Simultaneous spatial evaluation of IDH1, EGFR, IGF1R and Ki67 biomarkers in glioblastoma

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ABSTRACT

Each year about 14,000 people in the United States are diagnosed with glioblastoma (GBM), the most aggressive primary neuroepithelial tumor. Patients with GBM have a poor prognosis and only 5% of these patients can survive for more than 5 years. Various biomarkers are used for Immunohistochemical (IHC) diagnosis of GBM including: Isocitrate dehydrogenase 1 (IDH1), Epidermal Growth Factor Receptor (EGFR), Insulin Like Growth Factor 1 Receptor (IGF1R), and a cell proliferating marker Ki67. Typically, IHC is done by using a single-biomarker HRP-DAB detection technique which is not suitable for the simultaneous spatial analysis of multiple biomarkers. The aim of this study was to develop a multi-color immunofluorescence protocol allowing for the spatial biology analysis of distribution and co-localization of biomarkers in GBM tissues.

Paraffin-embedded tissue sections were incubated with a mixture of the following primary antibodies: monoclonal mouse anti-human IDH1 (MAB7049), polyclonal goat anti-human EGFR (AF231), monoclonal mouse anti-human IGF1R (MAB391), and monoclonal rabbit anti Ki67 (MAB7617). Primary antibodies were directly conjugated to fluorescent dyes (FL) with different excitation and emission spectra. At the qualitative level, we have observed about 80% of cells with strong immunoreactivity for EGFR and IDH-1 whereas IGF-1R labeling was confined to about 50% of cells. Immunoreactivity for Ki67 was the lowest and detected in about 5% of cells. About 80% and 50% of EGFR positive cells were also immunoreactive for IDH-1 and IGF-1R respectively. All Ki67 labeled cells were also immunoreactive for EGFGR and IDH1R but only 50% of Ki67 positive cells were immunoreactive for IGF1R.

We applied a spatial biology methodology for co-detection of multiple GBM biomarkers which allowed us to analyze their spatial distribution as well as the extent of colocalization at the single-cell level. Co-detection of multiple biomarkers has a strong diagnostic potential especially when analyzing limited small-size biopsies.

INTRODUCTION

Glioblastoma Multiforme is one of the most aggressive types of cancer with an average survival of 14 months after diagnosis (1). While there are currently a variety of brain tumors, glioblastomas account for approximately 49% of malignant brain tumors, and this progressive disease has a 5-year survival rate of 36% (2). Proper diagnosis and continuous research of this cancer can help improve treatment recommendations and a better understanding of this fatal disease. Diagnosis of glioblastoma requires tumor biopsies with consideration of histopathological and molecular characteristics (2). Immunohistochemistry (IHC) is an important technique used to diagnose and explore localization of different biomarkers. Specific markers involved with the histopathology of glioblastomas include Ki-67, EGFR, IDH-1 and IGF-R1. Ki-67 assessment of labeling using immunohistochemistry is the most widely used method for measuring proliferation in gliomas in the diagnostic setting (3). Additionally, EGFR & IGFR-1 are important markers as mutations or amplifications of these targets are present in more than 80% of primary glioblastomas (4). Mutations also occur in most low-grade gliomas and secondary high-grade gliomas for IDH-1, making this another important marker for glioblastoma (5). With the utilization of fluorescently conjugated primary antibodies for important markers like Ki-67, EGFR, IDH-1 and IGF-R1, multiplex IHC was performed to explore how these molecules are distributed spatially within glioblastoma tissue sections.

MATERIALS & METHODS

Tissues:

5 micron thick FFPE tissue sections of glioblastoma

R&D Systems' Primary Antibodies:

- Monoclonal Mouse anti Human Isocitrate Dehydrogenase 1/IDH1 (MAB7049) conjugated to Cy5.5™ (excitation/emission 675/694);
- Recombinant Monoclonal Rabbit anti-human/mouse Human Ki67/MKI67 (MAB7617) conjugated to Cy7™ (excitation/emission 743/767);
- Polyclonal Goat anti-human EGFR (AF231) conjugated to Alexa Fluor®488 (excitation/emission 495/525)
- Monoclonal Mouse Human/Mouse IGF-I R/IGF1R (MAB391) conjugated to (NorthernLights™557; excitation/emission 557/574)

Conjugation of Primary Antibodies to Fluorescent Dyes:

Labeling of primary antibodies to select fluorescent dyes has been done using an N-Hydroxy Succinimide (NHS) ester chemistry allowing the dyes reacting with the primary amines on the antibody molecule.

Validation of antibodies:

Unconjugated and FL-conjugated antibodies were tested on A172 glioblastoma cells fixed with 4% formaldehyde.

IHC Protocol:

1. 5 micron thick FFPE tissue sections of glioblastoma were cleared in alcohols and xylenes;
2. Blocked with 2% normal horse serum (15 min, RT)
3. Incubated with the cocktail of primary antibody conjugates overnight at 4°C;
4. Tissue sections were then washed with PBS, 3 x 15 min;
5. Washed tissue sections were mounted under coverslips into anti-fade mounting media containing DAPI.

Collection of Images and their processing:

Images were collected using an Olympus IX-83 fluorescence inverted microscope equipped with filters corresponding to excitation and emission properties of fluorophores conjugated to primary antibodies

Trademarks

RESULTS

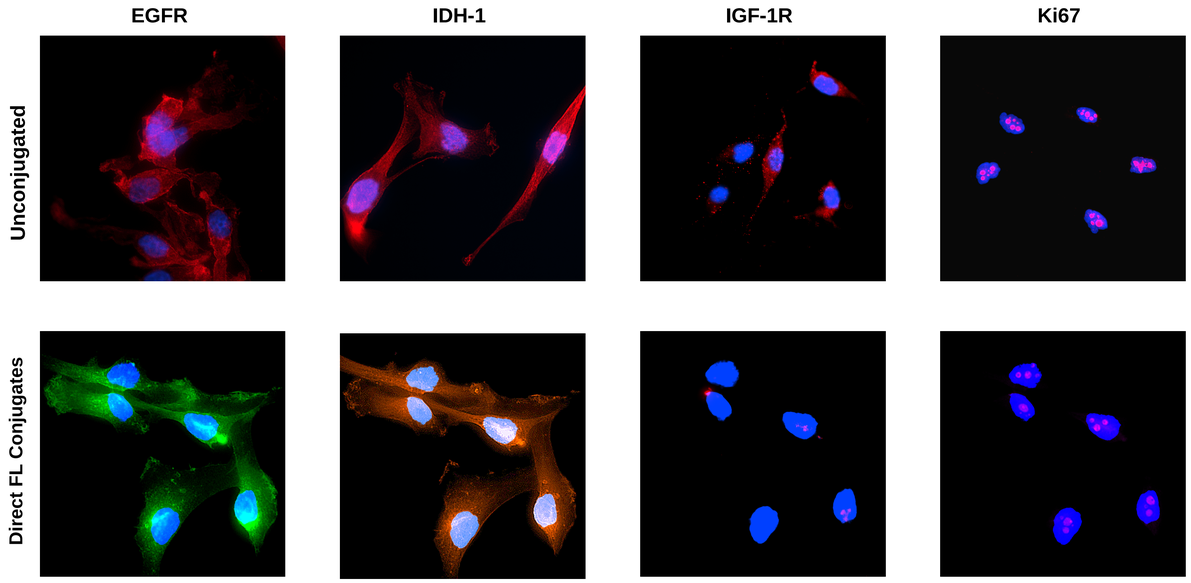

Figure 1. Comparison of unconjugated and FL conjugated primary antibodies on A172 glioblastoma cell line. Unconjugated primary anti using secondary antibodies (upper row) and then compared to staining produced by the same antibodies directly labeled with fluorescent (FL) dyes (bottom row). Note that direct conjugation did not affect the performance of primary antibodies which retained their strong antigen labeling activity.

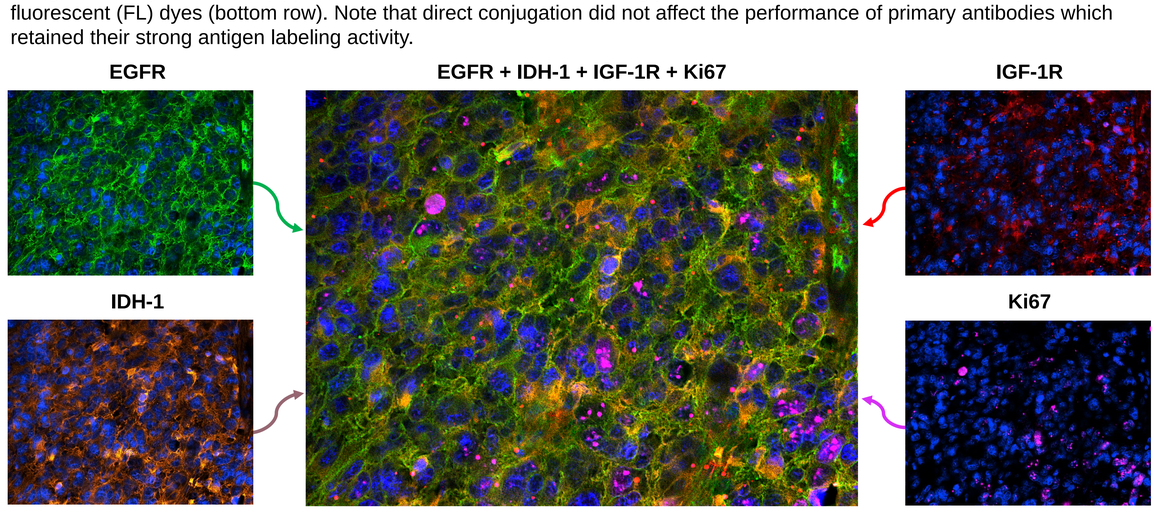

Figure 2. Spatial distribution of four targets observed by IHC in FFPE glioblastoma tissue section using direct FL antibodies conjugates. Single FL channel images are shown in left and right panels and merged four-plex image is shown in the center. Note strong spatial overlap in EGFR and IDH-1 biomarkers and their minimal co-localization with IGF-1R and Ki67 targets.

1. Spatial multi-omics of glioblastoma allows for characterization of different cancer subtypes to design precise treatment strategies.
2. Using primary antibodies directly labeled with fluorescent dyes allows for unambiguous distinction between IDH1, EGFR, IGF1R and a cell proliferating marker Ki67 in FFPE glioblastoma tissue sections.
3. Direct labeling with FL tags allows to utilize antibodies raised in the same host species to avoid cross-reactivity of secondary antibodies with irrelevant primary antibodies.
4. Direct FL conjugates can be utilized with other primary antibodies to analyze spatial distribution of many different biomarkers in various normal and pathological brain tissues.

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