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Feasibility assessment of *in vitro* chemoresponse assay on stereotactic biopsies of glioblastoma multiforms: a step towards personalized medicine

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ABSTRACT

Objective(s): P *In vitro* chemosensitivity and resistance assays (CSRAs) are a promising tool for personalized treatment of glioblastoma multiform (GBM). These assays require a minimum of 1 to 2 g of tumor specimen for testing, but this amount is not always accessible. We aimed to assess the feasibility and validity of utilizing stereotactic biopsies of GBM in CSRAs.

Materials and Methods: Single cell suspension was prepared from 1 g weight explants of the established xenograft tumor of GBM. Also, primary culture was carried out on 35 mg weight specimens, as a surrogate for stereotactic biopsies. Then, chemoresponse profile of cells obtained by direct cell disaggregation and primary culture was determined using temozolomide and carmustine by clonogenic assay.

Results: There was no statistically significant difference in the cytotoxicity of temozolomide and carmustine between cells obtained from both methods.

Conclusion: This work supports the feasibility of using stereotactic biopsies of GBM in CSRAs.

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Introduction

Glioblastoma multiform (GBM) is the most common subtype of primary brain malignancies in adults (1). This tumor is very invasive, highly aggressive, and often invades critical neurologic areas within the brain (2). Current standard care of GBM includes cytoreductive surgery followed by adjuvant radiotherapy and chemotherapy. However, the median survival time for newly diagnosed patients and recurrent cases is 8 to 15 months and 3 to 9 months, respectively (3). This failure in treatment has fueled attempts to shift the investigation of GBM therapies from non-specific one-size-fits-all approach to personalized medicine (4).

The goal of personalized medicine is to select the right chemotherapy regimen for the right person by predicting patient response to cancer chemotherapy (5). *In vitro* chemosensitivity and resistance assays (CSRAs) have been historically evaluated as a tool for predicting the clinical response to specific drugs and selecting proper drugs in clinical trials (6). Nowadays, these assays are sometimes used by clinicians to choose the appropriate treatment regimens for an individual patient. Several CSRAs have been developed that differ in processing of biological specimens and detection techniques. However, all techniques involve similar principles including: 1) isolation of cells, 2) incubation of cells with drugs, 3) assessment of cell survival, and 4) interpretation of the results (7). Clonogenic assay is probably the most common technique used. In this method, single cell suspension of tumor cells obtained by mechanical and enzymatic disaggregation are cultured in soft agar and

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then incubated with drugs (8). The tumor stem cell fraction, which accounts for less than 0.4% of total cell population, is able to divide and form colonies in this matrix. But other cells do not grow in this anchorage-independent medium (9). Success rate of CSRAs correlates with availability of an adequate amount of viable tumor cells (7). These assays require a minimum of 1 to 2 g of tumor specimen for testing (6). But this amount of tumor tissue is not always accessible. Sometimes the tumor of GBM is inoperable and the amount of specimen that can be obtained by stereotactic biopsy is very little (10). Primary culture of tumor cells by primary explant technique can be used to overcome this problem (11). But since genetic instability is a characteristic of most neoplastic cells, there could be a concern that tumor cells experience phenotypic alterations during primary culture (12).

The present study aimed to assess the preservation of chemoresponse profile of GBM neoplastic cells during primary explant technique. In the current work, we described the feasibility of utilizing small amount of GBM tumor material as weigh as those can be obtained by stereotactic biopsy in chemoresponse assay

Materials and Methods

Establishment of xenograft tumor model of GBM

Male BALB/c athymic nude mice (Six to 8-wk-old n=8) purchased from Omid Institute (Omid Institute for Advanced Biomodels, Tehran, Iran). The animals were treated according to guidelines outlined by Institutional Ethical Committee. All animals were kept under optimized hygienic conditions in individually ventilated cage system. The animals were fed with autoclaved commercial diet and water *ad libitum*. U-87 MG glioblastoma cell line was purchased from National Cell Bank of Iran (Pasteur Institute of Iran, Tehran, Iran). This cell line (5×10^6) in 200 µl of Matrigel/RPMI 1640 (1:1 v/v) (BD Matrigel™ Basement Membrane Matrix, BD Biosciences, USA) was inoculated subcutaneously into the both right and left flank regions. When the volume of xenograft tumors reached about 2000 mm^3, the animals were sacrificed in a humane manner using CO_2 gas. After harvesting under laminar flow conditions, the xenograft tumors were divided into three parts. To confirm the tumor type by hematoxylin and eosin (H&E) staining and immunohistochemical examination with glial fibrillary acidic protein (GFAP), a part of specimens was immediately fixed using 4% formaldehyde in 0.1 M phosphate-buffered saline (PBS) and sent to the Pathology Division of Cancer Institute of Iran. The other parts were put into cooled DMEM supplemented with 20% FBS, 100 U/ml of penicillin, 100 µg/ml of streptomycin, and 10 µg/ml of amphotericin B and devoted to primary culture or preparation of single cell suspension followed by clonogenic assay. All cell culture media and materials were obtained from Invitrogen (Carlsbad, CA, USA).

Primary culture with explant technique

To determine the preservation of chemoresponse profile of stereotactic biopsies during primary culture, about 35 mg of each tumor was devoted to primary explant technique. Since this amount of specimen is not sufficient to perform the clonogenic assay, primary explant technique was initiated from fresh tumor samples to obtain a sufficient number of cells prior to clonogenic assay. Briefly, tumor tissue was minced with scalpels into 1 mm^3 explants, washed with Hank's balanced salt solution supplemented with 100 U/ml of penicillin, 100 µg/ml of streptomycin, and 10 µg/ml of amphotericin B three times, and plated on 25 cm^2 culture flasks (BD Biosciences, NJ, USA) in 1 ml DMEM: F12 (1:1; V/V) medium supplemented with 20% FBS. After adhesion of tumor material, medium was raised to 5 ml. Medium was changed every second day. Once a substantial outgrowth was observed, the cells were harvested using trypsin-EDTA and prepared to clonogenic assay.

Preparation of single cell suspension

Tumor specimens with about 1 g weight were mechanically chopped with scalpels and subsequently incubated with an enzyme cocktail containing 41 U/ml collagenase, 125 U/ml DNase, and 100 U/ml hyaluronidase at 37˚C for 45 min. The cell clusters were passed through cell strainers of 75 µM and 50 µM (BD Biosciences, NJ, USA) mesh size and then washed. Cell viability was determined by trypan blue using a hemocytometer chamber.

Tumor clonogenic assay

For chemoresponse assessment, a modification of clonogenic assay as described by Hamburger and Salmon was used (8). The bottom layer consisted of 0.2 ml/well DMEM supplemented with 20% FBS and 0.75% agar; 20×10^4 cells obtained by primary culture or direct single-cell suspension were added to 0.2 ml of the medium containing 0.4% agar and plated in 24-multiwell dishes on top of the bottom layer. Temozolomide and carmustine (Sigma-Aldrich, USA) were added as drug overlays in 0.2 ml medium containing 1 to 100 µM of drugs in triplicate. Cultures were incubated at 37˚C under 5% CO_2 in a humidified atmosphere for up to 21 days and monitored closely for colony formation using an inverted microscope. In day 21, counting of colonies with more than 50 µm in diameter was performed, and surviving fraction was calculated by colony count of treated group divided by colony formation of control group. Finally, median inhibitory concentration (IC_{50}) was determined as the drug concentration that is required to reduce the colony formation to half that of the control.

Statistical analysis

Data were represented as mean ± standard error of the mean (SE). Statistical analyses were performed with Student's t-test. Linear regression analysis was used to calculate IC_{50}. Level of significance was set at $P<0.05$. The statistical analyses were carried out using BioStat 2008 software.

Results

Heterotopic xenograft tumor model of U-87MG was established with 70% take rate. In histopathological study, the nature of GBM xenograft tumor was approved by observing pseudopalisading necrosis as the landmark of GBM, and expression of GFAP.

Success rate of primary explant technique was 100% (Figure 1). In this method, the outgrowth covered at least 50% of the growth surface up to 7.21 ± 0.25 days.

Colony count data are summarized in Table 1. There was no statistically significant difference in the number of formed colonies between clonogenic assays performed by the cells obtained from primary explant technique and those prepared from direct disaggregation of tumor cells at any concentration of temozolomide or carmustine tested.

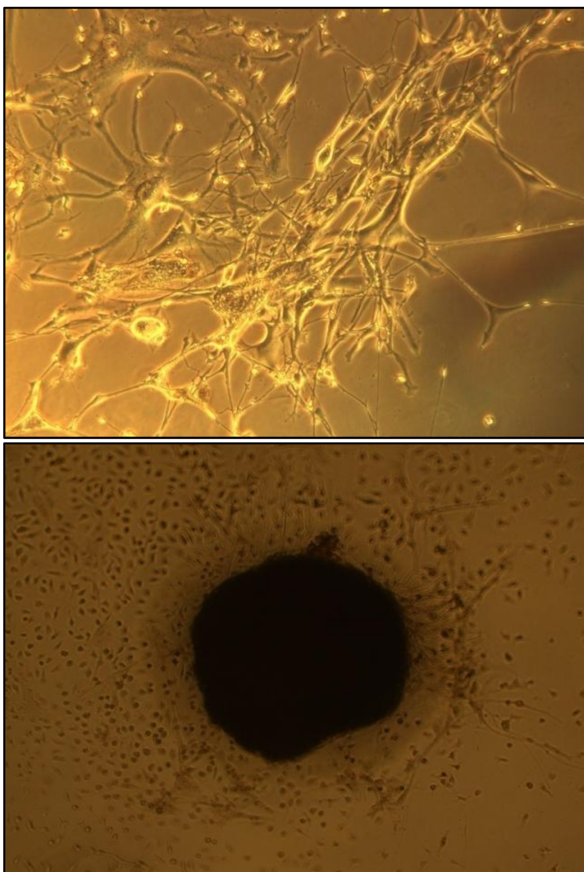

Figure 1. Primary explant culture of U-87MG xenograft tumor (A) Monolayer cell outgrowth was observed is observed around the tumor explant (original magnification, ×10); (B) Isolated cells from explants at 7th day of primary culture (original magnification, ×40)

In clonogenic assay with direct single cell suspension, the 95% confidence interval of IC_{50} for temozolomide and carmustine were from 29.55 µM to 87.46 µM and 65.16 µM to 135.91 µM, respectively. The IC_{50} of temozolomide and carmustine on the harvested cells of primary explant technique were calculated as 37.96 µM and 85.02 µM, respectively, which lied in the 95% confidence intervals of those obtained from the direct method.

Discussion

The present study aimed to assess the feasibility and validity of utilizing stereotactic biopsies of GBM in CSRAs via primary culture followed by tumor clonogenic assay. In this regard, we wanted to determine whether the chemoresponse profile of GBM neoplastic cells is preserved during primary explant technique. Our results demonstrated that the number of cells required to tumor clonogenic assay can be prepared from specimens equal to stereotactic biopsies in 7.21±0.25 days by primary explant method. Another finding of this work was that the IC_{50} of temozolomide and carmustine in cells harvested from primary explant technique lies in the 95% confidence intervals of those obtained from direct disaggregation of tumor cells.

In an attempt to prove that neoplastic cells of human solid tumors can be isolated and grown in primary explant culture, Ochs *et al* assessed telomerase activity and expression level of cytokeratins, tumor marker p53 and proliferation marker Ki-67 in cells isolated from primary culture. They demonstrated the presence of these markers in the cells obtained from monolayer outgrowth indicating the isolation of malignant cells and preservation of their phenotype in primary culture (13).

Primary explant technique has been previously used by Gallion *et al* for providing adequate amount of neoplastic cells from surgically excised ovarian carcinomas prior to chemosensitivity assay (14). They reported the good predictive value of CSRA indicating the maintenance of chemoresponse profile during primary explant method.

Brower *et al* described the use of primary explant method on ultrasound-guided core needle biopsies of breast cancer prior to CSRA (6). They showed the isolation of sufficient number of cells from 35 mg tumor specimens by this technique. They also proved the epithelial phenotype of isolated cells by immunocytochemical assessment of cytokeratins expression.

To establish new GBM cell lines, Mohd Ramli *et al* used primary explant method for isolation of malignant cells from tumor specimens. Using immunocytochemistry with GFAP, S-100 and vimentin they proved the malignant nature of isolated cells and stability of molecular profile of these GBM cells by this kind of primary culture (15).

Our results demonstrate that a sufficient number

Table 1. Tumor clonogenic assay of cells obtained from U-87MG xenograft model by primary explant culture or direct enzymatic disaggregation of tumor specimens. Colony counting was carried out in 21th day of cell seeding after treatment with different concentrations of temozolomide and carmustine. Data are represented as mean ± SE

Drug concentrations (μM)	Temozolomide		Carmustine	
	Primary explant culture	Direct disaggregation of tumor cells	Primary explant culture	Direct disaggregation of tumor cells
0	30.30 ± 2.03	28.92 ± 1.94	31.25 ± 2.33	27.64 ± 1.47
1	28.43 ± 2.46	29.95 ± 2.53	26.63 ± 1.21	28.12 ± 2.12
3.3	24.12 ± 1.98	26.17 ± 2.07	25.24 ± 1.24	28.49 ± 1.95
10	19.78 ± 1.76	24.54 ± 1.29	24.72 ± 0.86	25.17 ± 1.07
33	9.49 ± 1.30	6.52 ± 1.01	25.39 ± 1.25	16.75 ± 3.25
100	0.60 ± 0.12	0.30 ± 0.07	10.21 ± 1.63	8.12 ± 1.47

of GBM cells required for CSRA can be achieved by primary explant culture within a week. Another important finding of this work is that this monolayer cell outgrowth represents the same chemoresponse profile as seen in direct obtaining of cells from tumor. These results are consistent with the works indicating the preservation of molecular profile during primary explant culture (13, 15).

Conclusion

This work supports the feasibility of using stereotactic biopsies of GBM in CSRAs. Further research is needed to elucidate the clinical predictive value of this approach in personalized treatment of GBM.

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