Oncogenic extrachromosomal DNA identification using whole-genome sequencing from formalin-fixed glioblastomas

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Glioblastoma, an area of urgent unmet clinical need, is the most common intrinsic brain cancer and frequently contains extrachromosomal DNA (ecDNA). EcDNA is increasingly recognized as a driver of cancer evolution and a mediator of therapy resistance[1,2]. Recent studies have implicated ecDNA as a prognostic marker across many cancer types, and a driver of glioblastoma, with emerging clinical trials aiming to leverage knowledge of ecDNA status or therapeutically target ecDNA directly[3].

Current methods to detect copy number amplifications for oncogenes in clinical practice do not distinguish between intrachromosomal and extrachromosomal DNA. Although ecDNA is known to carry oncogenic drivers of glioblastomas (many including *EGFR* are direct targets of drugs in clinical trials) and cause drug resistance by increasing intratumoural heterogeneity, identification of ecDNA is seldom performed for stratification of patients into trials[4] due to lack of clinically accessible detection tools.

EcDNA has a unique and highly variable circular structure, reaching megabase-pair size. Targeted inhibition of intrachromosomal *EGFR* amplification is more effective than that of *EGFR* ecDNA in preclinical models, highlighting the importance of understanding the nature of oncogene amplifications[5]. The lack of optimal molecular stratification has been identified

as a reason for clinical trial failure[6], prompting the need for improved methods of ecDNA detection at clinical scale. One barrier to achieving this is the frequent lack of fresh frozen tumor material as a source of DNA for whole genome sequencing (WGS) which, in addition to the time and costs associated with WGS, limits widespread adoption of WGS for ecDNA identification and patient stratification in the clinic and hampers research efforts by precluding use of vast archival tissue banks. Moreover, DNA from formalin-fixed, paraffin-embedded (FFPE) tissue is generally of lower quality than from frozen tissue, given the detrimental effects of formalin fixation on DNA quality and quantity. The current gold standard for detecting focal copy number amplification is DNA fluorescence in-situ hybridization (FISH); however, routine assessment of FISH analyses of clinical tumour samples, in which cells are primarily in interphase, cannot always distinguish between intrachromosomal amplifications or amplifications on ecDNA, or determine genomic contents of focal amplifications in detail.

Here, we demonstrate that low coverage whole genome sequencing enables characterisation of the copy number, content and structure of oncogenic ecDNA from DNA extracted from FFPE samples routinely collected in clinical practice. We investigated *IDH1*-wildtype glioblastoma FFPE tissues obtained from surgical resections in 5 newly-diagnosed patients, Figure 1A. The *EGFR* amplification is diagnostic of *IDH1*-wildtype glioblastoma, yet its ecDNA status is not yet determined in practice. We performed DNA interphase FISH with probes for *EGFR* and centromere of chromosome 7, and confirmed high-level *EGFR* amplification, with a pattern of *EGFR* foci consistent with ecDNA in three glioblastoma patients' samples, Figure 1B. To elucidate the structures of these amplifications, we performed whole-genome sequencing using 15X coverage, 150 base-pair length, paired end reads on an Illumina platform. A single FFPE 20μm section was used for DNA extraction using a commercially available kit (yielding >

400ng of DNA). We analysed the WGS data and performed copy number calling with the tool CNVkit to identify seed regions of focal amplifications[7]. We then applied the AmpliconArchitect[8] method to these seed regions for identifying ecDNA and other complex focal amplifications. This confirmed the presence of ecDNA, the size of the ecDNA amplicons (0.49Mb, 1.04Mb and 1.92Mb), and identified the presence of oncogenic *EGFR* on circular ecDNA at high copy number (CN) in these three glioblastomas (median CN = 185.6, 35.0, 131.0), Figure 1C. In glioblastoma from patient 2, high-copy ecDNA containing the *EGFRvIII* variant (a clinically relevant activating mutation) was detected by identifying structural variants between *EGFR* introns 1 and 7 in the circular amplicon; notably, this glioblastoma harboured a mixed population of *EGFR* wt and *EGFRvIII* ecDNAs (total CN > 200), suggesting multiple *EGFR* driver events, Figure 1D.

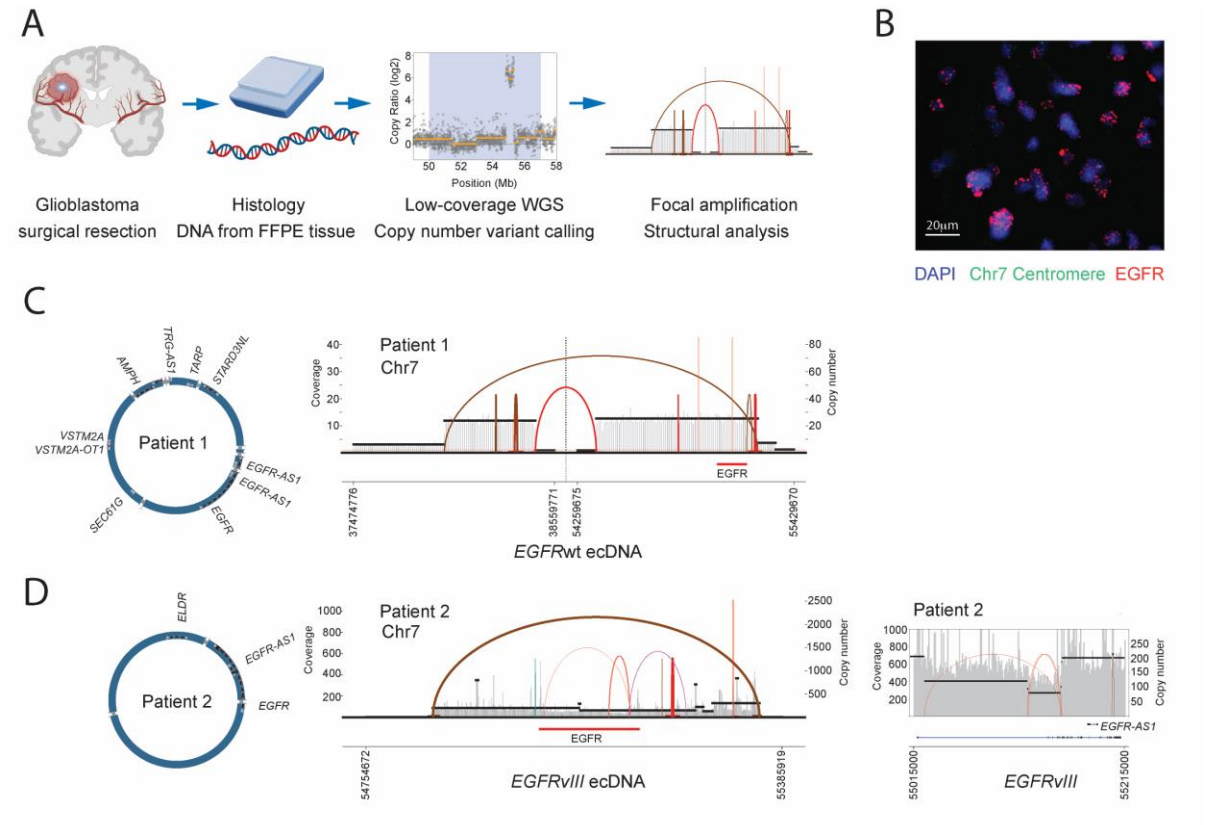

Figure 1. Oncogenic extrachromosomal DNA identification from routinely collected clinical glioblastoma samples. A. Outline of the workflow showing WGS analysis from FFPE tissues.

B. DNA FISH from a glioblastoma showing high-level focal amplification of *EGFR*, consistent with ecDNA. C. Left: The circular structure and gene contents of the ecDNA from the same glioblastoma in B. Right: The structure of this ecDNA from sample shown in panel B, identifying *EGFR* within the ecDNA and showing the breakpoints on chromosome 7. Left y-axis represents sequencing coverage, and right y-axis shows copy number, CN. D. Circular and breakpoint structure of ecDNA from glioblastoma of patient 2, showing copy number loss at exons 2-7 of *EGFR*, consistent with subclonal *EGFRvIII* ecDNAs; right-most panel shows the *EGFR* structural variants within this ecDNA.

This method can identify whether the amplification is derived from an intrachromosomal or extrachromosomal mechanism. For example, glioblastoma WGS data from patient 4 identified an intrachromosomal non-ecDNA amplification of *PDGFRA* and *KIT* oncogenes on chromosome 4 (CN = 3.4). WGS analysis of glioblastoma from patient 5 revealed an ecDNA-amplification containing the oncogene *MET* (CN = 8.4). Given ongoing clinical trials with MET inhibitors[9] such as tepotinib, this method should prove useful for stratifying patients based on the presence and structure of *MET*-amplifications.

We have demonstrated the feasibility of identifying ecDNA, a driver of treatment resistance, using WGS from FFPE tumour specimens collected in routine clinical practice. This approach may be particularly useful in cases where small biopsies are performed and frozen tissue is lacking. The low-depth of WGS also ensures reduced costs and can be performed with commercial sequencing services in under two weeks. Recent efforts have demonstrated FFPE-DNA can be used for mutational signature analysis[10]. We anticipate that copy number alteration, structural variant and ecDNA characterization with WGS of DNA from clinical

samples can be readily deployed to other cancer types, which is important given that oncogenic ecDNA is present across many solid tumours. This may open avenues for use of large archival tissue banks for cancer research, as well as for rapid molecular profiling of patients' tumours for optimal trial stratification based on copy number amplification with and without ecDNA.

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Competing Interests

C.S. acknowledges grant support from AstraZeneca, Boehringer-Ingelheim, BMS, Pfizer, Roche-Ventana, Invitae (previously Archer Dx (collaboration in minimal residual disease sequencing technologies)) and Ono Pharmaceutical. C.S. is an AstraZeneca Advisory Board member and Chief Investigator for the AZ MeRmaiD 1 and 2 clinical trials and is also Co-Chief Investigator of the NHS Galleri trial funded by GRAIL and a paid member of GRAIL's SAB. He receives consultant fees from Achilles Therapeutics (also a SABmember), Bicycle Therapeutics (also aSAB member), Genentech, Medicxi, Roche Innovation Centre–Shanghai, Metabomed (until July 2022), and the Sarah Cannon Research Institute. C.S. had stock options in Apogen Biotechnologies and GRAIL until June 2021, and currently has stock options in Epic Bioscience, Bicycle Therapeutics, and has stock options and is co-founder of Achilles Therapeutics. C.S. is an inventor on a European patent application relating to an assay technology to detect tumour recurrence(PCT/ GB2017/053289), the patent has been licensed to commercial entities and under his terms of employment, C.S. is due a revenue share of any revenue generated from such licence(s). C.S. holds patents relating to targeting neoantigens(PCT/EP2016/059401), identifying patient responses to immune checkpoint blockade(PCT/EP2016/071471), determining HLA LOH (PCT/ GB2018/052004), predicting survival rates of patients with cancer (PCT/GB2020/050221), identifying patients who respond to cancer treatment (PCT/GB2018/051912), a US patent relating to detecting tumour mutations (PCT/US2017/28013), methods for lung cancer detection(US20190106751A1) and both a European and US patent related to identifying indel mutation targets (PCT/GB2018/051892) and is a co-inventor on a patent application to determine methods and systems for tumour monitoring (PCT/EP2022/077987). C.S. is a named inventor on a provisional patent related to a ctDNA detection algorithm

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eDyNAmiC team lead and V.B. is a member of the eDyNAmiC team. Work supervised by V.B. was also funded by National Institutes of Health (NIH) grant R01-GM114362.

P.S.M., V.B., and J.L. are named co-inventors on patent application 'Methods and compositions for detecting ecDNA' (US patent application number 17/746,748).

Figure

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