Measurement of Plasma Cell-Free Mitochondrial Tumor DNA Improves Detection of Glioblastoma in Patient-Derived Orthotopic Xenograft Models

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Abstract

The factors responsible for the low detection rate of cell-free tumor DNA (ctDNA) in the plasma of patients with glioblastoma (GBM) are currently unknown. In this study, we measured circulating nucleic acids in patient-derived orthotopically implanted xenograft (PDOX) models of GBM (n = 64) and show that tumor size and cell proliferation, but not the integrity of the blood–brain barrier or cell death, affect the release of ctDNA in treatment-naïve GBM PDOX. Analysis of fragment length profiles by shallow genome-wide sequencing (<0.2× coverage) of host (rat) and tumor (human) circulating DNA identified a peak at 145 bp in the human DNA fragments, indicating a difference in the origin or processing of the ctDNA. The concentration of ctDNA correlated with cell death only after treatment with temozolomide and radiotherapy. Digital PCR detection of plasma tumor mitochondrial DNA (tmtDNA), an alternative to detection of nuclear ctDNA, improved plasma DNA detection rate (82% vs. 24%) and allowed detection in cerebrospinal fluid and urine. Mitochondrial mutations are prevalent across all cancers and can be detected with high sensitivity, at low cost, and without prior knowledge of tumor mutations via capture-panel sequencing. Coupled with the observation that mitochondrial copy number increases in glioma, these data suggest analyzing tmtDNA as a more sensitive method to detect and monitor tumor burden in cancer, specifically in GBM, where current methods have largely failed.

Significance: These findings show that detection of tumor mitochondrial DNA is more sensitive than circulating tumor DNA analysis to detect and monitor tumor burden in patient-derived orthotopic xenografts of glioblastoma.

Introduction

Release of DNA fragments from solid tumors, which can be collected in body fluids and used to identify and quantify tumor mutations, has created new possibilities for minimally invasive diagnosis and therapy monitoring (1, 2). The concentration of cell-free tumor DNA (ctDNA) varies with cancer type, with some, such as glioblastoma (GBM), showing extremely low plasma concentrations (3), which has hindered clinical translation. Although ctDNA levels have been correlated with tumor burden (2, 4), an understanding of the relationship between tumor biology and the release of ctDNA into the circulation is lacking, most notably for GBM. Detection and measurement of ctDNA may be affected by both technical and biological factors (1, 5). Recent work has related necrosis, tumor volume, and proliferation to detection of ctDNA in patients with non–small cell lung cancer (2). However, no investigation of the effect of tumor biology on ctDNA release in GBM has been performed.

Using a large cohort of patient-derived orthotopically implanted xenografts (PDOX; n = 64), we investigated combined detection of circulating tumor mitochondrial DNA (tmtDNA) and ctDNA. Custom digital PCR (dPCR) was used to differentiate human mitochondrial DNA, originating from grafted tumor cells, from the host rat mitochondrial DNA. We demonstrated a higher frequency of detection and higher copy number for tmtDNA when compared with ctDNA in the plasma, cerebrospinal fluid (CSF), and urine of the xenografted rats. We used this improved yield to analyze the factors affecting tumor DNA release.

Release of ctDNA and tmtDNA in treatment-naïve GBM was associated with tumor volume and cell proliferation but not cell death. However, following treatment with temozolomide and radiotherapy (6), plasma tmtDNA was correlated with the levels of tumor cell death. Finally, bypassing blood–brain...
barrier (BBB) integrity did not significantly affect the yield of ctDNA or tmtDNA.

**Materials and Methods**

**Cell culture**

Cells were obtained either locally or from the American Type Culture Collection (ATCC) and mycoplasma tested using RNA-capture ELISA. Cell line authentication was performed using short tandem repeat genotyping contemporaneously with the experiments. U87 cells (ATCC) were cultured in DMEM, 2 mmol/L l-glutamine (Gibco), and 10% FBS (Gibco). Patient-derived cell lines were derived using protocols compliant with the UK Human Tissue Act 2004 (HTA licence ref. 12315), approved by the Local Regional Ethics Committee (LREC ref. 04/Q0108/60), and in accordance with the Declaration of Helsinki. GBM tissue was filtered (40 μm; Falcon) and washed with red blood cell lysis buffer. Live cells were seeded at 1.5 × 10^4 cm⁻² and grown as monolayer cultures on extracellular matrix coatings. L-glutamine (Gibco), and 10% FBS (Gibco). Patient-derived cell lines were derived using protocols compliant with the UK Human Tissue Act 2004 (HTA licence ref. 12315), approved by the Local Regional Ethics Committee (LREC ref. 04/Q0108/60), and in accordance with the Declaration of Helsinki. GBM tissue was minced, and cells were filtered (40 μm; Falcon) and washed with red blood cell lysis buffer. Live cells were seeded at 1.5 × 10^4 cm⁻² and grown as monolayer cultures on extracellular matrix-coated flasks (Engelbreth-Holm-Swarm murine sarcoma—1:10 dilution, Sigma) in Neurobasal A (Gibco), 2 mmol/L l-glutamine (Sigma), 1% streptomycin/penicillin/amphotericin B (Invitrogen), 20 ng/mL hEGF (Sigma), 20 ng/mL hFGF (R&D Systems), 2% B27 (Invitrogen), and 1% N2 (Invitrogen) at 37.5°C in 5% CO₂.

**Orthotopic tumor model**

Procedures were performed in compliance with project and personal licenses issued under the United Kingdom Animals (Scientific Procedures) Act, 1986, and approved by the local Animal Welfare and Ethical Review Board. Patient-derived cells, below passage 20, were resuspended at 2.5 × 10⁶ cells L⁻¹, and 5 μL were implanted stereotactically (2 mm anterior and 3 mm lateral to the bregma, right side) in 6 week-old female nu/nu athymic nude rats (Charles River; Harlan; n = 64).

**Subcutaneous tumor model**

Patient-derived cells (GBM4) were resuspended at 2.5 × 10⁶ cells L⁻¹, and 200 μL were injected subcutaneously into the right flank of 6 athymic nude rats.

**Sample collection**

Whole blood was taken via tail vein cannulation or peripherally by cardiac puncture. Coagulation was inhibited by adding 4.5 mmol/L EDTA to a maximum of 10 mL of blood. CSF was collected peri-mortem via cisterna magna puncture (7) and urine by direct bladder cannulation. Samples were centrifuged (4°C, 1,500 × g for 10 minutes then 20,000 × g for 10 minutes) before freezing (~80°C).

**DNA extraction**

DNA from plasma (~1 mL), CSF (~100 μL), and urine (~100 μL) was extracted with the QIAamp Circulating Nucleic Acids Kit (QIAGEN) and elution volume of 50 μL. Fragments of the Xenopus Tropicalis genome were spiked into the samples to estimate DNA extraction efficiency ([Forward PCR primer: 5’-GTGATCATGGGATTTGTAGCTGTT 3’; Reverse PCR primer: 5’ AAACCAACTCGAACCATGGA-3’]).

**Western blot**

Cell or tissue samples were lysed in RIPA buffer with 1% protease inhibitor (Thermo Fisher), run on BIS-TRIS gels (Thermo Fisher) transferred onto nitrocellulose membranes, and incubated with nestin (Atlas, 1:100) and β-actin (Abcam; 1:5,000) antibodies in LI-COR Odyssey blocking buffer (LI-COR Biotechnology) overnight at 4°C. Primary antibodies were visualized using fluorescently-labeled anti-mouse or anti-rabbit LI-COR secondary antibodies and a LI-COR Odyssey CLx imaging system (LI-COR biotechnology).

**Chemoradiation**

Rats were anesthetized with 1% to 2% isoflurane (Isoflo, Abbotts Laboratories Ltd.), and tumors were irradiated via a lead collimator [15 Gy; Cs-137 irradiator (IBL 637; CIS Bio International)]. Temozolomide (100 mg kg⁻¹) was given by oral gavage 1 hour prior to radiotherapy.

**Histopathology and immunohistochemistry**

Brains were placed in 10% formalin (Sigma-Aldrich) for 24 hours, and then sectioned. Hematoxylin and eosin staining ([St020 Multistainer; Leica Microsystems] was performed on 5 μm sections. TUNEL staining and IHC were performed on 10 μm sections. TUNEL staining used Leica’s Polymer Kit (Leica Microsystems) and Promega’s DeadEnd Colorimetric TUNEL System (Promega). IHC was performed using Leica’s Polymer Refine Kit and human-specific antibodies: Ki67 (1:200 dilution; M7240; Dako), cleaved caspase 3 (CC3; 1:200 dilution; 9664; Cell Signaling Technology), Glial Filibrillary Acid Protein (GFAP; 1:10,000 dilution; Z0334; Dako), and Carboxy Anhydrase 9 (CAIX; 1:1,000 dilution; AB1001; BioScience, Slovakia).

**In situ hybridization**

**Pecam1** (CD31) mRNA was detected on 5 μm formalin-fixed paraffin-embedded tissue sections with a probe for rat Pecam1 (NM_031591.1, region 861–1766; RNAscope 2.5 LS red detection kit, 322150, Advanced Cell Diagnostics) on a Leica Bond Rx (Leica Biosystems). Hybridization was detected using the Bond Polymer Refine Red detection Kit (Leica Biosystems, DS9390) followed by counterstaining with hematoxylin. Probes targeting peptidylprolyl isomerase B (PPIB) (NM_022536.2, region 95–830) and Dabp (EF191515, region 414–86) were used as positive and negative controls, respectively.

**Image analysis**

Images were annotated manually and analyzed using in-house algorithms (Aperio, Leica)

**Digital PCR**

dPCR was performed using Fluidigm 12.765 and 37k dPCR chips (Fluidigm). For targeting human nuclear DNA, 5 μL of TaqMan Gene Expression Master Mix, 0.5 μL of buffer, 0.5 μL of EVAGREEN (Biotium), and 1 μL of 10 μmol/L forward primer (5’TACACTCAAGCGCGCTCAACTAC-3’; Invitrogen) and 10 μmol/L reverse primer (5’TCCGCTATTCGTTATGACC-3’; Invitrogen) were mixed with 3.5 μL of DNA. Primers for identifying human mitochondrial DNA were forward 5’-ATACC- CATTGGCAACCTCCT-3’ and reverse 5’-GGCCCTTGCGTAGTGTT-3’. Primers for identifying rat DNA were forward 5’- CCACCCCTGGGCCTGTGTT-3’ and reverse 5’-CCTGCGATCCCTGGCTGAGA-3’. Assays for human DNA (ctDNA) and rat DNA [ectoplasm-free DNA (ntDNA)] targeted the human (RPP30 gene) and rat (RPP30 gene) sequences, respectively, in copy-
number–neutral regions where there was no homology with the reciprocal rat and human genomes.

**Shallow whole-genome sequencing**

Libraries were prepared using an NEB ultra v2 kit (New England Biolabs). Ten ng of tumor tissue DNA was sheared to 150 to 200 bp with an ultrasonic sonicator (Covaris). For plasma and CSF samples, we selected rats with concentrations of ctDNA greater than 1,000 copies/mL, as determined by dPCR. Libraries were pooled in equimolar amounts and sequenced on a HiSeq 2500 (Illumina) generating 125 bp paired-end reads. Reads were aligned, and localization of somatic copy-number aberrations was estimated by QDNAseq (8).

**Magnetic resonance imaging**

We used a 7T spectrometer (Agilent) and a 72 mm inner-diameter 1H quadrature birdcage coil (Rapid Biomedical GMBH). Animals were anesthetized with 1% to 2% isoflurane in O2. Axial T1-weighted images were acquired using a fast spin-echo sequence [TR 1.5 seconds, TE 40 ms, 256 × 256 data points over a 40 × 40 mm field-of-view (FOV)], 4–8 averages] from 15 2-mm-thick slices. A T1-weighted spoiled gradient echo sequence (27° flip angle, TR 43 ms, TE 4.6 ms, FOV40 mm × 40 mm, 256 × 128 data points) was used to acquire images before and after injection of contrast agent (100 µmol/kg Dotarem; Guerbert). Five axial slices, 1.5 mm thick and with a 0.3 mm gap between them, were acquired. Images were transferred to MATLAB (Mathworks), and difference maps calculated, on a voxel-by-voxel basis, as the postcontrast image minus the precontrast image divided by the precontrast image.

**Disruption of the BBB**

Mannitol (2.5 mL of a 25% solution in 0.9% saline) was administered via a tail vein cannula. Rats immediately underwent diuresis, evident from urinary incontinence under anesthesia.

**Demonstration of BBB opening using dynamic contrast–enhanced magnetic resonance imaging**

Images were acquired using the 72 mm diameter 1H transmit coil and a 2-channel rat-head 1H receive coil placed over the brain. A fast spin-echo sequence (TR 2 seconds, TE 48 ms, FOV 4 cm × 4 cm, 2 mm thick slice, 256 × 256 data points) was used to acquire 4 axial brain slices from the same region where tumors were implanted in the other animals. Baseline T1 measurements used an inversion recovery-spoiled gradient echo sequence (adiabatic inversion pulse, 8 inversion times between 0.05 and 10 seconds, scan repeat time 12 seconds, TR 2.08 ms, TE 0.92 ms, flip angle 10°, 4 × 1.8 mm thick slices with a 0.2 mm gap between slices). Dynamic contrast–enhanced (DCE) images were acquired using a gradient echo sequence (TR 25 ms, TE 2.85 ms, flip angle 30°). A series of 100 images (2 averages, 6.4 seconds per set of 4 images) were acquired. Dotarem (0.2 mmol/kg; Gadoteric acid, Guerbet) was injected via a tail vein after the 10th image. Mannitol was administered immediately prior to the start of DCE image acquisition. Signals from the DCE time course were converted, on a pixel-by-pixel basis, to a contrast-agent concentration by assuming an R1 relaxivity for Dotarem of 3.1 s⁻¹ mmol/L⁻¹ (9). An elliptical region of interest was drawn in each of the four slices, covering the thalamus to the prefrontal cortex, and an average DCE profile was calculated (10) using the same population-derived double-exponential arterial-input-function for each data-set (11). The calculated extravascular and extracellular spaces per unit volume of tissue (Ve) accessible to the contrast agent were used as an indicator of BBB permeability.

**Statistical analysis**

Statistics were performed using GraphPad Prism (GraphPad Software Inc.) and R (www.r-project.org). Principal Component Analysis (PCA) was performed with R using the factoextra package.

**Results**

**tmtDNA is a more sensitive marker of systemic tumor nucleic acids than ctDNA and is detected in multiple body fluids**

There are 10⁷ to 10⁹ copies of the 16.5 kb mitochondrial genome per human cancer cell (12), and therefore, tmtDNA released into the circulation may be a more sensitive marker of tumor burden than ctDNA (13). We used dPCR to investigate the levels of tmtDNA and ctDNA in different rat PDOX models of GBM, which were derived from tumor material taken from different patients with GBM. The selected dPCR assays were chosen from among 9 dPCR assays. Specificity for human (in the PDOX models, this represents tumor DNA) and rat (host) DNA was determined using plasma DNA from 4 healthy human individuals and 4 nongrafted rat controls (Fig. 1A). Human nuclear DNA levels averaged 7,469 copies/mL, and human mitochondrial DNA averaged 38,091 copies/mL in the human plasma samples, where copies/mL represents the number of amplifiable copies in the dPCR reaction. Rat nuclear DNA was not detected in the human plasma, and human nuclear DNA was not detected in rat plasma, despite a high concentration of rat nuclear DNA (15,610 copies/mL). Only very low amounts of human mitochondrial DNA (mean 3 copies/mL, <0.02%) were detected in rat plasma.

The sensitivity of our selected ctDNA and tmtDNA assays was determined with a duplicate dilution series of human DNA in rat plasma DNA. The tmtDNA assay could detect the presence of human DNA at dilution levels 100x greater than the ctDNA assay, and could detect the presence of human mitochondrial DNA even when human nuclear DNA could no longer be detected (Fig. 1B).

Six representative PDOX models of GBM (GBM1, 8 rats; GBM2, 8 rats; GBM3, 3 rats; GBM4, 36 rats; GBM5, 6 rats; and GBM6, 3 rats) were studied. In total, 64 animals were analyzed using the dPCR assay. As shown previously (14), these models showed much slower growth rates than tumors arising from implantation of a GBM cell line (U87; Supplementary Fig. S1A and Supplementary Table S1) and much higher levels of expression of glioblastoma acidic protein in vivo (Supplementary Fig. S1B; ref. 15) and nestin, a neural stem cell marker, in vitro (16), which were largely absent from U87 tumors and cells, respectively (Supplementary Fig S1C). All showed histologic features of GBM (Supplementary Fig. S1D).

Plasma ctDNA was detected in all but one cohort (GBM1), with a detection rate of 24% across all animals (15/64) and at an average concentration of 27 tumor haploid genome equivalents per mL (copies/mL of the targeted human sequence; Fig. 2A and B). Plasma tmtDNA was identified in all the PDOX cohorts with a detection rate of 82% (52/64) and an average concentration of 5,081 copies/mL (~190-fold higher than the mean value for ctDNA; Fig. 2A and B). Nontumor (rat host) cell-free nuclear DNA was detected in all the animals at considerably higher
Figure 1.
Validation of the specificity and sensitivity of the ctDNA and tmtDNA dPCR assays. A, dPCR assays designed to detect ctDNA, tmtDNA, and nt cfDNA were tested with human and rat plasma DNA. Samples were tested in quadruplicate for each assay. ND, nondetectable. B, Dilution series of human (tumor) DNA in rat (nontumor) DNA, which was used to evaluate the sensitivity of tmtDNA detection in comparison with detection of ctDNA. tmtDNA was detected at 100× greater dilution than ctDNA. Each sample was measured in duplicate.

Figure 2.
tmtDNA was detected more frequently in plasma than ctDNA. A, Detection rates for plasma circulating ctDNA, tmtDNA, and nt cfDNA in 64 animals implanted orthotopically with cells derived from tumors from 6 different patients. B, Concentration (copies/mL) of circulating nucleic acids in the samples where these were detected. ctDNA (C) and tmtDNA concentrations (D) in CSF, plasma, and urine from the tumor-bearing animals. In the ratios shown below the plots in C and D, the numerator represents the number of samples containing the indicated DNA and the denominator the number of samples.
Figure 3.
Factors affecting the levels of ctDNA and tmtDNA in the plasma of treatment-naive tumor-bearing rats. PCA of variables associated with tumor histology and circulating nucleic acids in the plasma of rats with GBM4 tumors (n = 36). The vectors represent ctDNA, tmtDNA, and nontumor cfDNA concentrations, tumor volume, tumor proliferation (Ki67), necrosis (TUNEL), and apoptosis (CC3). PC1 (44.9%) and PC2 (25.1%) indicate the % variance accounted for by the two principal components.

cDNA and tmtDNA levels correlate with cell death following treatment with temozolomide and radiotherapy
In GBM4 (n = 36), tmtDNA and ctDNA were highly correlated with tumor volume (R² = 0.8; P ≤ 0.0001; Fig. 4A), suggesting that tmtDNA, like ctDNA, could be used to track tumor burden and monitor treatment response.

We analyzed plasma from GBM4 PDX models 72 hours after treatment with temozolomide plus radiotherapy (15 Gy, n = 7). ctDNA detection frequency and concentration increased (from 40%, 7 copies/mL to 75%, 54 copies/mL, P = 0.051; Fig. 4B). tmtDNA concentration also increased (from a median 121 copies/mL to 256 copies/mL, P = 0.094; Fig. 4B), but detection frequency remained unchanged (6/7 cases). These increases in ctDNA and tmtDNA concentrations were associated with an increase in tumor cell death, as assessed by TUNEL (P = 0.039; Fig. 4C) and CC3 staining (P = 0.037) of tumor sections (n = 7; Fig. 4D), with a correlation being observed between ctDNA and CC3 staining (R² = 0.58, P = 0.074, Pearson analysis), which is a marker of early apoptosis (18).

These data indicate that in treatment-naive models, tumor DNA release was related to tumor burden and cell proliferation, whereas following treatment, tumor DNA was released primarily through tumor cell death.

Genome-wide sequencing showed a different fragmentation pattern for ctDNA and host DNA in treatment-naive PDXs
We used genome-wide sequencing at low coverage (<0.2×) to determine copy-number profiles of host rat and human (tumor) nuclear genomes in plasma, CSF, and tumor tissue. Paired-end sequencing reads were aligned to rat (RGSC 6.0/rn6) and human (hg19) genomes, and assigned to the appropriate species (Fig. 5A). Similar copy-number profiles were found in tumor DNA from the different fluid compartments and from tumor tissue (Fig. 5B), even though the plasma compartment exhibited a lower tumor DNA fraction, relative to host DNA, when compared with tumor tissue and CSF. We also determined the size distribution of human (tumor) and rat (host) circulating nuclear DNA fragments (Fig. 5C–E) in the plasma of animals grafted with GBM6 (Fig. 5C) and with GBM4 (Fig. 5D). We also determined, for one animal implanted with GBM4, the size distribution of the DNA fragments from CSF (Fig. 5E). The fragment size distribution in plasma and CSF showed a peak at 133–145 bp for human (tumor) DNA, and a different fragmentation pattern for host rat DNA, with a peak at 167 bp (Fig. 5C and D). Mitochondrial DNA showed a peak below 100 bp for both human (tumor) and rat circulating mitochondrial DNA (Fig. 5F), in agreement with previous work (19).

The BBB has a limited effect on plasma ctDNA and tmtDNA concentrations
Despite extensive disruption of the BBB during gliomagenesis (20), the low levels of ctDNA observed in the plasma of patients with GBM and the apparent enrichment of tumor DNA in the CSF have been attributed to the impermeability of the BBB (3). This was supported by sequencing, where tumor mutations in DNA concentrations than ctDNA (t test; P < 0.001) with a mean concentration of 6,393 copies/mL (Fig. 2A and B). Variable detection rates were observed between the different PDX models, with tmtDNA detected in 66% of some models (GBM1; n = 8) and 100% in others (GBM5; n = 6; Supplementary Fig. S2A). ctDNA and tmtDNA were not detected in plasma from nongrafted animals (n = 4; Supplementary Fig. S2B).

cDNA has been detected at low concentrations in urine from patients with nonbrain tumors (17). Urine samples from 11 tumor-bearing animals (10 GBM4 and 1 GBM5) had undetectable levels of ctDNA. However, tmtDNA was identified in 60% of samples with a median concentration of 660 copies/mL (Fig. 2C and D). The CSF presents another possible source of cell-free DNA and tmtDNA was detected in all samples (median concentration of 760 copies/mL; Fig. 2C and D). Rat host cell-free nuclear DNA was detected in all samples with a median concentration of 215 copies/mL.

cDNA and tmtDNA levels correlate with tumor size and cell proliferation in treatment-naive PDXs
We performed PCA on 8 tumor-related variables in treatment-naive GBM4 models (n = 36). The first component included plasma ctDNA and tmtDNA concentrations, tumor volume, and Ki67 staining, a marker of cell proliferation, and the second component included plasma concentrations of host nontumor cell-free DNA (tmtDNA), staining for TUNEL and CC3, which are cell death markers, and carbonic anhydrase 9 (CAIX), a marker of hypoxia (Fig. 3). Correlations (Pearson analysis) were observed between tmtDNA and ctDNA (R² = 0.83, P < 0.001), tumor-derived DNA and tumor volume (tmtDNA R² = 0.86, P < 0.001; ctDNA R² = 0.83, P < 0.001), and tmtDNA and ctDNA and the number of proliferating cells (Ki67-positive cells; tmtDNA R² = 0.54, P < 0.001; ctDNA R² = 0.54, P < 0.001). We also observed a correlation between nt cfDNA and cell death (TUNEL R² = 0.62, P < 0.001 and CC3 R² = 0.47, P < 0.01). Tumor microvessel density was not significantly different between the different PDX models (P = 0.27; Supplementary Fig. S3).
from the CSF of patients with GBM were detected more frequently than in plasma and at higher mutant allele fractions (21, 22). However, the absolute concentrations of tumor and nontumor DNA in CSF and in plasma of patients with GBM have not been reported previously. The data shown in Fig. 2 show that the higher detection rate of tumor DNA in CSF is due to a higher concentration of ctDNA relative to host nt cfDNA in CSF (222 copies/mL ctDNA vs. 215 copies/mL nt cfDNA) when compared with plasma (27 copies/mL ctDNA vs. 6,989 copies/mL nt cfDNA). We investigated this further by using dPCR to quantify the concentrations of tmtDNA in plasma and CSF samples collected from 12 of the tumor models [GBM1 (n = 1), GBM2 (n = 1), GBM4 (n = 10)]. tmtDNA concentration was higher in CSF as compared with plasma in each of the tumor models (Fig. 6A), with a median of 476 copies/mL in CSF and 93 copies/mL in plasma. However, CSF volume in the rat is approximately 90 μL and the plasma volume is approximately 6 mL (23), and therefore, the total amount of tmtDNA in the plasma (558 copies) is approximately 13 times higher than in the CSF (43 copies), showing therefore that the BBB does not prevent significant amounts of tumor DNA, at least tmtDNA, from reaching the circulation. Whereas the concentration of tumor-derived ctDNA was 5 to 8 times higher in CSF compared with plasma, the concentration of nt cfDNA was nearly 25 times higher in plasma compared with CSF. Therefore, lower detection rates of tumor-derived DNA in plasma are due, at least in part, to the presence of higher levels of background host DNA in plasma.

To investigate more directly the effect of the BBB on plasma tmtDNA and ctDNA concentrations, we used subcutaneous implantation of GBM4 cells to generate a GBM model that was outside the BBB. We also disrupted the BBB by intravenous administration of mannitol (24). Following mannitol injection, 60 minutes were allowed for ctDNA to escape into the circulation before plasma collection. If the BBB blocks release of tumor DNA into the circulation, then 60 minutes after mannitol injection, there should be an increase in tumor DNA levels in the circulation,
Figure 5.
The fragmentation patterns of plasma DNA in tumor-bearing animals. A, Sequencing reads were obtained by paired-end shallow WGS of plasma DNA and aligned to the human (tumor) and rat (host) genomes. B, Copy-number profiles obtained from sWGS of DNA from tumor tissue, CSF, and plasma from the GBM4 model (n = 3), separated into reads that aligned with the human and rat genome. C, Size distribution of DNA fragments of nuclear origin from a plasma sample from the GBM6 model. ctDNA fragments originating from tumor cells, aligned to the human genome, are shown in red, whereas nt cfDNA fragments from host cells, aligned to the rat genome, are shown in blue. A vertical line (at 167 bp) indicates fragment sizes associated with nt cfDNA of apoptotic origin. D, Size distribution of the DNA fragments of nuclear origin from a plasma sample from a GBM4 tumor-bearing animal. E, Size distribution of the DNA fragments of nuclear origin from a CSF sample from a GBM4 tumor-bearing animal. F, Size distribution of mitochondrial DNA fragments from a plasma sample. tmtDNA originating from tumor cells, aligned to the human mitochondrial genome, are shown in purple, and nontumor mitochondrial DNA, aligned to the rat genome, are shown in green.
given that maximal BBB opening occurs 5 minutes following mannitol infusion (24), and the circulating DNA half-life is 16 minutes (25). Gadolinium-based contrast agents do not cross the intact BBB and are used commonly for MR imaging of BBB breakdown in GBM (26). We confirmed in 3 control rats that mannitol infusion caused BBB disruption using DCE MRI measurements. Within 10 minutes of mannitol administration, there was an increase in the fraction of tissue accessible to the contrast agent (\(P < 0.02\)) and in the contrast agent concentration [untreated, 6.3 \(\pm\) 4.0 \(\mu\)mol/L (SD); post mannitol, 14.8 \(\pm\) 1.8 \(\mu\)mol/L (SD), \(P < 0.025\) (one sided Welch \(t\) test; Supplementary Fig. S4A and S4B)]. There were no significant differences in the concentrations of ctDNA or tmtDNA between these groups (Fig. 6C); however, there was no significant difference in the ctDNA or tmtDNA levels (Fig. 6B), suggesting that release from the subcutaneous tumors was not affected by vascular density. Comparison of ctDNA and tmtDNA concentrations in contrast agent enhancing (GBM4, GBM3) and nonenhancing (GBM1, GBM5) tumors showed no differences in ctDNA (\(P = 0.65\)) or tmtDNA concentrations (\(P = 0.49\)) between these groups (Fig. 6D).

**Discussion**

Detection of ctDNA in patients with GBM is challenging because of low plasma concentrations (3). Sampling of CSF has been proposed as a method for detecting ctDNA in GBM (21, 22, 29); however, lumbar puncture is contraindicated in patients with intracerebral space occupying lesions, and thus routine use of this technique is not clinically feasible (30, 31). Nevertheless, the requirement for minimally invasive techniques that avoid...
repeated biopsies in patients with GBM remains due to current inadequacies in identifying response/escape (32) and the evolving nature of the disease during treatment (33–35). We therefore pursued methods to improve detection of circulating tumor-derived nucleic acids through the use of PDOX models of GBM and used these methods to identify factors affecting DNA release.

dPCR was used to estimate plasma tmtDNA and ctDNA concentrations in a large number of PDOX models of GBM. The detection rate for tmtDNA was 82% in plasma samples (n = 64), at an average concentration of 5,081 copies/mL, versus a detection rate for ctDNA of 24%, at an average concentration of 27 copies/mL. Host cell-free nuclear DNA concentrations have a broad range, and the values we report are within the range reported previously for animal models (27, 28). tmtDNA was also detected in 60% of urine samples in which ctDNA was undetectable. Because mtDNA is highly fragmented in plasma (Fig. 5F), in vitro or in silico size selection of fragments below 100 bp could be used to sieve tmtDNA from nuclear ctDNA, enriching the sample for tmtDNA and further enhancing the sensitivity of detection (36). The potential for tmtDNA to be used to detect smaller tumors, either at diagnosis or at recurrence, would be important clinically.

Using both ctDNA and tmtDNA, we investigated the factors influencing release of tumor-derived nucleic acids into the circulation. The levels of both were correlated with tumor size, in agreement with previous preclinical (27, 28) and clinical (3, 25) studies. Previous analyses of ctDNA fragment sizes in plasma showed these to be mostly distributed around 167 bp, and multiples thereof, characteristic of caspase-dependent cleavage and suggesting that the majority of ctDNA originates from apoptosis (37, 38). In patients with cancer, a shortening of ctDNA was observed (39, 40), which could reflect modifications in chromatin organization (41, 42). Recent work on fetal ctDNA suggested that methylation-related chromatin reorganization can result in shortening of fragment length (38, 43). The first comprehensive analysis of the relationship between tumor physiology and ctDNA in patients indicated that cell proliferation and tumor volume are more strongly correlated with ctDNA concentration than cell death (2). Here, we have shown, in treatment-naïve PDOX models, that there is a correlation between nontumor (host) ctDNA levels and cell death. Fragmentation analysis showed a distribution centered around 167 bp, consistent with release from apoptotic host cells. We observed a correlation between ctDNA levels and tumor volume, and to a lesser extent with cell proliferation, but not with cell death, as was observed previously (2). Analysis of ctDNA fragment sizes revealed a shift toward shorter fragment sizes, with a distribution centered around 145 bp, corresponding to the core nucleosome. These findings suggest that size selection could potentially be used to improve the yield of ctDNA fragments (36).

The concentrations of plasma ctDNA and tmtDNA were increased following temozolomide and radiotherapy treatment and, in this instance, were related to an increase in tumor cell death. However, CC3 and TUNEL staining only inform upon a proportion of dying cells and not those affected by mitotic catastrophe or senescence for example. In these treated animals, there was no longer any correlation between plasma levels of ctDNA and mtDNA and cell proliferation. Therefore, it appears that release of tumor DNA before and after treatment occurs via different processes. DNA release via cell death after treatment may be explained by the requirement for tumor cells to be in close proximity to viable blood vessels, which provide the oxygen necessary for radiotherapy-induced tumor cell kill (44). Thus, when these cells die, they do so in a vessel-rich microenvironment, and are distinct from dying tumor cells in treatment-naïve GBM, where cell death may occur predominantly in cells with a poor blood supply.

The BBB has been proposed as the main reason for reduced ctDNA detection in GBM (21). Our experiments, in which we circumvented the BBB via heterotopic tumor engraftment or opened the BBB using mannitol, suggest that the effect of the BBB on release of tumor-derived DNA into the plasma may be less significant than previously thought. Recent studies have shown higher relative levels of mutant DNA in CSF compared with plasma of patients with GBM (21, 22), which has been interpreted as being due to enrichment of tumor DNA in the CSF. Using dPCR to measure absolute concentrations of tumor and host DNA, we found that higher relative levels of tumor DNA in CSF resulted primarily from lower concentrations of nontumor host DNA together with more modest increases in the quantity of tumor-derived DNA.

Although we used single-copy human mitochondrial sequences to identify tmtDNA in the PDOX models, this strategy is not directly applicable to a human patient. However, mitochondrial mutations are present in the majority of cancers, with frequencies depending upon the tumor of origin, and mutational ‘hotspot’ regions have been identified (45), suggesting that mutated mitochondrial sequences could be used to detect tmtDNA in the clinic (12). Whole-genome sequencing (WGS) has enabled detection of mitochondrial DNA variants at fractions down to 1% (46); moreover, studies have shown that certain tumors positively select for nonsynonymous mitochondrial DNA mutations (47). Although WGS is expensive, the small size of the mitochondrial genome means that targeted and/or capture-sequencing-based methods could provide a more affordable alternative and may enable improved sequencing depth (48).

Several cancers have higher mitochondrial copy numbers, thus further increasing the probability of detecting tmtDNA (49). Detection of ctDNA in IDH1-mutant glioma, for example, has demonstrated limited clinical efficacy (3); however, the high tmtDNA copy number in these tumors may make circulating tmtDNA analysis achievable (49). Recent studies have identified certain cancers with functional tmtDNA mutations that affect metabolism (46). This could be used to target metabolic therapies to tumors with known metabolic weaknesses (46). tmtDNA mutations have also been described that confer specific chemoresistant properties (50). Thus, their monitoring via serial liquid biopsy may enable therapy modulation, as has been demonstrated with the use of ctDNA (4).
patients with gliomas, where detection rates for ctDNA have so far been very low.

Disclosure of Potential Conflicts of Interest
D. Gale is co-founder of, and has an ownership interest (including stock, patents, etc.) in, Inivata Ltd. F. Marass has an ownership interest (including stock, patents, etc.) in patent and shares of Inivata Ltd. D.W.Y. Tsui received honoraria from the speakers’ bureau of Astrazeneca and National Taiwan University, and is a consultant/advisory board member for Inivata Ltd. N. Rosenfeld is CSO at Inivata Ltd., reports receiving commercial research grant from Astrazeneca, and has an ownership interest (including stock, patents, etc.) in patents/applications. No potential conflicts of interest were disclosed by the other authors.

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References


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