkers. Here we report, for the first time, proteins secreted by three GBM cell lines, HNGC2, LN229 and U87MG. Analysis of the conditioned media of these cell lines by LC-MS/MS using ESI-IT mass spectrometer (LTQ) resulted in the confident identification of 102, 119 and 64 proteins, respectively. Integration of the results from all the three cell lines lead to a dataset of 148 non-redundant proteins. Subcellular classification using Genome Ontology indicated that 42% of the proteins identified belonged to extracellular or membrane proteins, viz. Vinculin, Tenascin XB, SERPIN F1 and TIMP-1. 52 proteins matched with the secretomes of 11 major cancer types reported earlier whereas remaining 96 are unique to our study. 25 protein identifications from the dataset represent proteins related to GBM or other cancer tissues as per Human Protein Atlas; at least 22 are detectable in plasma, 11 of them being reported even in cerebrospinal fluid. Our study thus provides a valuable resource of GBM cell secretome with potential for further investigation as GBM biomarkers.

1. Introduction

Glioblastoma multiforme (GBM) is the most common of the primary brain tumors in adults. It develops *de novo* or progresses from the lower grade astrocytoma. It is highly malignant with weak response to chemo or radiotherapy and resultant poor prognosis [1]. Diagnosis is primarily based on imaging and histological analysis of the surgical biopsies but for deep-seated, irresectable tumors diagnosis is done using stereotactic biopsies. Identification of plasma or serum based biomarkers would help in diagnosis or prognosis of the disease.

Association of many genes with glioblastoma has been reported earlier. A recent study of genome-wide profiling indicated as many as 27 mutations in TP53, a tumor suppressor gene and proved it as a common event in primary glioblastoma [2]. The study also reported deregulation of RB and PI3K pathways in most of the GBM tumors. Similarly in Proteomic studies, several proteins show abnormal expression in the tumor tissues [3]. Some of them such as GFAP, YKL-40, and Haptoglobin are associated with the serum or plasma [4–6]. However, increasing the panel of serum or plasma protein markers would be highly useful for effective assessment of the tumor progression or recurrence.

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Direct analysis of patient plasma or serum to investigate protein biomarkers is difficult due to technical challenges in their analysis [7]. Tumor cell lines therefore offer a useful alternative for this purpose. Protein secreted by cells into the culture medium (secretome) under minimal cell death conditions, identified by mass spectrometry approaches could be regarded as potential candidates for investigation in the CSF or plasma in a targeted manner. The cell secretome approach has been used for various cancer cell types [8]. For example, secretome study using M-BE cell line from lung cancer identified 47 deregulated proteins by 2DE MS approach [9]. In pancreatic cancer secretome study, 145 differentially secreted proteins were identified using SILAC-based differential proteomics approach [10]. Several glioblastoma cell lines are available and have been used for molecular and genetic analysis of GBMs. In the present study, we have explored the secretomes of three glioblastoma cell lines – U87MG, LN229 and HNGC2. The cell lines U87MG, LN229 are well-established cell lines and have been used earlier in several studies although not for the secretome analysis [11]. The third cell line HNGC2 derived from high-grade tumor is a more recently developed cell line, which has been shown to possess characteristics of glioblastoma [12]. The HNGC2 cells enriched with brain tumor initiating cells have been extensively characterized. All these cell lines carry p53, pTEN mutations implicated in pathways important for gliomagenesis and are capable of inducing tumors when injected in immune competent mice [13,14]. Our study resulted in non-redundant identification of a set of 148 proteins from the three cell lines that include extracellular and membrane localised proteins; GBM or other tumor associated proteins and those detectable in cerebrospinal fluid and plasma thus providing a reference GBM secretome for further investigation.

2. Materials and methods

2.1. Cell culture and collection of conditioned media

Glioblastoma cell lines HNGC2, U87MG and LN229 were grown in 75 cm^2 flasks in DMEM medium (Invitrogen, Carlsbad, CA) supplemented with 10% FCS (CSL, Melbourne) and in the presence of penicillin 50 μg/mL and streptomycin 60 μg/mL (Sigma, St. Louis, MO), in 5% CO_2 incubator at 37 °C. Cells were first grown to 80% confluence and then washed with PBS twice. To remove the adhered serum proteins from the cells, cells were incubated in serum free DMEM media for 1 h and washed again with serum free media twice. Cells were subsequently incubated in serum-free media for 24 h. About 60 ml of conditioned medium was collected from each cell line, centrifuged at 500×*g* for 5 min to sediment cells followed by further centrifugation at 2000×*g* for 10 min to remove any cell debris. To the resultant supernatant, protease inhibitor cocktail (Roche, Nutley, NJ, USA) was added and the medium was filtered through a 0.2 μm membrane (Millipore Billerica, MA). The filtrate was concentrated to 1 ml using a 5 kDa Amicon Ultra centrifugal filter device (Millipore Billerica, MA). The concentrated samples were dried by speed vac and resuspended in 200 μl of deionized water. The protein content of the samples was determined using amido black method [15].

2.2. Cell viability assay

Cell culture medium after incubation in serum free conditions, was collected and centrifuged at 500×*g* to collect non-adherent cells. Adherent cells were trypsinised and harvested by centrifugation as above. Both the adherent and non-adherent cells were combined and viability assessed in triplicate by Trypan Blue exclusion test. Trypan blue dye in PBS (100 μl of 0.4% w/v) was mixed with 100 μl of cell suspension and observed under microscope. The percentage of cell viability was expressed as the ratio of total viable cells to the sum of total viable and dead cells. Conditioned media was examined and compared to cell lysate for the presence of tubulin by Western Blot analysis. In brief, 20 μg of the protein extract was separated by 12% SDS PAGE and transferred to PVDF membrane (Millipore, Bedford, MA, USA). The blot was probed with rabbit polyclonal anti tubulin antibody (1:1000; Pierce Thermo Scientific) and developed using DAB system (Bangalore Genei, India) with anti rabbit goat antibody conjugated with horse raddish peroxidase (Bangalore Genei, India).

2.3. LC-MS/MS

Conditioned medium collected from each cell line was concentrated and proteins present were reduced with 10 mM DTT at 60 °C for 45 min followed by alkylation with 50 mM iodoacetamide in 25 mM ammonium bicarbonate, at room temperature for 1 h. Samples were in-gel digested with trypsin (Promega, Madison, WI, USA) (1:100) in 25 mM ammonium bicarbonate at 37 °C, overnight. Tryptic peptides were extracted; freeze dried, resuspended in buffer-A (see below) and subjected to on-line LC-MS analysis. Peptide separation was carried out in a Bio Basic C18 micro capillary column (Thermo Fisher, Waltham, MA, USA) using a 210 min linear gradient of the mobile phases [5% ACN containing 0.1% formic acid (buffer-A) and 95% ACN containing 0.1% formic acid (buffer-B)] at a flow rate of 2 μl/min. The RP HPLC was coupled on-line with an ESI-linear ion-Trap mass spectrometer (LTQ, Thermo Fisher Scientific, Waltham, MA, U.S.A.) for MS and MS/MS analysis. Each cycle comprised of one full MS scan (m/z 300–2000) followed by data dependent MS/MS analysis of the top seven intense precursor ions. Data was acquired with mass accuracy within 1 Da for MS and 0.35 Da for MS/MS.

2.4. Bioinformatic analysis

Identical triplicate LC-MS runs were carried out for the conditioned media proteins generating three RAW files for each cell line. Each of the MS files was analyzed and peptide sequences identified with SEQUEST algorithm based Bioworks software version 3.1 (Thermo Fisher Scientific, Waltham, MA, USA) using NCBI human curated database. Prior to the search, the NCBI non-redundant human protein database was modified so that the description lines from NCBI Entrez gene annotation were incorporated into the NCBI Human protein database. The search parameters were as follows: 1) variable modifications – methionine oxidation (+16 Da), carbamidomethylation of cysteine (+58 Da). 2) Peptide mass