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Article

Next-Generation Sequencing Comparative Analysis of DNA Mutations between Blood-derived Extracellular Vesicles and Matched Cancer Tissue in Patients with IV Grade Glioblastoma

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Abstract: The biological heterogeneity of glioblastoma (GBM), the most aggressive type of brain cancer, is a critical hallmark, caused by changes in the genomic mutational asset and influencing the clinical progression over the time. The understanding and monitoring of the mutational profile is important not only to reveal novel therapeutic targets in this set of patients, but also to ameliorate the clinical stratification of subjects and the prognostic significance. As neurosurgery represents the primary technique to manage GBM, it is of outmost importance to optimize alternative and less invasive methods to monitor the dynamic mutation profile of these patients. Extracellular vesicles (EV) are included in the liquid biopsy analysis and have emerged as the biological mirror of escaping and surviving mechanisms by many tumors as well as glioblastoma. Very few studies have investigated the technical feasibility to detect and analyze the genomic profile by Next Generation Sequencing (NGS) in circulating EV of patients with grade IV glioblastoma. Here, we attempted to characterize and to compare with the corresponding matched tissue samples, potential variants with pathogenic significance of the DNA contained in peripheral blood derived EV. The NGS analysis has revealed that patients with grade IV glioblastoma, exhibited lesser DNA content in EV than controls and that both in EV and matched cancer tissues, the NF1 gene was consistently mutated in all patients with the c.2568C>G as the most common pathogenic variant expressed. This study supports the clinical utility of the circulating EV in glioblastoma and as eligible tool for personalized medicine.

Keywords: glioblastoma; extracellular vesicles; Next Generation Sequencing; pathogenic mutations; NF1

1. Introduction

Gliomas are brain tumors of glial origin with about 100,000 new diagnoses every year worldwide (1). This is the most frequent malignancy of the brain being associated with a dismal prognosis and quality of life (2). Gliomas are generally diagnosed by clinical assessment and imaging evaluations, and histological analysis which is mandatory to

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confirm the diagnosis (3). In the last decades, this approach has allowed to distinguish between low (WHO grades I, II, III) and high grade of glioma (glioblastoma, GBM, WHO grade IV) considered the most aggressive form. Despite the best of the cares still based on a combination of surgery, radio- and chemotherapy with temozolomide (TMZ) (4), GBM is still an untreatable tumor with a median survival of about 12-15 months (3). Recently, the World Health Organization (WHO) has suggested to integrate the mutational analysis of the isocitrate dehydrogenase gene (IDH1) in the glioma classification, therefore improving tumor diagnostics and patient's prognosis (5). Nevertheless, the early diagnosis, and in particular the possibility to monitor the progression and the molecular rearrangement of the GBM without recurring to invasive procedures, is clearly more complex compared to other types of tumors.

Extracellular vesicles (EVs) have been found in biofluids and represent an intercellular mechanism of biomolecules transport (6, 7) including by lipids (8), proteins (9) and nucleic acids (10). Glioblastoma cells release EVs in the local microenvironment and can be detected in the bloodstream, being able to trespass the blood brain barrier, which is often compromised in these patients, mediating angiogenesis, proliferation, immunomodulation, carcinogenesis, and invasiveness (11-13). Importantly, EVs are acknowledged as a useful tool to follow the molecular profile of brain tumors over the time, as the molecular cargo of EVs in patients with GBM can foster non only specific miRNAs and mRNAs, but also a defined genomic signature of the corresponding parental cancer tissue. This property may include the recent observation regarding the extrachromosomal DNA (14), potentially responsible of the molecular heterogeneity and further complexity of GBM. Genomic mutations have been extensively found in circulating DNA and from EVs extracted from cerebrospinal fluid (15, 16), which is an invasive procedure for GBM patients, who normally display high intracranial pressure. Thus, the employment of peripheral blood derived EVs could be more useful to patients with GBM in order to strictly monitor the evolution of the disease.

Studies regarding genomic mutations in the EVs originating from blood of patients with grade IV GBM are lacking in literature, except for those regarding in vitro GBM cell-derived EVs (17-19), the characterization of both EVs and circulating free DNA of blood origin (20, 21).

Here, we firstly compared the genomic mutational profile of peripheral blood derived EVs from patients with grade IV GBM with the matched cancer tissues by Next-Generation Sequencing (NGS) analysis. We found a high correlation in the mutational status of both NF1 and H3F3A genes which were mainly represented in both type of samples.

2. Materials and Methods

2.1. Study design

The present investigation consists in a perspective observational study, conducted at a single academic institution. The ethical committee approval was not necessary according to the study design, the non-modification of the standard of care and national legislation. A properly designed informed consent, consistently matching institutional guidelines for scientific investigations, was collected from every included subject. The present study agrees with the WMA Helsinki declaration of Human Rights. The time range for conducting the present investigation was set from January 2019 through June 2019, consisting in 6 months.

2.2. Inclusion and exclusion criteria

Patients group: consecutive patients admitted at our tertiary hospital for brain tumors were considered for eligibility. Pre-operative Karnofsky performance status (KPS)>70%, total-body contrast-enhanced CT scan negative for solid tumors, and pathological diagnosis of GBM (WHO-2016) were considered as inclusion criteria. A control group was selected from patients scheduled for brain MRI and blood samples for

non-specific cephalgia at our Neurologic Department. Their images were retrieved anonymously from the institutional PACS viewer, and their stored blood samples were processed for EX extractions. No modifications of the study protocol were necessary for conducting these evaluations. Subjects were considered for inclusion in the control group in case of negativity of their brain MRI for intracranial solid tumors. The KPS was assessed pre-operatively and before hospital discharge. Complications, recurrence-free survival, and overall survival were registered for every patient.

2.3. Radiologic assessment

A gadolinium enhanced MRI (gh-MRI) and a non-gh-MRI were performed in patients and controls groups, respectively. Radiological study was conducted according to the glioma-and-neurodegenerative disorders study protocol, as standardized at the Neuroradiology Department, using the same MRI for all patients.

In patients' group, gh-t1-weighted and FLAIR sequences were processed for calculating the volume of the nodular lesion and the perilesional edema, respectively. The grade of resection was calculated on post-operative gh-MRI, and rated as gross-total removal, partial removal, excisional biopsy. A single senior Neuroradiologist personally evaluated images, using the workstation connected to the Picture archiving and communicating system (PACS), and the institutional certified PACS-viewer.

2.4. Tissue sampling and histopathologic analysis

During surgery, tumor samples were collected and fixed in 4% buffered formaldehyde before being embedded in paraffin. 2µm sections were stained with a fully automated staining system (Leica Microsystems) for Hematoxylin and Eosin. The sections were also incubated with mouse monoclonal antibodies against human GFAP, OLIG2, ATRX, EGFR, IDH1^{R132H}, Ki67. Diagnosis of certainty was performed according to the World Health Organization Gliomas scheme within 3 weeks from surgery.

2.5. Tissue processing and DNA extraction

Tumor areas were circumscribed under a light microscope on stained Hematoxylin and Eosin (H&E) slide sections and manually macrodissected from 10 µm unstained FFPE sections prior to DNA extraction with the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. DNA concentration was measured with the Qbit4 (Invitrogen, Waltham, MA, United States). DNA samples with concentrations higher than 2 ng/µl received the approval to be processed for NGS analysis.

2.6. Plasma separation, EV isolation and DNA extraction

Whole blood samples from 26 glioma patients (24h before surgery) and 10 healthy donors were collected in PAXgene blood ccfDNA collection tubes (Qiagen) and centrifuged at 2000g for 10min at 4°C within 4 hours. Then, the separated plasma was collected and centrifuged at 3000g for 15min at 4°C prior to storage at -80°C. EVs were isolated from blood plasma with the exoEasy maxi kit (Qiagen) following the manufacturer's instructions. DNA was extracted from the isolated EVs by the QIAamp MinElute Virus spin Kit (Qiagen) according to the manufacturer's instructions. Due to the lower yield of DNA extracted from GBM plasma EVs, we considered suitable for our analysis only those samples with a DNA concentration above 0.5 ng/µl.

2.7. Next-Generation Sequencing analysis

Next-Generation Sequencing analysis was performed with the GeneReader instrument (Qiagen) considering a custom U.M.I. (Unique Molecular Indexes) panel, the QIAact GliomaProject DNA Panel CP153 (Qiagen), which considers the following genes: ATRX (NM_000489.4 NP_000480.3), CDKN2A (NM_000077.4 NP_000068.1), H3F3A (NM_002107.4 NP_002098.1), IDH1 (NM_001282386.1 NP_001269315.1), IDH2

(NM_002168.3 NP_002159.2), NF1 (NM_000267.3 NP_000258.1), PTEN (NM_000314.6 NP_000305.3), TERT (NM_198253.2 NP_937983.2) and TP53 (NM_000546.5 NP_000537.3). Results were analyzed with the Clinical Insight (QCI, Qiagen), a variant analysis, interpretation and decision support tool for research and clinical labs analyzing human genetics data and is not intended to be used for diagnostic purposes. QCI Interpret software includes the following underlying databases, data reference sets and tools; QIAGEN Clinical Insight-Interpret (5.5.20190807), Ingenuity Knowledge Base (Utopia 190824.002), CADD (v1.4), Allele Frequency Community (2018-12-15), EVS (ESP6500SI-V2), Refseq Gene Model (2018-07-10), JASPAR (2013-11), Ingenuity Knowledge Base Snapshot Timestamp (2019-08-24 18:08:27.0), Vista Enhancer hg18 (2012-07), Vista Enhancer hg19 (2012-07), Clinical Trials (Utopia 190824.002), PolyPhen-2 (v2.2.2), 1000 Genome Frequency (phase3v5b), ExAC (0.3.1), iva (Jun 28 11:10 iva-1.0.1085.jar), PhyloP hg18 (2009-11), PhyloP hg19 (2009-11), DbSNP (151), TargetScan (7.2), GENCODE (Release 28), CentoMD (5.3), OMIM (May 26, 2017), gnomAD (2.0.1), BSIFT (2016-02-23), TCGA (2013-09-05), Clinvar (2019-01-02), DGV (2016-05-15), COSMIC (v87), HGMD (2018.4), SIFT4G (2016-02-23).

2.8. Statistical analysis

Values were reported as mean ± standard deviation (SD). The Student's t-test was used to compare the quantitative continuous variables. A p value <0.05 was considered statistically significant. The Statistical Analysis was performed by GraphPad PRISM 5 software.

3. Results

In order to compare potential mutations between plasma-derived EV and cancer tissue in patients with glioblastoma, we analyzed a total of 26 subjects. Blood was withdrawn before surgery. Patients' characteristics and inclusion criteria are described in Table 1 and methods, respectively. The mean age was 60.1 years, the male/female ratio was 1.4/1, and the mean pre-operative KPS was 89.5 ±16.8%. Ten subjects were used as reference for blood-derived EV with a mean age 42±11 years and male/female ratio of 1/1.5 and were referred to the hospital for headache.

In the recruited set of patients, the mean post-operative KPS was 91±15.7%) ($p>0.05$). Gross-total removal was reported in 12 (46.2%), near-total in 11(42.3%), partial removal in 0 (0%) and biopsy was performed in 3 (11.5%). All patients underwent post-surgical adjuvant therapies according to Stupp et al. (4). The mean recurrence-free survival was 13.2±9.5 months, and the mean overall survival was 15.9±9.72 months. The mean solid lesion volume calculated on gh-t1-weighted sequences was 16.1±18.7 cm^3. The mean necrotic lesion volume calculated on gh-t1-weighted sequences was 6.63±16.11 cm^3. Total volume measurement on gh-t1 weighted sequences was 23.45±30.6 cm^3 while the mean volume of the infiltrating non-enhancing component, calculated on FLAIR images, was 63.4±56.6 cm^3, thus a solid-to-infiltrative volume ratio of 17.7%.

Table 1. Baseline characteristics of the adult glioma cohort enrolled in the study.

Characteristics	Patients (N=26)
Median age, years (range)	60.1 (22-88)
Gender, n (%)	
Male	15 (57.7)
Female	11 (42.3)
Tumor location, n (%)	
Frontal lobe	8 (30.8)
Right temporal lobe	3 (11.5)
Left temporal lobe	5 (19.2)
Frontoparietal lobe	1 (3.9)
Temporo-insular	1 (3.9)
Occipital	3 (11.5)
Frontoparietal lobe	2 (7.7)
Parietal	3 (11.5)
Histology (WHO 2016), n (%)	
IV	20 (77.0)
III	3 (11.5)
II	3 (11.5)
I	0 (0)
Surgical resection, n (%)	
Gross total	12 (46.2)
Near total	11 (42.3)
Partial	0 (0)
Biopsy	3 (11.5)
Median tumor volume, cm³ (±SD)	
Total Flair	63.4 (±56.3)
Non-enhancing (t1 Post-contrast)	6.6 (±16.8)
Enhancing (t1 Post-contrast)	16.1 (±18.7)
Enhancing+Non-enhancing (t1 Post-contrast)	23.4 (±30.6)
Karnofsky Performance Status (KPS), n (%) preoperative	
90\100	20 (76.9)
80\70	5 (19.2)
<60	1 (3.9)
Karnofsky Performance Status (KPS), n (%) preoperative	
90\100	22 (84.6)
80\70	3(19.2)
<60	1 (11.5)
Progression, n (%)	
Yes	21 (80.8)
No	5 (19.2)
Free survival, months (range)	13.23 (0-63)
Survival, months (range)	15.92 (3-63)

Out of 26 patients recruited, only the 20 patients with grade IV glioblastoma were sorted. All histopathological features for the main diagnostic markers are reported in Table 2.

Table 2. Immunohistochemical features of the adult glioma cohort enrolled.

Immunohistochemical Characteristics	Nucleotide change
GFAP	23
OLIG2	15
ATRX	2
EGFR	7
IDH1	3
p53	19
ki67 (mean % all patients)	28
mitosis (mean over 10 HPF all patients)	22.5

Next Generation Sequencing analysis was in parallel performed on both peripheral blood-derived EV and on the selected neoplastic area of the matched paraffin-embedded sections as depicted in the experimental plan (Figure 1A).

We performed the NGS analysis including the following genes: ATRX, CDKN2A, H3F3A, IDH1, IDH2, NF1, PTEN, TERT and TP53. The genomic sequencing was performed only in samples (N=10 patients and N=5 controls) where we achieved high DNA standards in terms of quality and quantity (DNA threshold >0.5 ng/µl). Accordingly, results showed a significant lower DNA concentration contained in EV isolated from plasma of patients with grade IV GBM compared to controls (Figure 1B, $p<0.01$).

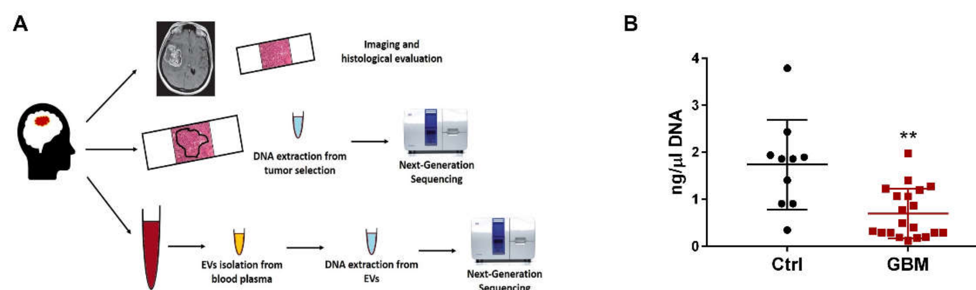

Figure 1a-b. (a) Experimental workflow of the study. Brain tumor imaging and histopathological analysis were performed for cancer staging. A peripheral blood sample was also obtained from patients to isolate EV. Afterwards, DNA was isolated in parallel from both paraffin-embedded sections and matched EVs and genomic mutations assessed by NGS analysis. (b) Blood plasma-derived EVs DNA concentration yield in patients with glioblastoma (N=20) compared to healthy donors (N=10). **p<0,01. GBM, patients with glioblastoma, Ctrl, controls.

Interestingly, the mutational analysis by NGS on the same blood-derived EV samples, has revealed that all patients displayed pathogenic mutations of the NF1 gene (10/10 patients) and that mutations of TERT were also moderately represented (3/10 patients, Figure 2A). Oppositely, peripheral blood-derived EV samples from controls showed a homogenous wild-type genomic profile (Figure 2A). Results also have highlighted that out of 10 patients, 7 exhibited the specific pathogenic variant of NF1 c.2568C>G corresponding to the p.S856R (Substitution - Missense, position 856, S➞R) in blood-derived EV with a Variant allele frequency (VAF) >0.5% (Figure 2B). According to Wikipathway database, the pathway affected was related to the MAPK signaling (https://cancer.sanger.ac.uk/cosmic/mutation/overview?id=109750332).

The full list of the genomic variants classified as pathogenic that we found, is reported in Table 3.

When the NGS analysis was performed in parallel on the corresponding cancer tissue, we observed that the mutational status of NF1 and TERT in the EV, correlated with that from matched tumor tissue (8/10 and 2/10 patients for NF1 and TERT, respectively. Figure 2C).

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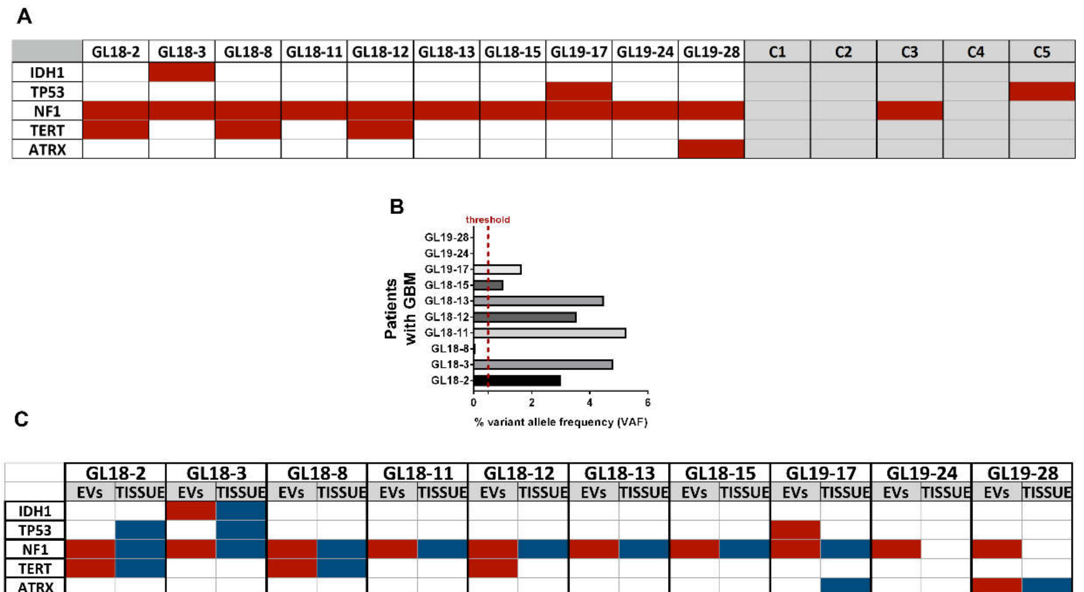

Figure 2a-c. (a) Comparative analysis of pathogenic mutations (highlighted in red) found in peripheral blood derived EVs of patients with glioblastoma (N=10) and healthy donors (N=5). Acronyms GL18-2/19-28 and C1-C5 indicate patients and controls, respectively. (b) Variant allele frequency (VAF, expressed as percentage) of the pathogenic variant S856R/N of the NF1 gene in blood-derived EV of patients (N=10). A >0.5% VAF cut-off threshold was set. Results are expressed as the mean±SD. (c) Comparative analysis of pathogenic mutations (highlighted in red) found in peripheral blood derived EVs and matched cancer tissue (highlighted in blue) of patients with GBM (N=10). Acronyms GL18-2/19-28 and C1-C5 indicate patients and controls, respectively.

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Table 3. Genomic variants with pathogenic significance found in blood-derived EV of patients with IV grade glioblastoma.

Gene	Nucleotide change	Amino acid change	Type of tumor
NF1	c.233delA	p.Asn78fs	Neurofibromatosis Type 1
	c.1466A>G	p.Tyr489Cys	
	c.1658A>G	p.His553Arg	
	c.2027delC	p.Pro678fs	
	c.2568C>G	p.Ser856Arg	
	c.3033delA	p.Thr1013fs	
	c.3859T>C	p.Phe1287Leu	
	c.2297T>C	p.Ile766Thr	
IDH1	c.395G>A	p.Arg132His	CNS
TP53	c.700T>A	p.Tyr234Asn	Hematopoietic
	c.1146delA	p.Lys382fs	CNS
	c.841G>T	p.Asp281Tyr	Glioblastoma, Intestine, skin, Breast
ATRX	c.1074delA	p.Lys358fs	CNS, Intestine
	c.2658_2659delGA	p.Glu886fs	Intestine
TERT	c.336delC	p.Glu113fs	Intestine

*CNS, central nervous system

4. Discussion

To date, the development of novel biological and non-invasive markers are urgently required for all tumours but mainly for those as GBM challenged by the anatomical localization. Besides, the biology of GBM is poorly understood, hence there is still an increasing need to find novel markers to help classify this tumor, to follow its progression and to drive therapy.

From a diagnostic standpoint, the extracellular vesicles are contextualized in the scope of the liquid biopsy. The rich cargo of nucleic acid including DNA, is a very useful tool that can be exploited either to reveal insights of the progression of the disease, but also to understand the potential evolution of the genomic mutations caused by clinical treatments which are known to induce resistance and accelerate the aggressiveness over the time. Accordingly, GBM is well acknowledged to resist to conventional therapies due to multiple mechanisms spanning from stem cells-like features to immunosuppression (22-25) and to secrete a very high number of EVs (26). Moreover, EVs can be released at any stage of cancer, carrying a DNA cargo more stable than the circulating free form due to the protection exerted by the cell membrane. This represents an important advantage as genomic sequencing technique requires the integrity of the DNA, although, potential contamination may arise from multiple cell sources, when free nucleic acids are isolated from the blood (27).

In our preliminary study, we have already observed a correlation between the DNA content of blood-derived EVs obtained from patients with glioma and both tumour volume and mitotic activity (28). In this report we have increased the number of observations with specific focus on grade IV GBM, the most aggressive brain tumor.

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Although it is acknowledged that patients with GBM exhibit an enrichment of EVs (29), insights in their DNA cargo are currently considered more critical to follow the evolution of a very heterogenous and dynamic tumor. The number itself of circulating EVs in patients with GBM has its own diagnostic significance during the follow up of patients, but it is subjected to profound variations post-surgery and chemotherapy or metastasis (30), strengthening the role of the molecular analysis of the cargo respect to the sole quantitative analysis of EVs. Hence, we found a decreased content of DNA in circulating EVs derived from our set of patients compared to controls. This result is quite in line with a previous publication showing that the amount of DNA localized inside the GBM cells-derived EVs is lower than the DNA on the outer membrane (31), although other reports have highlighted that cancer exosomes including GBM, carry a higher amount of DNA (32, 33). These discrepancies may origin from the heterogeneity and diversity of the DNA content in circulating EVs and from the different methodology of isolation.

Notably, we also found a very high correlation between the genomic mutational profile of EVs and that found in matched tissue samples, suggesting that the DNA-based cargo of circulating EVs might be equally useful to reflect the profound intra-heterogeneity of GBM (34, 35), allowing a potential quantitative and qualitative characterisation of the tumour genome. To the best of our knowledge, only a couple of studies report the mutational analysis of genomic DNA isolated from peripheral blood-derived EVs of patients with GBM (31, 36).

Generally, the mutational analysis of EVs from patients with GBM refers to a panel of well recognized mutations, including IDH1 and EGFR (36-38). In our study we have focused on additional genes of current interest in GBM (39). We observed a homogenous tendence of all patients to display pathogenic mutations of the NF1 gene in circulating EVs as in matched cancer tissue. In other studies, mutations of NF1 are the expression of a subpopulation of the tumor (31). Notably, NF1 has been recently discovered as negative regulator of the RAS/MAK signalling and controls the mesenchymal signature in GBM (40). This is a key finding, as difference of contribution between cancer cells and tumor microenvironment (also represented by EVs) has not clearly elucidated yet in GBM. In line with this article, we have found that the most representative pathogenic genomic variant of NF1 was the c.2568C>G, which has been described only in liver neoplasm and affecting the MAPK signalling pathway, whose role is acknowledged in tumor cell proliferation. We also found additional pathogenic variants that so far have been described for other type of cancers and that require to be fully verified in GBM.

A prognostic value of circulating microparticles in patients with glioblastoma has been suggested (41), strengthen also by key observations including the transformation of astrocytes, and the horizontal transferring of nucleic acid within the tumor microenvironment, resulting in proliferation of the cancer counterpart (42). Based on the biological role of EVs in GBM, the tumor progression index (TPI) is currently proposed as the novel predictive marker to include the molecular signature of the EVs, allowing a better distinction between cancer cells and healthy tissue (43). Thus, it is conceivable that additional pathogenic genetic alterations will be included in future.

This study has several limitations. To reach a suitable pro diagnostic validation of circulating EVs in GBM to potentially correlate the result with the clinical parameters of the patients, a higher sample size is required. More importantly, we have not followed up patients, therefore it will be important to investigate potential changes of the molecular cargo of the DNA contained in the EVs in parallel to a more profound biological stratification of the patients and subtypes of GBM, a frequent phenomenon occurring in the same tissue area of this type of tumor.

This study demonstrates the technical feasibility of the mutational analysis of the genomic cargo in circulating EVs and its utility as clinical biomarkers.

Author Contributions: P.R. and E.D.F. performed main experiments and wrote the paper; L.P. performed NGS analysis; A.P, P.C., F. F. and S.T. selected patients and elaborate all clinical data. L.R.

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performed statistical analysis; M.M and A.R. performed neurosurgery, A.C. conceived the study and revised the manuscript.

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Informed Consent Statement: samples were obtained from patients that had given their informed consent for use of the remaining samples after diagnosis for non-interventional, observational research studies. Patients understood that data resulting from the analysis could be published.

Data Availability Statement: Main data generated or analysed in this study are included in this article. Details are available from the corresponding authors on reasonable request.
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Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

References

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2018;68(6):394-424.
2. Stupp R, Hegi ME, Mason WP, van den Bent MJ, Taphoorn MJ, Janzer RC, et al. Effects of radiotherapy with concomitant and adjuvant temozolomide versus radiotherapy alone on survival in glioblastoma in a randomised phase III study: 5-year analysis of the EORTC-NCIC trial. Lancet Oncol. 2009;10(5):459-66.
3. Chinot OL, Wick W, Mason W, Henriksson R, Saran F, Nishikawa R, et al. Bevacizumab plus radiotherapy-temozolomide for newly diagnosed glioblastoma. N Engl J Med. 2014;370(8):709-22.
4. Stupp R, Mason WP, van den Bent MJ, Weller M, Fisher B, Taphoorn MJ, et al. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. N Engl J Med. 2005;352(10):987-96.
5. Louis DN, Perry A, Reifenberger G, von Deimling A, Figarella-Branger D, Cavenee WK, et al. The 2016 World Health Organization Classification of Tumors of the Central Nervous System: a summary. Acta Neuropathol. 2016;131(6):803-20.
6. Mangino G, Iuliano M, Carlomagno S, Bernardini N, Rosa P, Chiantore MV, et al. Interleukin-17A affects extracellular vesicles release and cargo in human keratinocytes. Exp Dermatol. 2019;28(9):1066-73.
7. Bordin A, Chirivì M, Pagano F, Milan M, Iuliano M, Scaccia E, et al. Human platelet lysate derived extracellular vesicles enhance angiogenesis through miR-126. bioRxiv. 2022:2022.05.10.491341.
8. Llorente A, Skotland T, Sylvanne T, Kauhanen D, Rog T, Orlowski A, et al. Molecular lipidomics of exosomes released by PC-3 prostate cancer cells. Biochim Biophys Acta. 2013;1831(7):1302-9.
9. Hallal S, Azimi A, Wei H, Ho N, Lee MYT, Sim HW, et al. A Comprehensive Proteomic SWATH-MS Workflow for Profiling Blood Extracellular Vesicles: A New Avenue for Glioma Tumour Surveillance. Int J Mol Sci. 2020;21(13).
10. Thakur BK, Zhang H, Becker A, Matei I, Huang Y, Costa-Silva B, et al. Double-stranded DNA in exosomes: a novel biomarker in cancer detection. Cell Res. 2014;24(6):766-9.
11. Giusti I, Di Francesco M, Dolo V. Extracellular Vesicles in Glioblastoma: Role in Biological Processes and in Therapeutic Applications. Curr Cancer Drug Targets. 2017;17(3):221-35.
12. Chistiakov DA, Chekhonin VP. Extracellular vesicles shed by glioma cells: pathogenic role and clinical value. Tumour Biol. 2014;35(9):8425-38.
13. van der Vos KE, Balaj L, Skog J, Breakefield XO. Brain tumor microvesicles: insights into intercellular communication in the nervous system. Cell Mol Neurobiol. 2011;31(6):949-59.
14. Shiras A, Mondal A. Extrachromosomal DNA: Redefining the pathogenesis of glioblastoma. Biochim Biophys Acta Rev Cancer. 2021;1876(1):188551.
15. Figueroa JM, Skog J, Akers J, Li H, Komotar R, Jensen R, et al. Detection of wild-type EGFR amplification and EGFRvIII mutation in CSF-derived extracellular vesicles of glioblastoma patients. Neuro Oncol. 2017;19(11):1494-502.
16. Heitzer E, Haque IS, Roberts CES, Speicher MR. Current and future perspectives of liquid biopsies in genomics-driven oncology. Nat Rev Genet. 2019;20(2):71-88.
17. Andre-Gregoire G, Bidere N, Gavard J. Temozolomide affects Extracellular Vesicles Released by Glioblastoma Cells. Biochimie. 2018;155:11-5.
18. de Vrij J, Maas SL, Kwappenberg KM, Schnoor R, Kleijn A, Dekker L, et al. Glioblastoma-derived extracellular vesicles modify the phenotype of monocytic cells. Int J Cancer. 2015;137(7):1630-42.
19. Naryzhny S, Volnitskiy A, Kopylov A, Zorina E, Kamyshinsky R, Bairamukov V, et al. Proteome of Glioblastoma-Derived Exosomes as a Source of Biomarkers. Biomedicines. 2020;8(7).
20. Bagley SJ, Nabavizadeh SA, Mays JJ, Till JE, Ware JB, Levy S, et al. Clinical Utility of Plasma Cell-Free DNA in Adult Patients with Newly Diagnosed Glioblastoma: A Pilot Prospective Study. Clin Cancer Res. 2020;26(2):397-407.

21. Fraser K, Jo A, Giedt J, Vinegoni C, Yang KS, Peruzzi P, et al. Characterization of single microvesicles in plasma from glioblastoma patients. Neuro Oncol. 2019;21(5):606-15.
22. Cenciarini M, Valentino M, Belia S, Sforna L, Rosa P, Ronchetti S, et al. Dexamethasone in Glioblastoma Multiforme Therapy: Mechanisms and Controversies. Front Mol Neurosci. 2019;12:65.
23. Lee SY. Temozolomide resistance in glioblastoma multiforme. Genes Dis. 2016;3(3):198-210.
24. Rosa P, Catacuzzeno L, Sforna L, Mangino G, Carlomagno S, Mincione G, et al. BK channels blockage inhibits hypoxia-induced migration and chemoresistance to cisplatin in human glioblastoma cells. J Cell Physiol. 2018;233(9):6866-77.
25. Noch EK, Ramakrishna R, Magge R. Challenges in the Treatment of Glioblastoma: Multisystem Mechanisms of Therapeutic Resistance. World Neurosurg. 2018;116:505-17.
26. Whitehead CA, Kaye AH, Drummond KJ, Widodo SS, Mantamadiotis T, Vella LJ, et al. Extracellular vesicles and their role in glioblastoma. Crit Rev Clin Lab Sci. 2019:1-26.
27. Salvianti F, Gelmini S, Costanza F, Mancini I, Sonnati G, Simi L, et al. The pre-analytical phase of the liquid biopsy. N Biotechnol. 2020;55:19-29.
28. Piazza A, Rosa P, Ricciardi L, Mangraviti A, Pacini L, Calogero A, et al. Circulating Exosomal-DNA in Glioma Patients: A Quantitative Study and Histopathological Correlations-A Preliminary Study. Brain Sci. 2022;12(4).
29. Del Bene M, Osti D, Faletti S, Beznoussenko GV, DiMeco F, Pelicci G. Extracellular vesicles: The key for precision medicine in glioblastoma. Neuro Oncol. 2022;24(2):184-96.
30. Sabbagh Q, Andre-Gregoire G, Guevel L, Gavard J. Vesiclemia: counting on extracellular vesicles for glioblastoma patients. Oncogene. 2020;39(38):6043-52.
31. Maire CL, Fuh MM, Kaulich K, Fita KD, Stevic I, Heiland DH, et al. Genome-wide methylation profiling of glioblastoma cell-derived extracellular vesicle DNA allows tumor classification. Neuro Oncol. 2021;23(7):1087-99.
32. Santiago-Dieppa DR, Steinberg J, Gonda D, Cheung VJ, Carter BS, Chen CC. Extracellular vesicles as a platform for 'liquid biopsy' in glioblastoma patients. Expert Rev Mol Diagn. 2014;14(7):819-25.
33. Fernando MR, Jiang C, Krzyzanowski GD, Ryan WL. New evidence that a large proportion of human blood plasma cell-free DNA is localized in exosomes. PLoS One. 2017;12(8):e0183915.
34. Torrisi F, Alberghina C, D'Aprile S, Pavone AM, Longhitano L, Giallongo S, et al. The Hallmarks of Glioblastoma: Heterogeneity, Intercellular Crosstalk and Molecular Signature of Invasiveness and Progression. Biomedicines. 2022;10(4).
35. Patel AP, Tirosh I, Trombetta JJ, Shalek AK, Gillespie SM, Wakimoto H, et al. Single-cell RNA-seq highlights intratumoral heterogeneity in primary glioblastoma. Science. 2014;344(6190):1396-401.
36. Garcia-Romero N, Carrion-Navarro J, Esteban-Rubio S, Lazaro-Ibanez E, Peris-Celda M, Alonso MM, et al. DNA sequences within glioma-derived extracellular vesicles can cross the intact blood-brain barrier and be detected in peripheral blood of patients. Oncotarget. 2017;8(1):1416-28.
37. Degli Esposti C, Iadarola B, Maestri S, Beltrami C, Lavezzari D, Morini M, et al. Exosomes from Plasma of Neuroblastoma Patients Contain Doublestranded DNA Reflecting the Mutational Status of Parental Tumor Cells. Int J Mol Sci. 2021;22(7).
38. Yan H, Parsons DW, Jin G, McLendon R, Rasheed BA, Yuan W, et al. IDH1 and IDH2 mutations in gliomas. N Engl J Med. 2009;360(8):765-73.
39. Liu J, Zhang X, Yan X, Sun M, Fan Y, Huang Y. Significance of TERT and ATRX mutations in glioma. Oncol Lett. 2019;17(1):95-102.
40. Marques C, Unterkircher T, Kroon P, Oldrini B, Izzo A, Dramaretska Y, et al. NF1 regulates mesenchymal glioblastoma plasticity and aggressiveness through the AP-1 transcription factor FOSL1. Elife. 2021;10.
41. Reynes G, Vila V, Fleitas T, Reganon E, Font de Mora J, Jorda M, et al. Circulating endothelial cells and procoagulant microparticles in patients with glioblastoma: prognostic value. PLoS One. 2013;8(7):e69034.
42. Zeng A, Wei Z, Rabinovsky R, Jun HJ, El Fatimy R, Deforzh E, et al. Glioblastoma-Derived Extracellular Vesicles Facilitate Transformation of Astrocytes via Reprogramming Oncogenic Metabolism. iScience. 2020;23(8):101420.
43. Shao H, Chung J, Balaj L, Charest A, Bigner DD, Carter BS, et al. Protein typing of circulating microvesicles allows real-time monitoring of glioblastoma therapy. Nat Med. 2012;18(12):1835-40.