Characterization and classification of glioblastoma multiforme using the novel MACSima™ Imaging Platform enabling multiparametric cyclic immunofluorescence analysis

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Introduction

Glioblastoma multiforme (GBM), a highly malignant, incurable type of brain tumor, has been subclassified into several distinct subtypes using a multitude of analysis methods. Here we introduce the MACSima™ Imaging Platform which allows for fully automated, multiparametric, cyclic immunofluorescence analysis of specimens based on hundreds of antibodies. We applied this method to characterize and categorize glioblastoma according to published classification schemes and to identify new glioblastoma-specific markers.

A selection of 96 markers, identified through flow cytometric analysis of cell surface expression on primary glioblastoma–derived xenografts, was used for characterization of eight primary glioblastoma samples using the MACSima™ Imaging Platform. Cryosections were fixed using acetone, and each specimen was exposed to the 96 fluorochrome-conjugated antibodies by cycles of i) staining with antibody, ii) image acquisition, and iii) erasure of the fluorescence signal. Previously published markers, such as PDGFRα, p53, synaptophysin, CD44, nestin, podoplanin, GFAP, and EGFR were used to subclassify the glioblastoma into oligodendrocyte precursor (OPC), differentiated oligodendrocyte (DOC), astrocytic and mesenchymal (AsMes) or mixed subtype, according to Motomura *et al.*[1] Analysis of well-established glioblastoma markers already used in CAR T cell–based clinical trials, such as IL-13Rα2[2], EGFRvIII[3], or ErbB2[4] revealed a broad inter- and intratumor diversity of expression. Infiltrating immune cells were present in most of the tumors, but showed varying percentages. Finally, segmentation and correlation analysis enabled identification of new markers, which might be candidates for future CAR T cell–based therapies.

In summary, our analysis using the MACSima Imaging Platform showed a high heterogeneity of protein expression in glioblastoma and enabled the classification of the diverse tumors. The new imaging platform also allowed a selective detection of tumor cells and subsequent segmentation, clustering, and correlation analysis of tumor-related markers for their potential use in immunotherapy.

Methods

1 Characterization and classification of glioblastoma multiforme using the MACSima™ Imaging Platform

Patient-derived glioblastoma xenografts were dissociated into single cells using the Tumor Dissociation Kit, human and the gentleMACS™ Octo Dissociator with Heaters. The Mouse Cell Depletion Kit was applied to remove contaminating mouse cells and to obtain a pure xenograft cell suspension. Single cells were analyzed by flow cytometry for cell surface marker expression using 523 fluorochrome-conjugated antibodies (MACS® Marker Screen and an additional 152 antibodies). A ranking was applied according to the percentage of positive cells and the stain index, leading to a selection of 96 markers for further immunohistochemical characterization of eight primary glioblastoma samples using the MACSima™ Imaging Platform. To this end, fresh frozen glioblastoma cryosections were fixed with acetone. Each specimen was exposed to 96 fluorescently labeled antibodies by repeated cycles of antibody staining, image acquisition of each region of interest (ROI), and erasure of the fluorescence signal (fig. 1A). This approach resulted in a 2D image stack displaying the staining profiles of multiple markers. Both, pixel-based and segmented single-cell imaging data were used for protein expression profiling and pattern recognition. Finally, the data were used for classification of the different glioblastoma specimens, for characterization of different tumor-specific cells, and for clustering and correlation analysis to identify new glioblastoma-specific markers (fig. 1B).

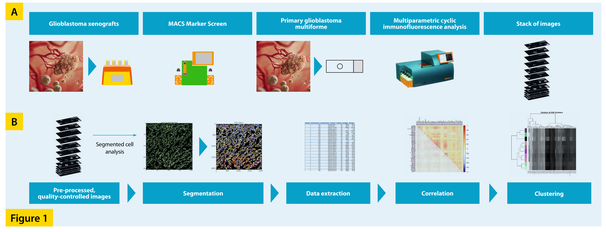

Figure 1

Results

1 Classification of different primary glioblastoma tumors

A subclassification of primary glioblastoma based on expression patterns determined by immunohistochemistry in combination with the DNA copy number and DNA methylation patterns was described by Motomura *et al.*[1] Based on the expression of GFAP, podoplanin, p53, CD44, synaptophysin, nestin, PDGFRα, and EGFR four distinct glioblastoma subtypes were defined: the oligodendrocyte precursor (OPC), differentiated oligodendrocytes (DOC), astrocytic/mesenchymal (AsMes) and mixed type. The OPC type was characterized by high levels of PDGFRα and p53 in most of the tumors, whereas levels of CD44, podoplanin, and nestin expression varied. Tumors that were assigned to the DOC type showed expression of PDGFRα and CD44, but were negative for GFAP, p53, and EGFR. The AsMes type was generally characterized by a strong expression of GFAP, podoplanin, CD44, and nestin and moderate expression of p53 and PDGFRα. The main characteristic of the mixed type was a high expression level of EGFR. In addition, GFAP, podoplanin, p53, and CD44 were detected on most tumors of this type (table 1).

We classified eight different primary glioblastoma tumors using the MACSima Imaging Platform and analyzed them according to their expression of PDGFRα, p53, synaptophysin, CD44, nestin, podoplanin, GFAP, and EGFR. Figure 2 shows representative immunohistochemical stainings of four tumors. According to table 1, glioblastoma samples 1, 3, and 4 were classified as mixed type and glioblastoma 2 as AsMes type. Four other tumors were also assigned to the mixed and AsMes types.

Marker	OPC type (oligodendrocyte precursor cells)	DOC type (differentiated oligodendrocytes)	AsMes type (astrocytic mesenchymal)	Mixed type
GFAP	●	●	●	●
Podoplanin	●	●	●	●
p53	●	●	●	●
CD44	●	●	●	●
Synaptophysin	●	●	●	●
Nestin	●	●	●	●
PDGFRα	●	●	●	●
EGFR	●	●	●	●

Table 1 — Legend: ● expressed ● occasionally expressed ● not expressed

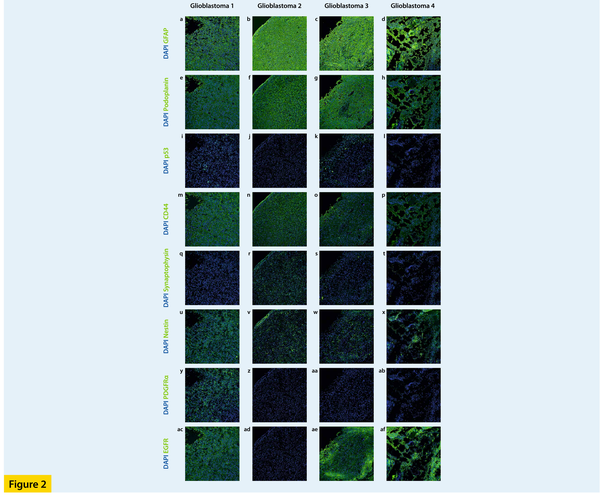

Figure 2

2 Characterization of the immune cell environment in glioblastoma multiforme

Primary glioblastoma tumors were further characterized regarding infiltration of immune cells and expression of tumor-related markers.

Figure 3 shows staining of various immune cell markers to analyze immune cell infiltration. CD45⁺ lymphocytes were detected in each tumor, but showed varying percentages. Only a small number of CD3⁺ T cells and only very few PD-1⁺ T cells were detected (fig. 3, a–h). In contrast, all tumors showed up-regulation of PD-L1 (fig. 3, i–l), indicating that immune escape mechanisms via the PD-1/PD-L1 pathway play an important role in glioblastoma. Analysis of monocytes and macrophages showed different percentages and distribution among the tumors (fig. 3, m–t). Only very few infiltrating B cells were detected (fig. 3, u–x).

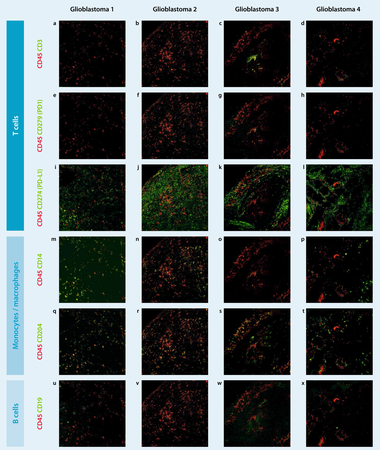

Figure 3

3 Identification of new glioblastoma-specific markers

Eight glioblastoma specimens were analyzed with regard to the expression of well-established glioblastoma markers used in CAR T–cell based clinical trials, such as EGFRvIII, ErbB2 (HER2), and IL-13Rα2. Multiparametric cyclic immunofluorescence analysis revealed only a restricted expression of ErbB2 and IL-13Rα2 in these tumors (fig. 4A, a–h). None of the tumors showed a specific signal for EGFRvIII (data not shown).

Screening for so far unknown tumor-specific markers resulted in the identification of new marker candidates (fig. 4A, i–x; four new candidates are shown), which were validated by cell segmentation and determination of the mean fluorescence intensity (MFI) per cell (fig. 4B and C). Compared to known glioblastoma markers, the new marker candidates showed higher expression levels and percentages of positive cells. Figure 4B displays the average MFI per ROI, and fig. 4C the percentage of cells per ROI with an MFI above background.

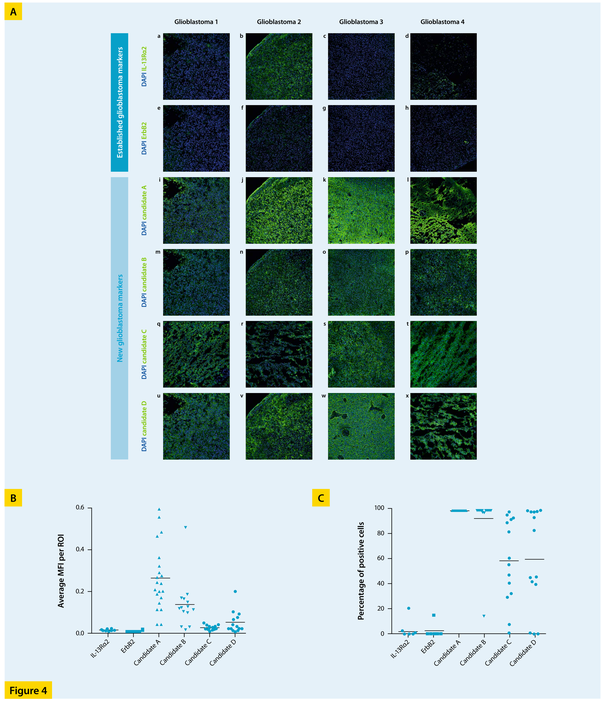

Figure 4

Conclusion and outlook

- We introduce the new MACSima Imaging Platform that allows for multiparametric immunofluorescence analysis and thereby enables extensive analysis of the expression pattern of hundreds of antigens on tumor cells.
- The new system was used for comprehensive characterization of the diversity of primary glioblastoma tumors and in-depth classification of different glioblastoma subtypes.
- Screening of an expanded antibody library led to the identification of new glioblastoma-specific marker candidates which showed broader expression on tumor samples and higher expression levels on tumor cells compared to clinical markers like IL-13Rα2 and ErbB2.
- Further correlation and clustering as well as expression analysis on healthy tissue are necessary to validate the markers for their potential use in glioblastoma immunotherapy.

References
1. Motomura, K. *et al.* (2012) Cancer Sci. 103: 1871–1879.
2. Brown, C.E. *et al.* (2016) N. Engl. J. Med. 375: 2561–2569.
3. O'Rourke, D.M. *et al.* (2017) Sci. Transl. Med. 9: eaaa0984.
4. Ahmed, N. *et al.* (2017) JAMA Oncol. 3: 1094–1101.