Immunohistochemical and flow cytometric analysis of intracellular and membrane-bound Hsp70, as a putative biomarker of glioblastoma multiforme, using the cmHsp70.1 monoclonal antibody

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Abstract

The major stress-inducible 70 kDa heat shock (stress) protein 70 (Hsp70) is frequently overexpressed in highly aggressive tumor cells and thus might serve as a tumor-specific biomarker of aggressive disease. We have previously shown that, in contrast to normal cells, tumor cells present Hsp70 on their plasma membrane. In order to elucidate the role of intracellular and membrane-bound Hsp70 as a potential tumor biomarker in glioblastoma multiforme, herein, we describe protocols for the staining of cytosolic Hsp70 in tumor formalin fixed paraffin embedded (FFPE) sections using immunohistochemistry, and for plasma membrane-bound Hsp70 by multi-parametric flow cytometry using the cmHsp70.1 monoclonal antibody (mAb).

Key words intracellular and membrane-bound heat shock protein 70 (Hsp70), glioblastoma multiforme, formalin fixed paraffin embedded (FFPE) sections, immunohistochemistry, flow cytometry, Hsp70 antibody epitope

1. Introduction

Although great progress in the treatment of many cancers has been achieved, glioblastoma multiforme (GBM) remains a debilitating and life-threatening disease. Comprising nearly 75% of all cases, glioblastoma is the most frequent primary malignant tumor of the central nervous system (CNS) and continues to be associated with very poor long-term survival and the worst prognosis of all glial brain tumors (gliomas). Despite improvements in the treatment of glioblastoma using advanced neurosurgical techniques and radiation therapy, there remains no effective cure for this tumor [1]. The average life-expectancy

after diagnosis is less than 1 year, and fewer than 10% of patients survive longer than 5 years after diagnosis, even after total tumor resection [1-3]. Administration of the alkylating chemotherapeutic agent Temozolomide (TMZ) only increases median survival rates to approximately 15 months [4-11]. Death from glioblastoma is due to a rapid, aggressive local infiltration of glioblastoma cells in the brain. Moreover, a high genetic tumor heterogeneity can mediate resistance to radio(chemo)therapy. An inherent or acquired resistance to radio(chemo)therapy drastically reduces life expectancy. It is therefore essential to have reliable tumor biomarkers which can identify patients who are likely to acquire resistance to TMZ at an early time point.

The major stress-inducible 70 kDa heat shock (stress) protein Hsp70 is frequently overexpressed in the cytosol for a large variety of different tumor cells and elevated cytosolic Hsp70 levels are often associated with therapy resistance. Therefore, we propose Hsp70 as a potential biomarker of disease in GBM.

Using a unique monoclonal antibody (mAb, cmHsp70.1), the Multhoff laboratory made the seminal discovery that, in addition to its cytosolic localization, Hsp70 is also selectively expressed on the plasma membrane of tumor cells (but not normal tissue) [12-14]. The same laboratory also showed that the expression density of membrane Hsp70 on a broad profile of cancer cell lines is further increased *in vitro* by standard treatments such as radio(chemo)therapy [15,16] where it also can confer therapy resistance. Furthermore, membrane Hsp70 is more highly expressed on metastatic disease than its corresponding primary tumor [17], and its expression is associated with an unfavourable prognosis and a reduced overall survival for some tumors [18]. An ongoing screening program of over 1,500 patients with various solid tumors in the Multhoff laboratory reveals that more than 50% of all patients have membrane Hsp70 positive tumors, as identified by flow cytometric analysis of isolated viable tumor cells using the cmHsp70.1 mAb.

These findings indicate that membrane-bound Hsp70 might act as a universal, selective tumor-specific marker of ‘aggressive’ disease. Therefore, it is of importance that, apart from cytosolic Hsp70 levels also the membrane status of Hsp70 should be determined to describe a tumor. Furthermore, membrane-bound Hsp70 also could act as a tumor-specific therapeutic target which enables the development of new approaches for the treatment of GBM such as

cell based therapies. A number of pre-clinical studies have shown the capacity of *ex vivo* activated (low-dose IL-2 plus a 14-mer Hsp70-derived peptide, IL-2/TKD) activated NK cells to target a range of cancer cell types expressing the membrane form of Hsp70 [19,12,13,20-24]. Studies have also demonstrated that this cytotoxic effect is manifested by the release and uptake of granzyme B into target cells via membrane Hsp70 [25]. The clinical safety and tolerability of IL-2/TKD activated NK cells has been demonstrated in a Phase I clinical trial [26], and its efficacy for the treatment of patients with non-small cell lung carcinoma after radiochemotherapy is currently being assessed in a multi-centre Phase II clinical trial [27]

We have also previously reported that recombinant human granzyme B binds to membrane Hsp70 on cancer cells and that its subsequent uptake, which results in the selective killing of membrane Hsp70 expressing cells and tumors, occurs via an Hsp70-mediated endocytosis process which is independent of perforin [28,17]. Taken together, these findings make membrane Hsp70 an excellent and broadly-applicable molecule on which to develop a portfolio of new immunotherapeutics based on *ex vivo* IL-2/TKD activated NK cells [21,22,26,24,29,27], human recombinant granzyme B [25,28,17] or Hsp70-targeting antibodies. The membrane-Hsp70 specific antibody (cmHsp70.1) itself can induce antibody-dependent cellular cytotoxicity (ADCC) [14], or can be used for a targeted delivery of therapeutics in the form of antibody-drug conjugates (ADCs) or as a therapeutic ‘cargo’ delivered by nanoparticles.

Membrane Hsp70 has also been used as a basis for the development of innovative imaging platforms for tumor detection and outcome monitoring which employ the cmHsp70.1 monoclonal antibody or a 14-mer tumor penetrating peptide (TPP) [30,15,31,32].

Given the established association between membrane Hsp70 expression and disease aggressiveness, and the portfolio of theranostic approaches that are being developed to target tumors expressing membrane Hsp70, techniques for determining the membrane Hsp70 expression by human glioblastoma tissue, and cell lines derived from primary glioblastomas have been established using the cmHsp70.1 mAb. These immunohistochemical and flow cytometry-based approaches are described herein.

2. Materials

2.1. Patient-derived glioblastoma multiforme cell lines

The human, low passage glioblastoma (UP007, UP029, and SEBTA027) and brain metastatic lung carcinoma (SEBTA028) cell lines, were established in culture at the University of Portsmouth from biopsy tissue obtained at Kings College Hospital London under ethics permission, 11/SC/0048. All cell lines were cultured in DMEM growth medium supplemented with 10% v/v fetal bovine serum (FBS).

- **UP-007** was derived obtained from a 71 year old treatment naive male *de novo* GBM patient with wild type IDH1
- **UP-029** was derived from a 66 year old treatment naïve female *de novo* GBM patient with wild type IDH1 and IDH2
- **SEBTA-027** was derived from a 59 year old female with recurrent GBM in the right parieto-occipital region, with sarcomatous elements, grade IV, wild type IDH1, unmethylated MGMT and a 40% Ki67 labelling in dex and who had previously received radiotherapy and Temozolomide chemotherapy.
- **SEBTA-028** was derived from a 52 year old male with brain metastatic lung carcinoma in the right temporal-occipital-parietal region,

2.2. Patient- and pre-clinical model-derived paraffin-embedded tissue

The diagnosis of glioblastoma multiforme was based on haematoloxylin/eosin staining of FFPE sections (2 µm) of two patients with glioblastoma multiforme WHO stage IV. Additionally, sections (2 µm) of orthotopically implanted GL261 mouse glioblastoma were used for the analysis. Ethical approval for access to patient tissue was obtained from the local ethical committee of the Technische Universität München, and informed consent was provided.

2.3. cmHsp70.1 anti-Hsp70 monoclonal antibody (mAb)

The cmHsp70.1 monoclonal antibody (mAb, multimmune GmbH, Munich, Germany), which selectively binds to membrane Hsp70 on tumor cells was generated by immunization of mice with the 14-mer peptide TKDNNLLGRFELSG, termed “TKD”, comprising amino acids 450–461 ($aa_{450–461}$) in the C terminus of the inducible Hsp70. Since the human and murine “TKD” sequences only differ in one amino acid (T**K**DNNLLGRFELSG; mouse T**R**DNNLLGRFELSG), [33] and the minimal antibody epitope (NNLLGRF) is

identical among different species [14], the human the cmHsp70.1 mAb shows cross-reactivity for human, mouse, canine and feline tumors.

The “TKD” sequence which is exposed to the extracellular milieu of tumors resides in the C-terminally localized oligomerization domain which is part of the substrate binding domain of the Hsp70 molecule [34]. In contrast to other commercially available Hsp70 antibodies, the cmHsp70.1 mAb uniquely identifies the membrane form of Hsp70 on viable human and mouse tumor cells not only *in vitro* but also *in vivo* in tumor-bearing mice [30].

In contrast to the cmHsp70.1 mAb, other commercially available mouse antibodies directed against Hsp70 fail to stain the surface of viable tumor cells.

2.4. Immunohistochemistry (IHC)

Deparaffinization reagents

1. Xylene
2. Ethanol absolute 100% v/v
3. Ethanol 96% v/v
4. Ethanol 70% v/v
5. Ethanol 50% v/v
6. H_2O_{dd}

Target retrieval and staining reagents

1. Target retrieval solution: 1x / H_2O_{dd}, 0.5x H_2O_{dd} (DAKO cat #: S1699)
2. Peroxidase blocking solution: 3% v/v H_2O_2 / 0.1% w/v sodium azide / Phosphate buffered saline (PBS)
3. Protein blocking solution: 5% v/v rabbit serum / antibody diluent (DAKO REAL antibody diluent, cat #: S2022)
4. Phosphate buffered saline (PBS)
5. Murine IgG1 anti-Hsp70 monoclonal antibody cmHsp70.1: working solution 1: 500 in antibody diluent (multimmune GmbH, Munich)
6. Horseradish peroxidase (HRP)-conjugated rabbit anti-mouse polyclonal antibody: DAKO EnVision+ system HRP-conjugated rabbit anti-mouse polyclonal antibody (cat #: DAKO K4001)
7. DAB (3,3'-diaminobenzidine): DAKO liquid DAB+ Substrate and Chromogen System (cat #: K3468)

2.3. Flow cytometry

1. Cells of interest
2. Fc receptor (FcR)-blocking immunoglobulin (Ig; e.g., normal rat IgG) solution, 200 µg/ml or specific Fc blocking reagents that are suitable for use in the species of interest
3. Carboxyfluorescein or FITC-conjugated cmHsp70.1 IgG1 murine anti-Hsp70 monoclonal antibody (multimmune GmbH)
4. Carboxyfluorescein or FITC-conjugated IgG1 murine monoclonal antibody to Hsp70 (clone C92F3A, StressMarq Biosciences Inc. See **Notes**)
5. Appropriately conjugated murine immunoglobulin (Ig) isotype controls
6. Phosphate buffered saline (PBS) supplemented with 0.1% w/v sodium azide (NaN_3) and bovine serum albumin (BSA) (PAB; see recipe below)
7. Nuclear / membrane integrity (viability) staining compound dissolved in PBS: 200 µg/ml PI [alternative options include 250 µg/ml 7-AAD, 250 µg/ml TO-PRO-3 (Molecular Probes), or 200 µg/ml PY(G)] – see **Notes**

PBS, pH 7.2, supplemented with sodium azide (NaN_3) and fetal bovine serum (FBS)

- Dulbecco's PBS, Ca^{2+}- and Mg^{2+}-free (e.g., Life Technologies), containing:
- 0.1% (w/v) sodium azide
- 10% (w/v) fetal bovine serum (FBS)
- Adjust pH to 7.2 using 1 N NaOH or 1 N HCl
- Filter solution through 0.22-µm membrane
- Store up to 1 month at 4°C
- EDTA (1 mM) can be added to reduce cellular aggregation

CAUTION: Always keep cells and reagents at 4°C. Be extremely careful when handling sodium azide.

3. Methods

3.1. Immunohistochemistry (IHC)

IHC must be performed on formalin-fixed, paraffin-embedded (FFPE) tissue sections (2 µm) using a rabbit anti-mouse polyclonal antibody staining kit (DAKO). In brief, tissue sections were boiled by microwaving for 30 minutes in

target retrieval buffer (DAKO) to unmask the epitope. Between each of the following incubation steps, sections were washed in PBS (Sigma). Non-specific binding must be blocked in protein blocking solution for 60 minutes. Slides must be incubated overnight at 4ºC with cmHsp70.1 mAb (multimmune GmbH) or with a corresponding IgG control (Sigma). After washing, sections are incubated with the HRP-conjugated anti-mouse polyclonal antibody, followed by DAB chromogen reaction to detect the binding of the primary antibody. Nuclei are counterstained with haematoxylin and sections embedded in embedding medium.

Rehydration

1.	Xylene I & II	2 x 15 min
2.	Ethanol absolute I & II	2 x 10 min
3.	Ethanol 96%	2 x 5 min
4.	Ethanol 70%	1 x 5 min
5.	Ethanol 50%	1 x 5 min
6.	H_2O_{dd}	1 x 5 min

Target retrieval and staining

1.	Target retrieval solution	1 x 30 min boiling (microwave)
2.	Cooling down period	1 x 10 min in target retrieval solution
3.	H_2O_{dd}	1 x 2 min
4.	Marking of the section with a hydrophobic pen	
5.	Peroxidase blocking solution	1 x 30 min
6.	H_2O_{dd}	3 x 5 min
7.	Protein blocking solution	1 x 60 min
8.	PBS	2 x 5 min
9.	cmHsp70.1 mAb (1:500)	overnight (4°C)
10.	PBS	2 x 5 min
11.	Envision+ System HRP labelled Anti-mouse polymer	1 x 30 min (RT)
12.	PBS	1 x 5 min
13.	DAB	1 x 4 min
14.	H_2O_{dd}	1 x 5 min
15.	Counterstain with haematoxylin	1 x 30 sec

16. Rinse with warm tab water 1 x 5 min

Dehydration and embedding

1. Ethanol 50% 1 x 5 min
2. Ethanol 70% 1 x 5 min
3. Ethanol 96% II & III 2 x 5 min
4. Ethanol absolute III & IV 2 x 5 min
5. Xylene III & IV 2 x 10 min
6. Embedding (Eukitt)

CAUTION: Keep the time of the chromogen reaction exactly at 4 min and use sections of the same thickness (2 µm) in order to be able to compare staining intensities of different experiments. Always run reference sections with a defined staining intensity as an internal control.

Scoring criteria of IHC using cmHsp70.1 mAb

The staining intensity must be analysed semi-quantitatively within the tumor area. The staining intensity of tumor cells is graded into different scores: normal (+), intermediate (++), strong (+++). Normal tissues are graded as weak (+/-). The localization of the staining (cytosolic, nuclear) as well as the percentage of positively stained cells must be considered. The scoring must be performed by at least two independent researchers.

3.3. Flow cytometry

1. Prepare a single-cell suspensions of tumor cells in ice-cold PBS supplemented with 10% (v/v) FBS (PBS/FBS)
2. Dispense 1×10^5 cells into microcentrifuge tubes
3. Pellet cells by centrifugation for 5 min at 500 × *g*, 4°C
4. Remove the supernatant by vacuum aspiration
5. Add 4 µl ice-cold cmHsp70.1 mAb (1 µg/µl) to the pellet containing approximately 50 µl PBS/FBS, resuspend and vortex tubes slightly
6. Incubate tubes for 30 minutes on ice and wash in ice-cold PBS/FBS
7. Resuspend cell pellet in 300 µl PBS/FBS

8. Add 3µl PI to tube (*Alternatively, add 4 µl 7-AAD, 4 µl TO-PRO-3, or 5 µl PY(G)*
9. Incubate cells on ice for 5 min
10. Analyze cells using a flow cytometer immediately thereafter
11. Keep samples on ice during analysis
12. *prolonged periods before analyzing them is generally not recommended.*

4. Notes

4.1. Immunohistochemistry (IHC) scoring

Every nucleated human cell type expresses the stress-inducible Hsp70 at low levels in the cytosol, whereas the membrane-bound form of Hsp70 appears to be restricted to tumor cells. Given that the cmHsp70.1 antibody can stain both, the membrane-bound and cytosolic forms of Hsp70 and that approximately 70% to 90% of the total Hsp70 is residing intracellularly, it is nearly impossible to distinguish between the membrane-bound and cytosolic localizations of Hsp70 by IHC. Therefore, membrane-bound Hsp70 must be determined by flow cytometry of viable cells with intact plasma membranes, whereas cytosolic Hsp70 can be determined by IHC. It is well known that highly aggressive tumor cells show a higher expression density and a different subcellular localization of Hsp70 compared to normal cells. Therefore, the intensity and the subcellular distribution (cytosol, membrane, nucleus) of Hsp70 can be considered as tumor-specific characteristics. Since no human cell type is completely negative for cytosolic Hsp70, different Hsp70 staining intensities rather than Hsp70 negative and Hsp70 positive phenotypes can be distinguished. For a comparable scoring of different staining intensities in different IHC staining procedures, it is key to run control sections (tumor and normal tissue sections) as a reference. The thickness of the sections must be exactly 2 µm to be able to correctly score different staining intensities. Apart from the staining intensity, many glioblastomas show a heterogenous staining pattern within the tumor tissue. Therefore, it is also necessary to determine the percentage of the field which shows a certain staining intensity. Finally, the subcellular distribution of the Hsp70 staining also needs to be considered. Figure 1 provides a typical example of strong and weak staining intensity in the nucleus and the cytosol in the tumor of a single patient with GBM.

4.2. Flow cytometry

The inclusion of the IgG1 murine monoclonal antibody to Hsp70 (clone C92F3A, StressMarq Biosciences Inc) in the staining protocol is essential to determine the amount of Hsp70 which is bound to receptors such as TLRs or scavenger receptors on the cell surface of tumor cells. This antibody does not stain the confirmation of lipid bound Hsp70 in the plasma membrane of tumor cells.

Although cell viability should routinely be assessed in all flow cytometric analyses, as non-viable cells and debris can non-specifically take up and bind probes and antibodies and so must be excluded from phenotypic analysis, it is particularly essential for the specific analysis of membrane Hsp70 expression by flow cytometry, as cells having a compromised cell membrane will be highly positive for intracellular Hsp70 expression. Non-viable cells might also exhibit a higher level of autofluorescence and / or release DNA which can promote cell clumping and aggregation. Cell aggregation can be inhibited by including a small amount of DNAse in the staining solution, and non-viable cells can be excluded from the analysis using viability stains.

The viability of cultured cells can be quite low, however cell death during sample processing can be minimized by performing all cell preparations and staining procedures (including wash steps) at 4°C or on ice and in the presence of bovine serum albumin (BSA) or heat-inactivated fetal bovine serum (FBS). Cells should be analyzed in a flow cytometer as soon as possible. **Cells should not be fixed when analyzing the expression of membrane Hsp70.**

The best method for excluding dead cells from data analysis is to use a vital DNA dye in all samples. Although some of the more common vital dyes used in multicolor analyses are propidium iodide (PI), 7-aminoactinomycin D (7-AAD), TO-PRO-3, and pyronin Y(G) [PY(G)], a range of options are now available from commercial suppliers. A note of caution is that dyes such as PI and 7-AAD can be taken up into viable cells over time, and so these stains should be added immediately prior to analysis.

The relatively broad emission spectrum of 7-AAD can result in spectral overlap into other detectors and require considerable compensation in large multi-parameter panels. It is also quite a ‘dim’ fluorescent molecule.

The far-red viability dye DRAQ7™ (Biostatus Ltd, UK) can be used in similar settings to PI and 7-AAD. A particularly useful feature of DRAQ7™ is that its dual excitation characteristics allows multibeam excitation and the exclusion of dead (DRAQ7+) cells without 'consuming' what could be a vital additional fluorescent channel.

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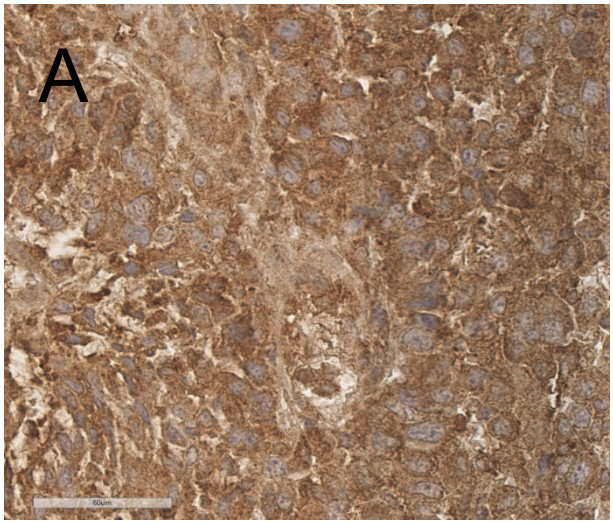
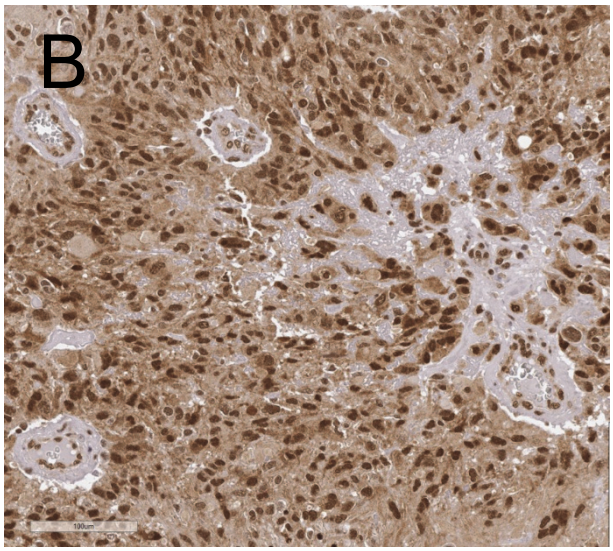
Figure Legends

Fig 1: Representative immunohistochemical (IHC) staining patterns of sections (2 µm) of GL261 mouse glioblastoma (A), human glioblastoma multiforme WHO grade IV (B, C) with cmHsp70.1 mAb. Staining intensity scores are as follows: A: +++, B: +++, C: +. Panel A shows a typical cytosolic staining pattern, panels B and C show representative examples of a cytosolic and nuclear staining pattern.

Fig 2: Representative flow cytometric histograms illustrating membrane Hsp70 expression by a panel of patient-derived cell lines, as described in the main body of the text. GL261 tumors were induced via intracranial injection of methylcholanthrene followed by serial intracranial and subcutaneous transplantations of tumor fragments into syngeneic C57BL/6 mice. Green – unstained control; black – stained using the C92F3A clone mAb; blue – stained using the cmHsp70.1 clone mAb.





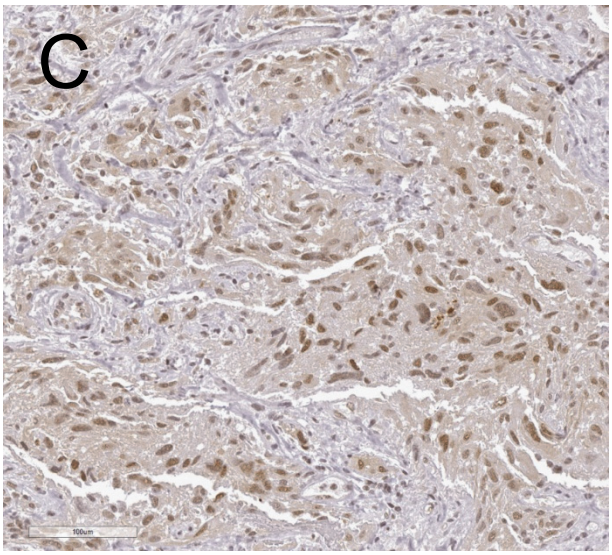


Figure 1

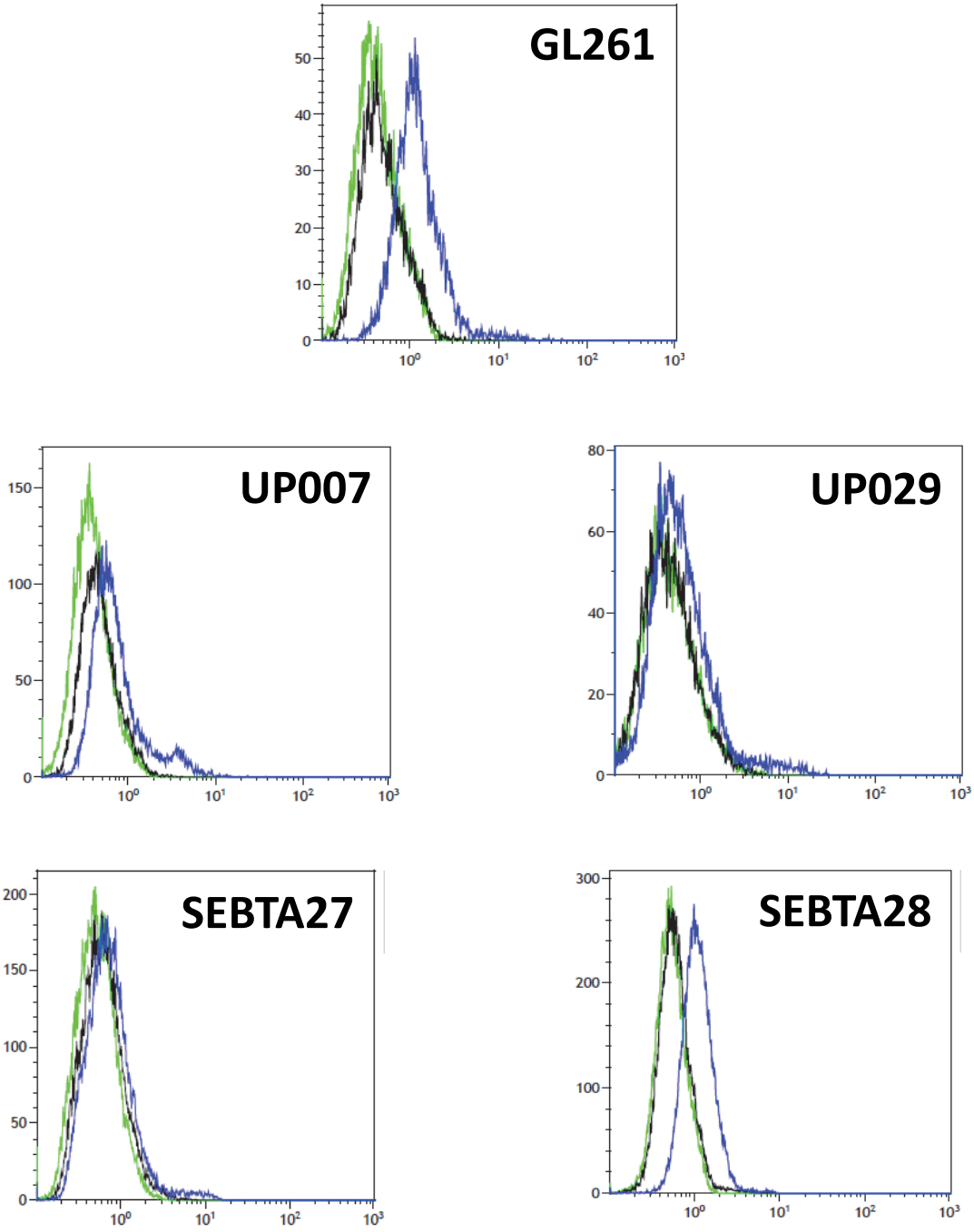


Figure 2