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Dr. Richard Roe
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Patient	Doe, Jane
Date of birth	DD.MM.YYYY
Sex	Female
Patient-ID	#
Report date	DD.MM.YYYY

Report of Immunofluorescent Stains – Doe, Jane (*DD.MM.YYYY)

Order and Clinical Information

Requesting institution/physician	Dr. Richard Roe
Tissue sample supplied by	Department of Pathology, Hospital Model City
Diagnosis	glioblastoma, IDH wildtype, CNS WHO grade 4
Previously reported molecular findings	relevant variants on DNA level: *PTEN* (missense)*, TP53* (frameshift)*, TERT promoter* relevant amplifications on DNA level: *EGFR* relevant homozygous deletions on DNA level: *RB1* overexpressed genes on RNA level: *VEGFA* genes with reduced expression on RNA level: *RB1, TP53* (see sequencing report from MM/DD/YYYY, report ID #)
Sample material	FFPE block external ID: N24-555 A3 date of sample collection: MM/DD/YYYY
Order/request	Immune and Oncoprotein Panel

Summary

Marker	Cell type	Result
PD-L1	immune cells (IC Score)	(+)
	tumor cells (TP Score)	(+)
LAG3	immune cells	-
p-mTOR	tumor cells	+++
	tumor cells (H Score)	+++
p-ERK	tumor cells	+++
p16	tumor cells	+++ (no loss)
p53	tumor cells	- (compatible with *TP53* frameshift variant)
HER2	tumor cells	-
TROP2	tumor cells	-
	tumor cells (H Score)	-

- negative | **(+)** very low | **+** low | **++** moderate | **+++** strong

Detailed Results of the Requested Stains

Marker	Cell type	Subcellular location	Evaluation system*	Result*
CD45/LCA	immune cells	cell membrane/ cytoplasm	descriptive (IF)	positive staining of diffusely distributed immune cells covering 38% of the evaluated tumor area
PD-L1	immune cells	cell membrane	IC Score (IF)	< 1%
	tumor cells	cell membrane	TP Score (IF)	< 1%
LAG3	immune cells	cell membrane	descriptive (IF)	no staining observed
p-mTOR	tumor cells	cytoplasm	descriptive (IF)	80% of tumor cells positive
	tumor cells	cytoplasm	H Score (IF)	235
	tumor cells	nuclear	descriptive (IF)	10% of tumor cells positive
	tumor cells	nuclear	H Score (IF)	15
p-ERK	tumor cells	cytoplasm	descriptive (IF)	70% of tumor cells positive
	tumor cells	nuclear	descriptive (IF)	40% of tumor cells positive
p16	tumor cells	cytoplasm	descriptive (IF)	95% of tumor cells positive
	tumor cells	nuclear	descriptive (IF)	95% of tumor cells positive
p53	tumor cells	nuclear	descriptive (IF)	no staining observed

HER2	tumor cells	cell membrane	descriptive (IF)	weak, incomplete membrane staining in < 1% of the tumor cells
	tumor cells	cytoplasm	descriptive (IF)	cytoplasmic staining in < 1% of the tumor cells
TROP2	tumor cells	cell membrane	descriptive (IF)	no staining observed
	tumor cells	cell membrane	H Score (IF)	0.0

Interpretation

Marker	Interpretation*
CD45/LCA	a moderate fraction of the evaluated tumor area is covered by positively stained immune cells
PD-L1	< 1% of immune cells in the evaluated areas positive, very low expression in the immune cells < 1% of tumor cells in the evaluated areas positive, very low expression in the tumor cells
LAG3	negative in the evaluated tumor area
p-mTOR	predominantly strong cytoplasmic expression in the majority of tumor cells unclear, predominantly slight nuclear expression in a small fraction of tumor cells
p-ERK	cytoplasmic expression in a large fraction of tumor cells nuclear expression in a moderate fraction of tumor cells
p16	retained nuclear expression in the majority of tumor cells (no loss) cytoplasmic expression in the majority of tumor cells
p53	negative
HER2	weak, incomplete membrane staining in < 1% of tumor cells (according to score 0) < 1% of the tumor cells show cytoplasmic positivity (very low cytoplasmic expression)
TROP2	negative

Supplementary Findings

Please find below the results for those markers which were not requested, but were either part of the applied panel, or stained for tissue identification purposes.

Marker	Cell type	Subcellular location	Evaluation system*	Result*
Ki67	tumor cells	nuclear	descriptive (IF)	25% of tumor cells positive

Quality Control

A Hematoxylin and Eosin (HE) staining has been evaluated by a board-certified pathologist/neuropathologist.

Tissue specimen	External ID	Tumor content in the evaluated areas	Suitability for antibody-based stains
FFPE	N24-555 A3	75%	yes

Staining quality has been evaluated by a board-certified neuropathologist and a biologist.

Marker	Staining method/ platform	Evaluated fraction of the sample	Staining quality	Control
CD45/LCA	manual staining	60%	ok	external positive control
PD-L1	manual staining	60%	ok	external positive control
LAG3	manual staining	60%	ok	external positive control
p-mTOR	manual staining	60%	ok	external positive control
p-ERK	manual staining	60%	ok	external positive control
p16	manual staining	60%	ok	external positive control
p53	manual staining	60%	ok	external positive control
HER2	manual staining	60%	ok	external positive control
TROP2	manual staining	60%	ok	external positive control
Ki67	manual staining	60%	ok	external positive control

Disclaimer

This immunofluorescence panel is a laboratory-developed test that has been established at Zentrum für Humangenetik Tübingen. The immunofluorescence stainings have been validated against traditional chromogenic immunohistochemistry.

***The scoring systems for the immunofluorescence markers have been assigned according to standards proposed for chromogenic immunohistochemistry. However, due to the higher sensitivity and wider dynamic range of immunofluorescence analysis, fluorescence intensity values may not be exactly linear to the intensities observed in chromogenic immunohistochemical stainings. Fluorescence signals were checked for plausibility, compared with and adjusted to chromogenic immunohistochemistry during antibody establishment and validation. However, differences**

between the results of immunofluorescence and immunohistochemical analysis cannot be fully excluded. Therefore, the names of the scoring systems in this analysis were given the suffix "(IF)" (immunofluorescence).

Utilization of the results is the sole responsibility of the attending physician.

For further questions and information, please do not hesitate to contact us.

Report written by:

Dr. med. John Q. Public
Consultant for Neuropathology

This report was issued electronically and is therefore valid without signature.

Methods

Pathological review	The pathological services including the confirmation of the histological diagnosis and determination of the tumor content were carried out on our behalf by a board-certified pathologist and/or neuropathologist. The suitability of a sample for immunofluorescent analysis depends on the tumor content as well as on the overall material quality (e.g. inhomogeneous fixation may result in quality impairment). To obtain representative results, a minimal tumor content of 10% is required. In cases with low material quality, evaluation of the staining results may be impaired or even impossible. **In the present case, the quality criteria were fulfilled.**
Staining method	One FFPE section was stained with the listed antibodies. DAPI was used to visualize nuclei. Afterwards the same FFPE section was stained with HE.
Controls	External controls were included in the same staining run. For internal controls, the staining result was positive in at least some structures of the patient sample itself.
Data processing and data evaluation	The results of the immunofluorescent stains were evaluated in the context of the corresponding HE, controls, and the autofluorescent properties of the tissue using a VS200 Research Slide Scanner and the OlyVIA Software (EVIDENT). Applied scoring systems: • H score (IF): Score that represents the extent of immunoreactivity of a tissue for an antibody that targets a certain marker protein. It is obtained by the formula: 3 x percentage of strongly positive cells + 2 x percentage of moderately positive cells + percentage of weakly positive cells, giving a range of 0 to 300. H score thresholds: 0 – negative; 1-99 – weak expression; 100-199 – moderate expression; 200-300 – strong expression. • TP score (IF): Percentage of PD-L1-positive tumor cells relating to all vital tumor cells. • IC score (IF): Percentage of the tumor area covered by PD-L1-positive immune cells. Immune cells comprise lymphocytes, macrophages, granulocytes and dendritic cells. • descriptive (IF): a) Percentage of positively stained cells. b) Percentage of the tumor area or normal tissue area that is covered by positively stained immune cells.
Data availability	Screenshots of representative areas can be provided on request.

Appendix – Information on the Applied Markers

Immune cell identification markers		
CD45/LCA	positive in	leukocytes including granulocytes, lymphocytes, monocytes, macrophages/histiocytes, mast cells and plasma cells
	relevance	identification of leukocytes

Immune checkpoint markers		
PD-L1	definition / function	inhibitory immune checkpoint protein; binds to its receptor PD-1 on T cells; inhibits autoimmunity and T cell activation; expression on tumor cells is an immune escape mechanism
	positive in	variably expressed on immune and tumor cells
	relevance	estimation of the extent of inhibition of T cell mediated anticancer activity
LAG3	definition / function	inhibitory co-receptor expressed on lymphoid cells; suppresses T cell mediated immune response
	positive in	exhausted T cells (antigen-activated T cells)
	relevance	identification of exhausted T cells; estimation of the extent of inhibition of T cell mediated anticancer immunity

Markers to characterize signal transduction pathways		
p-mTOR	definition / function	protein which promotes cell growth, proliferation, differentiation, protein synthesis, angiogenesis, autophagy, and apoptosis; phosphorylated (activated) state of mTOR; dysregulation of the mTOR pathway has been linked with malignant transformation/ tumorigenesis
	positive in	cells expressing activated mTOR protein
	relevance	evaluation of Pi3K/AKT/mTOR pathway activation in tumor cells (H Score for cytoplasmic expression)
p-ERK	definition / function	extracellular signal-regulated kinases (ERKs) are members of the mitogen-activated protein kinase superfamily (MAPK); ERKs require phosphorylation for their full activity; when activated, ERKs regulate cell proliferation, differentiation, and survival; ERK signalling is frequently dysregulated in cancer cells
	positive in	cells expressing activated ERK protein
	relevance	evaluation of MAPK pathway activation in tumor cells (H Score for cytoplasmic expression)
p16	definition / function	tumor suppressor protein encoded by the *CDKN2A* gene; inhibitor of the cell cycle activating proteins CDK4 and CDK6; suppresses cell growth and cell division; loss of p16 function contributes to the bypass cell senescence and is associated with progression of malignant disease
	positive in	cells with intact *CDKN2A*/p16; nuclear and cytoplasmic overexpression in HPV-associated tumors; loss of nuclear staining in tumors with biallelic *CDKN2A* inactivation
	relevance	evaluation of p16 expression in *CDKN2A* altered tumors; evaluation of activation of the CDK pathway; surrogate marker for HPV-association
p53	definition / function	tumor suppressor protein encoded by the *TP53* gene; activated in response to DNA damage; induces cell cycle arrest to allow for DNA repair mechanisms, directs cell senescence, initiates apoptosis
	positive in	normal tissues (scattered staining of few nuclei in case of wildtype p53 expression); tumor cells with *TP53* alteration (strong nuclear staining in a high fraction of tumor cells in case of most missense mutations, complete absence of nuclear staining in case of truncating, frameshift alterations and/or deletions)

	relevance	verification of a *TP53* alteration on the protein level; tumor cell identification in *TP53*-altered entities
HER2	definition / function	human epidermal growth factor receptor 2 encoded by the *ERBB2* gene; tyrosine kinase involved in cell growth, cell division and cell repair mechanisms; constitutively active, when overexpressed
	positive in	membranes of epithelial cells of several organs including breast, skin, gastrointestinal, respiratory, reproductive, and urinary tract; overexpression is found in up to 20% of breast cancers, but also in colon, stomach, and esophageal cancer
	relevance	assessment of membranous HER2 expression in tumor cells; verification of HER2 amplification or overexpression detected by DNA/RNA sequencing on the protein level
TROP2	definition / function	transmembrane glycoprotein encoded by the *TACSTD2* gene; modulates self-renewal, proliferation, invasion, and survival
	positive in	normal epithelial tissue, overexpressed in many tumor entities, predominantly in breast and bladder cancer
	relevance	verification of *TACSTD2* RNA overexpression on the protein level, categorization of TROP2 expression levels (e.g., low vs. intermediate vs. high), confirmation of target expression for clinical trials or translational research

Markers to characterize tissue and tumor types		
Ki67	definition / function	marker of cell proliferation; the extent of Ki67 staining is known as proliferation index and is oftentimes used as a measure of tumor malignancy
	positive in	cells that have entered the cell cycle
	relevance	assessment of the percentage of proliferating nuclei in a tissue type, cell type or tumor entity

This table provides an overview of the markers used in this analysis and an explanation of their application in the given context. The listed specifications may therefore not be comprehensive or complete.