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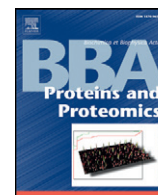

Evaluation of non-supervised MALDI mass spectrometry imaging combined with microproteomics for glioma grade III classification☆

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ABSTRACT

An integrated diagnosis using molecular features is recommended in the 2016 World Health Organization (WHO) classification. Our aim was to explore non-targeted molecular classification using MALDI mass spectrometry imaging (MALDI MSI) associated to microproteomics in order to classify anaplastic glioma by integration of clinical data. We used fresh-frozen tissue sections to perform MALDI MSI of proteins based on their digestion peptides after *in-situ* trypsin digestion of the tissue sections and matrix deposition by micro-spraying. The generated 70 μm spatial resolution image datasets were further processed by individual or global segmentation in order to cluster the tissues according to their molecular protein signature. The clustering gives 3 main distinct groups. Within the tissues the ROIs (regions of interest) defined by these groups were used for microproteomics by micro-extraction of the tryptic peptides after on-tissue enzymatic digestion. More than 2500 proteins including 22 alternative proteins (AltProt) are identified by the Shotgun microproteomics. Statistical analysis on the basis of the label free quantification of the proteins shows a similar classification to the MALDI MSI segmentation into 3 groups. Functional analysis performed on each group reveals sub-networks related to neoplasia for group 1, glioma with inflammation for group 2 and neurogenesis for group 3. This demonstrates the interest on these new non-targeted large molecular data combining both MALDI MSI and microproteomics data, for tumor classification. This analysis provides new insights into grade III glioma organization. This specific information could allow a more accurate classification of the biopsies according to the prognosis and the identification of potential new targeted therapeutic options. This article is part of a Special Issue entitled: MALDI Imaging, edited by Dr. Corinna Henkel and Prof. Peter Hoffmann.

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Abbreviations: A, Astrocytoma; ACN, Acetonitrile; ATRX, Alpha-Thalassemia/mental Retardation syndrome X-linked; CDKN2A, Cyclin-Dependent Kinase Inhibitor 2A; CGH-array, Comparative Genomic Hybridization; DNA, Deoxyribonucleic Acid; EGFR, Epidermal Growth Factor Receptor; F, Female; FDR, False Discovery Rate; FFPE, Formalin-Fixed Paraffin-Embedded; gCIMP, CpG Island Methylator Phenotype; HCD, Higher Energy Collision Dissociation; HES, Hematoxylin-Eosin Safran; IDH, Isocitrate Dehydrogenase; LC, Liquid Chromatography; H3F3A, H3 Histone, Family 3A; LESA, Liquid Extraction Surface Analysis; LFQ, Label-Free Quantification; M, Male; MALDI, Matrix-Assisted Laser Desorption/Ionization; MALDI MSI, MALDI Mass Spectrometry Imaging; TOF, Time-of-Flight; MeOH, Methanol; MGMT, O-6-Methylguanine-DNA Methyltransferase; MRI, Magnetic Resonance Imaging; MSI, Mass Spectrometry Imaging; O, Oligodendroglioma; OA, Oligo-Astrocytoma; PSM, Peptide Spectrum Matches; PTEN, Phosphatase and Tensin Homolog; ROI, Region of Interest; RNA, Ribonucleic Acid; SNEA, Subnetwork Enrichment Analysis; TERT, Telomerase Reverse Transcriptase; TFA, Trifluoroacetic Acid; TP53, Tumor Protein P53; WHO, World Health Organization.

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1. Introduction

Histological classification and clinical management of gliomas remain challenging. The World Health Organization (WHO) classification is the standard classification for gliomas and is used to guide clinical management according to the subtypes of tumors. It has been shown that molecular information can improve on the 2007 WHO classification [1]. A highly variable clinical outcome was observed between the same subtypes of gliomas, and a high intra and interobserver discrepancy between pathologists has been noted [2].

A new update of the WHO classification has recently been published [3]. The goal of these classifications is to provide information on the prognosis of patients in a pre-determined subtype of tumors and to guide clinical decisions. The integration of immunohistochemical markers and molecular markers to the standard pathological analysis adds objectivity and permits a better prognostic classification by improving the definition of prognostically distinct subtypes of gliomas [1,4]. In the WHO 2016 classification, grades II or III glioma with a glioblastoma-like profile, include *IDH1/2* wild-type status, frequent gains on 7q and losses on 10q and frequent telomerase reverse transcriptase (TERT) promoter mutations are mostly associated with a glioblastoma, an anaplastic astrocytoma or oligoastrocytoma pathological patterns have usually the worse outcome [1]. Thus, the determination of the presence of a +7q/−10q in patients with *IDH1/2* wild-type WHO grade II and III gliomas is relevant [1]. Tumors with an *IDH1/2* mutation, a 1p/19q codeletion, and an oligodendroglial histological subtype have the best prognosis [1,5,6]. Most of these tumors show a proneural glioblastoma-like expression profile, associated with a better outcome [1,7,8]. Yet *IDH1/2* mutation/CpG island methylator phenotype (gCIMP), 1p/19q co-deletion represents the main molecular features required for a better classification of gliomas [1,2,7,8]. Other biological markers of interest are often added, such as *TERT* promoter mutations, gain on chromosome arm 7q (+7q), loss on chromosome arm 10q (−10q), tumor protein p53 (*TP53*), alpha-thalassemia/mental retardation syndrome X-linked (*ATRX*) gene mutations, phosphatase and tensin homolog (PTEN), and Notch pathway gene mutations or (H3 Histone, Family 3A) H3F3A and BRAF mutations [1,9–20]. However, their implications and their relevance in refining prognostic information for grade II and grade III gliomas remain to be confirmed [1].

Immunohistochemistry and molecular-based classification of glioma have improved the determination of the prognosis and integrated diagnosis should now be required as part of tumor assessment in the clinical practice and help to guide the therapeutic decisions [17,18,21,22]. However, genomic approaches are limited, as some normal, upregulated or mutated genes may not be transcribed [23] and discrepancies have been identified between m-ribonucleic acid (mRNA) and proteomics expression profiles in gliomas [24,25]. The addition of proteomics findings could lead to the discovery of new prognostic information and a better characterization of tumor subtypes, improving thus the clinical decision making. Over the past years, technological advances have been realized in proteomics analysis. The proteomics approach gives access to many proteins' identification, relative quantification, and determination of post-translational modifications and could also help to identify personalized clinical strategies by determining specific molecular pathways and cellular functions [24,26–30].

In the present study, we aim to investigate glioma grade III molecular features and classification of patients by a non-targeted molecular approach using MALDI MS imaging (MALDI MSI) and spatially-resolved microproteomics. This is achieved by performing MALDI MSI of trypsin digested proteins [31] to retrieve protein distribution within tissues of patients with anaplastic glioma and further non-supervised spatial segmentation of the molecular data [32]. From such classification, regions of interest (ROIs) are selected for the spatially-resolved Shotgun proteomics [33,34]. Comparison between proteomics and histological data is investigated to search for concordance or discordance.

2. Materials and methods

2.1. Patient samples and consent

Samples were prospectively collected from histologically confirmed anaplastic glioma patients recruited between September 2014 and July 2015 at Lille University Hospital, France according to the gliomic study (NCT 02473484). The study adhered to the principles of the Declaration of Helsinki and the Guidelines for Good Clinical Practice. All patients gave written informed consent before enrollment. Patients enrolled in this cohort all had a newly diagnosed anaplastic glioma according to the WHO classification of the central nervous system [3] after pathological examination by the same pathologist (CAM) under routine practice conditions. Other criteria for inclusion included an age of 18 years or more, the absence of other prior cancer or cancer treatment, and the absence of genetic disease potentially leading to cancer. Tumor samples were processed within 2 h after sample extraction in the surgery room to limit the risk of protein degradation.

2.2. Glioma tissue samples for immunohistochemistry and molecular analyses

2.2.1. Immunohistochemistry analyses

IDH1-R132H mutations, ATRX, protein 53 (P53) and epidermal growth factor receptor (EGFR) statuses were determined by immunohistochemistry on formalin-fixed paraffin-embedded (FFPE) tumor tissue samples. 4 μm sections were labeled in an Ultra automate (Ventana-Roche Tissue Diagnostics, Tucson AZ), after antigen retrieval procedures (ATRX, IDH1, and P53: citrate pH 6.0; EGFR: proteinase 2), according to the suppliers' protocols. A punch of positive control tumor was stained on the same slide for IDH1 labeling (tumor tissue from an IDH1 R132H mutated, non-codeleted, astrocytoma). ATRX was determined as positive when cases with more than 10% positive tumor cells were scored positive (ATRX expression). P53 was considered expressed when 10% or more nuclei were deeply stained; EGFR expression was semi-quantified according to Hirsch score, allowed by comparison to the multiscale control. A multiscale positive control was stained in the same series for EGFR labeling. IDH1 R132H was explored using Diavona clone H09, mouse monoclonal at 1/40 dilution, ATRX using Sigma, ref HPA001906 Rabbit polyclonal, at 1/200 dilution, P53 using Dako, clone DO-7, mouse monoclonal, at 1/100 dilution, and EGFR using Invitrogen, clone 31G7, mouse monoclonal, at 1/20 dilution.

2.2.2. Deoxyribonucleic acid (DNA) extraction and quantification

Molecular analyses were performed on FFPE tissues. The following tests were performed: comparative genomic hybridization (CGH)-array, O-6-methylguanine-DNA methyltransferase (MGMT) promoter methylation and IDH1/IDH2 mutations. All tissues used for DNA extraction were histologically evaluated to determine the tumor cell content. Only tissue samples with a minimum tumor cell content of 70% or more were analyzed. DNA extraction from FFPE was performed using the kit QIAamp DNA FFPE Tissue (Qiagen). CGH profiles were determined using a SurePrint G3 Human CGH Microarray Kit, 8x60K (Aligent) and the CytoGenomics v2.7 software. The limit of the resolution was 1 Mb. The presence of 1p/19q codeletion, gain of chromosome 7, loss of chromosome 10, amplification of the EGFR gene and homozygous deletion of the Cyclin-Dependent Kinase Inhibitor 2A (CDKN2A) gene has been systematically evaluated. Mutations affecting codon 132 of *IHD1* (ref seq NM_005896.2), codon 172 of IDH2 (ref seq NM_ 002168.2) were validated by PCR-sequence Sanger when immunohistochemistry was negative. The MGMT promoter methylation status (CpGs 74–78) was determined after bisulfite treatment by pyrosequencing on a PyroMark Q96 with an MGMT PyroMark kit (Qiagen). The presence of methylation was scored as positive when a minimum of 8% of methylation was observed.

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