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A FLOW CYTOMETRIC COMPARISON OF TWO GLIOBLASTOMA CELL LINES

Constantin Daniel[1], Liana Toma[1]

[1] *National Institute of Research and Development for Biological Sciences, 0630031, Splaiul Incependentei 296, Bucharest, Romania, e-mail:constantin_daniel_bio@yahoo.com*

Abstract. Glioblastoma is the most common malignant neoplasm that occurs in the central nervous system in humans, it is very difficult to treat despite advanced diagnostic, imaging and surgical techniques. In recent decades, great efforts have been made to elucidate the cellular and molecular mechanisms of this disease. However, not much progress has been made due to the fact that the brain tumor is composed of heterogeneous populations of cells, many of them resistant to chemotherapy. It is clear that researchers need many in vitro test models (cell lines) in order to better understand and treat this disease. The purpose of this study is to compare in terms of the amount of DNA and cell cycle, a malignant glioma cell line, T11 obtained at INCDSB [1], with a stabilized glioblastoma cell line acquired from a reference center (U87 line). The flow cytometry analysis determines whether the cell cycle and DNA content of our local cell line, T11, are similar to those of the U87 line used worldwide.Our results indicate that are no significant differences between the two cell line regarding size, granularity, DNA content and cell cycle.

***Keywords*:** glioblastoma, cell cycle, flow cytometry.

Introduction

Glioblastoma multiforme (GBM) is the most malignant form of Grade IV astrocytoma according to the most recent neuropathological classification by World Health Organization.

It is the most prevalent primitive brain tumor among adults (undifferentiated small cells but with high differentiation potential) representing 40% of all tumors and 80% of primitive high–grade neoplasms of the central nervous system [OKAN DEMIR 2005, WRENSCH 2002].

In Europe, about 1 in 33 000 individuals is affected each year regardless of age, peaking between 65 and 75 years.

The tumor has an incidence of 40% higher in men and caucasian type than other ethnic groups.

Although GBM stands for only 1% of all adult cancers, it is responsible for 2% of annual deaths due to cancer.

Prognosis and survival of patients with GBM remains dismal, with an average survival rate of 12–14 months

The clinical data shows that the current treatment of patients (surgical resection, radiation, conventional chemotherapy) is palliative, inadequate and unable to remove the malignant tumor.

Although it has been shown that cytotoxic chemotherapy has given a chance of survival for patients with grade IV glioma, the results are modest [KADEMIR and GUL 2009].

The efficacy of usual chemotherapy drugs is conditioned by the release mechanism of the chemotherapeutic (for drugs that cross the blood brain barrier), by the toxicity to the nervous tissue and to the patient's whole body, and by the inherent resistance of high–grade brain tumors to chemotherapy [Mohamed A. 2009].To analyze the DNA content status of a population of cells, a fluorescent marker that binds stoichiometrically to DNA is used (Propidium Iodide, and the amount of marker is analyzed.

The DNA histograms are classified as normal or cancerous depending on the existence of aneuploid/polyploid cells, and on the percent of proliferating cells (S phase+G2/M).

Normal or diploid histograms contain between 5 to 14% proliferating cells (S phase+G2/M).

Abnormal (cancer) histograms show one of the following characteristics:

- (1)DNA diploidy and more than 15% proliferating cells (high proliferative fraction) and/or
- (2)DNA aneuploidy with a greater or lesser number of aneuploid and polyploid cells [Collins 1994].

In this paper we compared, in terms of cell cycle and DNA content, a glioblastoma cell line obtained in our laboratory (T11) with a glioblastoma cell line acquired from ATCC (U87 cell line).

Material and methods

The T11 cell line was obtained in our cell culture laboratory using enzymatic dissociation. [BACCIOCCH, 1992 and BAKIR 1998].

For cell cycle analysis by flow cytometry the T11 and U87 (acquired from ATCC cat. no. HTB–14) malignant glioma cells were seeded onto 6–well plates at a density of $5x10^5$cells/well and were left 24 hours to adhere and divide.

The cells were washed once with cold PBS and treated with 0.9 ml solution containing 0.02% trypsin (Sigma) and 0.2% EDTA at 37°C for 10 min.

The detached cells were transferred to cold glass centrifuge tubes containing 1.5 ml of fetal bovine serum, and centrifuged at 4°C and 1000rpm for 5min.

The samples were washed with PBS by centrifugation, and then suspended in 75% cold ethanol and left overnight at 4°C. The fixed cells were washed two times with PBS.

The final cell pellet was incubated with 0.5 ml of 0.5mg/ml RN–ase (Promega), at 37°C for 30min. After being washed twice by centrifugation the cells were incubated in 0.5ml PBS supplemented with 20µl propidium iodide (BD Pharmigen), for 30 min at 4°C. Flow cytometric measurements were performed with a LSR II flow cytometer (Becton Dickinson). MODFIT™ LT 3.0 software was used to analyze the cellular DNA content histograms to obtain quantitation of the percentage of cells in the respective phases (Go/G1, S and G2+M) of the cell cycle. All experiments were carried out in growth media (DMEM, Sigma–Aldrich) supplemented with 10% fetal bovine serum (Biochrom).

Results and Discussion

The populations of interest were established on a FSC–H/SSC–H dot plot (*Figure 1*) after removing dead cells and debris by setting polygonal gates.

Using a PI–H\PI–W dot plot (*Figure 2*) the doublets and other cell aggregates were excluded, so that we continued to analyze single cells. In the PE–A/count histograms (*Figure 3*), the G1 and G2 peaks are clearly visible and the area between the two peaks represents the cells in the DNA synthesis phase (S phase).

Peak height is directly proportional to the number of cells that are in that phase of the cell cycle. Since we removed doublets and other cell aggregates using FACS Diva software (the cytometer's software) gates, any registered signal higher the peak of the G2 is a cell with an aneuploid or polyploid karyotype (hyperdiploidy).

Based on this information we highlighted the hyperdiploidy signal in all dot plots and histograms using the color green.

Given the fact that FSC measures cell size and SSC measures cell granularity we can say that, as expected, hyperdiploid cells are larger and more granular compared with the ones in the diploid population. A problem in DNA analysis is the exclusion of cell aggregates. A cytometer can record two or more cells fused together as a single event. In other words, two fused singlets have the same amount of DNA that a tetraploid cell.

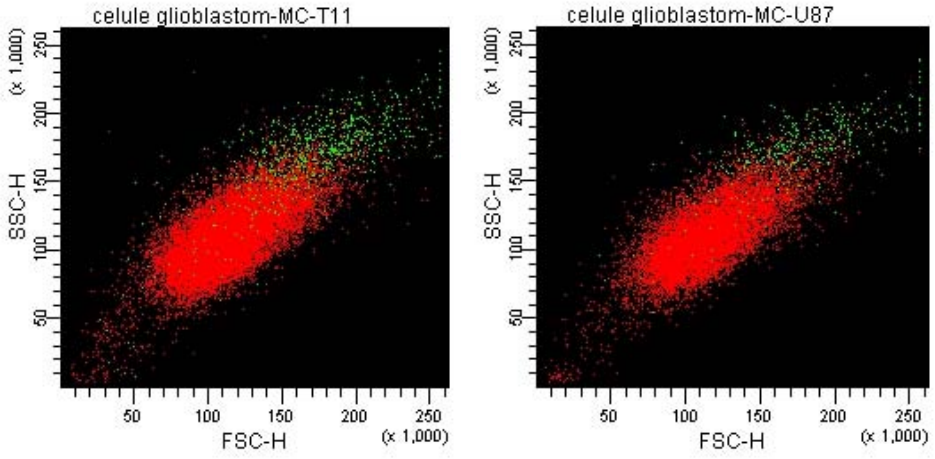

Figure 1. The population of interest of T11 cells (left) and U87 cells (right). The diploid population was red colored and the aneuploid/ polyploidy cells were green colored.

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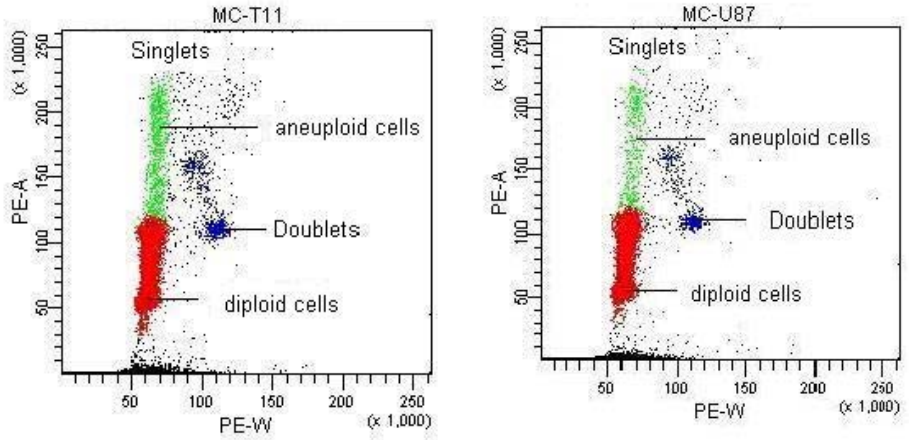

Figure 2. Doublet discrimination in T11 cells (left) and U87 (right), IP–A/IP–W dot plots, doublets are represented in blue. The singlet population is represented with red and polyploid/aneuploid cells are represented in green.

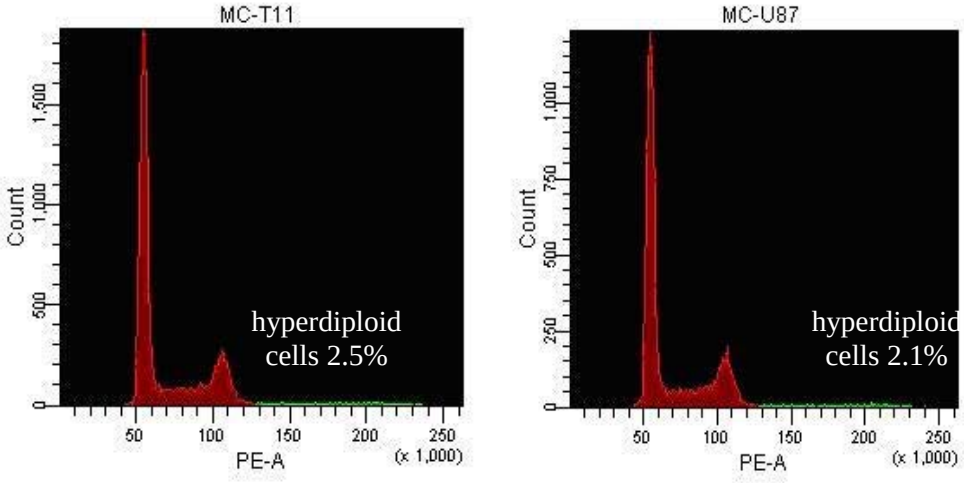

Figure 3. The histograms obtained by analyzing the T11 cells (left) and U87 cells (right).

The peaks that correspond to the G1 phases of cell cycle are around the 50 value of the histograms and the peaks of the G2 phases are found at around 100. Aneuploid and polyploid cells are found in the right side (higher than 100) of the histograms and are slightly more numerous in the case of the T11cell line.

The way to remove these false positive results from the acquisition of data is to set doublet discrimination gates on pulse width dot plots.

The cell clumps will have the same amount of marker in them as a tetraploid or polyploid cell but the time it takes them to cross the cytometer's laser beam is longer than the time necessary for a single cell to cross the same beam so the recorded pulse is much wider.

Analyzing the results using the MODFIT ™ LT 3.0 software (Verity Software) allows the user to determine exactly the percentage of cells found in each cell cycle phase. In *Figures 4* and *5* there are MODFIT analyzed histograms (PE–A) of the cell lines T11 and U87 respectively.

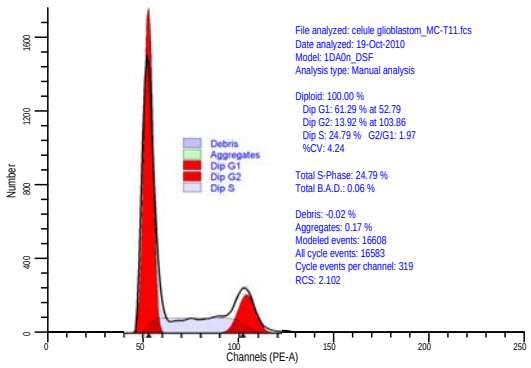

Figure 4. The histogram obtained by analyzing the T11 cells using ModFit software. The cells were divided into: G1: 61.29%, G2 and 13.92% S 24.79%.

This analysis was performed only on the relatively diploid (stabilized) cell populations of the two lines.

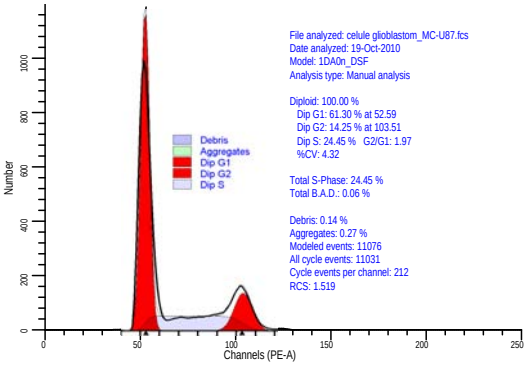

Figure 5. The histogram obtained by analyzing the U87 cells using ModFit software. The cell cycle percentages were: G1: 61.30%, G2 and 14.25% S 24.45%.

Conclusions

After performing the analysis we concluded that there are no major differences between the two glioblastoma cell lines regarding cell cycle and DNA content. The T11 and U87 cells have similar size and granularity, as evidenced in SSC–H/FSC–H dot plots.

In general, stabilized glioma cells have a relatively diploid karyotype and a variable number of aneuploid and polyploidy cells, T11 and U87 lines are no exception, as shown in PE–A histograms.

The percentage of aneuploid cells (hyperdiploid) of the two lines is similar (2.5% T11, 2.1% U87).

Regarding the cell cycle, the two lines showed similar percentages of cells in each phase of the cell cycle, T11: G1 61.29%, G213.92%, S 24.79% and the U87 cell population was divided into G1 61.30%, G2 14.25%, S 24.45%.

These percentages are typical for a cancer cell line (S and G2 phases put together are over 15% of the total cell population).

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