Recurrent glioblastoma in the era of molecular diagnostics: practice variation and practical implications
Opijnen, M.P. van
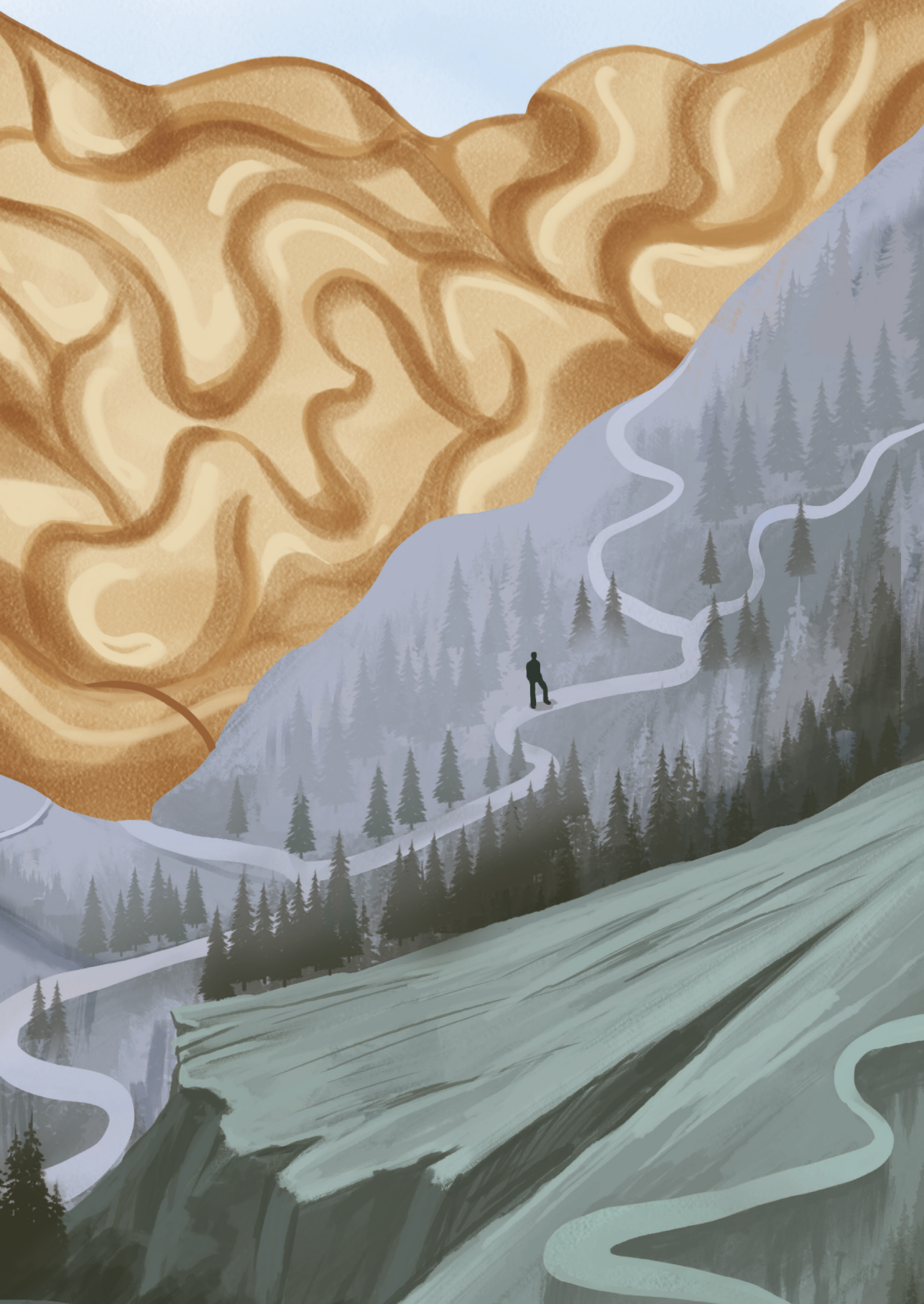
PART III

Practical implications

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Chapter 9

Genome sequencing-based analysis of genetic predisposition to adult glioblastoma

Under review (2025)

Mark P. van Opijnen, Devin R. van Valkengoed, Joep de Ligt, Filip Y.F. de Vos, Marike L.D. Broekman, Edwin Cuppen, Roelof Koster

ABSTRACT

The hereditary of adult glioblastoma is still largely unexplored. With the option of broad molecular testing, it is crucial that clinicians are aware of the a priori probability of finding germline predisposition in glioblastoma patients. Here, we studied the genetic predisposition to adult glioblastoma using paired tumor-normal WGS data in an unselected, average cohort of 98 glioma WHO grade 4 patients. In 11 patients (11%), 13 PGVs were found in genes strongly associated with familial glioblastoma (*MSH6* (3x), *PMS2* (5x), *MSH2*, *TP53*, *NF1*, *BRCA1*) or medulloblastoma (*SUFU*). In eight of these patients (73%), causality was supported by a second (somatic) event and/or a matching genome-wide mutational signature. Thus, germline predisposition does play a role in the development of adult glioblastoma, with mismatch repair deficiency being the main mechanism. Our results also highlight the benefits of tumor-normal WGS for glioblastoma patients and their families, beyond identifying actionable mutations for therapy.

Keywords. Glioblastoma, genome sequencing, genetic predisposition, germline

INTRODUCTION

Glioblastoma, a primary brain tumor, is the most common and most aggressive malignant brain tumor in adults. Despite intensive treatment consisting of surgical resection followed by radiotherapy with concurrent and sequential chemotherapy, the prognosis remains poor with a median survival of 15 months.[1, 2] One of the contributing factors challenging effective treatment strategies is the inter- and intratumoral heterogeneity of this devastating disease.[3] This becomes also apparent in the complexity revealed by genomics[4] and single-cell RNA sequencing.[5, 6] Nevertheless, genomic analysis of the tumor is considered a promising technological development that could enable personalized treatment strategies. The most comprehensive approach for genomic analysis is genome sequencing (GS), which has been clinically validated for diagnostic purposes.[7, 8] GS is not yet widely used in routine settings, especially for glioblastoma, mostly because of lack of evidence of clinical utility, costs, or both. Therefore, we have initiated the GLOW trial, a clinical study to explore potential added value of GS for recurrent glioblastoma patients.[9] As GS analyses typically include a control normal DNA sample (e.g. from blood) to distinguish somatic variants (acquired in the tumor cell) from germline variants (inherited), they may also reveal potential genetic predisposition to glioblastoma. This knowledge might be relevant to patients and their relatives, and the presence of familial predisposition is often an important question in the consulting room. Furthermore, variants in several predisposition genes are increasingly important for (immune- or targeted) therapy selection.[10-16]

In contrast to other (sub)types of cancer, for instance breast cancer and colon cancer, but also to pediatric gliomas, the prevalence of heredity in adult glioblastoma patients is still largely unexplored, mainly due to lower incidence and limited datasets that are available to investigate this topic.[17-19] In general, an estimate of approximately 5% of all glioma patients have a positive family history for glioma, with twofold to elevenfold increased incidence ratios in those families.[20-22] These cases show similarity to sporadic cases in terms of demographics (age, gender), morphology and tumor grade, and penetrance of hereditary glioma is suggested to be low.[23] Hereditary glioblastoma, also called familial glioblastoma, caused by single-gene hereditary disorders is very rare[24] and often involves predisposition of a range of tumor types. Current knowledge is limited to a few syndromes including neurofibromatosis type 1 (*NF1* mutation, autosomal dominant), Li Fraumeni syndrome (*TP53* mutation, autosomal dominant), Turcot syndrome type 1 (mismatch repair genes [MLH1 & PMS2] mutations, autosomal dominant) and Lynch or constitutional mismatch repair deficiency (mismatch repair genes

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mutations, autosomal dominant [Lynch] or recessive [constitutional mismatch repair deficiency]).[25-27] Furthermore, in enriched cohorts (i.e. selected for personal and/ or family history) pathogenic variants in *BRCA 1* and *2*, *CHEK2*, *HERC2*, *MUTYH*, *NF1*, *POT1* and *TERF2* have been associated with glioblastoma[20, 28-30], although their contribution to glioblastoma development remains unclear, since second-hit somatic variants were not observed for many.[29] Apart from these syndromes, familial glioblastoma is thought to be multifactorial and autosomal recessive.[31-33] Genome-wide association studies (GWAS) have identified several risk loci for glioblastoma, but causality of specific variants or genes in these regions remains unclear.[24, 34]

Taken together, in sporadic and/or late onset glioblastoma cases the prevalence and contribution of pathogenic germline variants (PGVs) remains unclear. It is, therefore, of interest to systematically analyze the complete germline genome of unselected glioblastoma patients, including small and structural variants, to identify genes with PGVs as potential candidates for cancer predisposition. This study thus aimed to gain novel insight into the prevalence of genetic predisposition to glioblastoma by retrospectively analyzing germline data of an unselected, adult glioblastoma patient population.

MATERIALS AND METHODS

Patient inclusion

For this retrospective, germline analysis study, genome sequencing data from the Hartwig Medical Foundation (Amsterdam, the Netherlands) database was used. All patients that contributed to this database have consented to reuse of their data, including germline data, for cancer research purposes. All adult patients (i.e. from 18 years and older) diagnosed with nervous system cancer (disease ontology ID: 3093) and whose data was stored in the database before November 1, 2023, were eligible. Patients were mainly collected in the context of the CPCT-02 (NCT01855477) and GLOW (NCT05186064) studies. Hereafter, the patient selection was further filtered based on tumor type, and only gliomas WHO grade 4 were included in final analyses (*n* = 98). Sampling in these patients was performed after recurrent disease. Family history of malignant neoplasms was not taken into account. Patient consent was based on a broad consent intending publicly available access-controlled data for academic cancer research related requests. For this study, a Data Access Request (DR-310) was signed to obtain the genome sequencing germline and somatic data. All samples were de-identified and keys between study number and patient number were stored solely locally in the hospitals.

Genome sequencing

All samples were sequenced at Hartwig Medical Foundation as per ISO-accredited diagnostic standards (ISO17025), as described previously.[35] Shortly, tumor samples with at least 20% tumor purity were deep-sequenced on Illumina Novaseq 6000 to an average depth of 90-100x. The blood control samples were sequenced to a depth of 30-35x. Somatic and germline variant calling was done using the open-source Hartwig WiGiTS toolset (https://github.com/hartwigmedical/hmftools v5_33). Also, tumor heterogeneity and presence of non-tumor cells in the tumor sample were computed (https://github.com/hartwigmedical/hmftools/tree/master/purple) and accounted for. The strategy for this germline analysis has been validated previously.[36]

Selection of relevant genes

Because of interpretation challenges and limited statistical power associated with the number of available patients compared to the vast search space of the genome, as well as the expected limited penetrance of individual genes, it was considered not feasible to perform a sufficiently powered genome-wide association study for analysis of variants that might be involved in glioblastoma predisposition. Hence, a manually curated list of known cancer-associated genes was created to first explore potential involvement of candidate genes. As a basis, the reportable germline gene list used as part of the pan-cancer routine diagnostic analysis pipeline from Hartwig was used.[36] This gene panel is based on national guidelines[37] and experience at the Netherlands Cancer Institute and was for this study expanded with genes from several other cancer predisposition gene panels: a germline driver catalogue previously described and curated by Priestley et al.[35], a subset of genes from the American College of Medical Genetics and Genomics (ACMG)[38], the Hereditary Cancer Gene Curation Expert Panel from ClinGen[39], the adult solid tumors cancer susceptibility panel created by National Health Service (NHS) and Genomics England[40], and from the literature.[20,30] After comparing these different gene lists, a comprehensive list of 170 genes was generated for the current germline predisposition analysis. For all of these genes the likely mechanism of action was determined as either oncogene or tumor suppressor gene (*Supplementary Table 1*).

Small variant calling

Small variants include stop-gain mutations, frameshifts due to small insertions or deletions, inframe deletions, inframe insertions, missense mutations and splice site mutations. Within the standard pipeline workflow of Hartwig (https://github.com/hartwigmedical/pipeline5), small variants in both tumor and germline are called by the algorithm 'Somatic Alterations in Genome' (SAGE; v3.2) (https://github.com/

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hartwigmedical/hmftools/tree/master/sage). SAGE is a precise and highly sensitive caller for single nucleotide variants (SNVs), multiple nucleotide variants ≤32 base pairs (MNVs) and small insertions and deletions (InDels). In the standard data processing workflow of Hartwig, SAGE is given a panel containing the regions of genes of interest for germline analysis in a Browser Extensible Data (BED) format (*Supplementary Table 1*). For our selected gene panel, a custom BED file (https://github.com/MvOglow/germlineGBM.git) was created using the in-house tool HMF Gene Utilities (v1.1, https://github.com/hartwigmedical/hmftools/tree/master/gene-utils) which used the GENCODE coordinates for the Genome Reference Consortium Human Build 37 (GRCh37) definitions. All raw compressed reference-oriented alignment map (CRAM) files containing the aligned sequencing reads for the included patients were re-processed with SAGE using the default germline run parameters (v3.4; *Supplementary Figure 1*) and these custom gene regions. Subsequently, this data was annotated and filtered using 'Prediction and Annotation of Variant Effects' (PAVE) germline (v1.6) (https://github.com/hartwigmedical/hmftools/tree/master/pave) using the default germline parameters (*Supplementary Figure 2*).

Hereafter, variants annotated as having only synonymous canonical coding effects were removed from the output files. To reduce inclusion of common variants and potential false positives, additional filters were used next to the default SAGE filters: (1) variants with a Genome Aggregation Database (gnomAD; v2.1.1[41]) population frequency >1% were removed and classified as population variance; (2) germline variants with a low recalibrated quality score (see below) were removed and (3) germline variants with a frequency ≥5% in the Hartwig database (n = 5,778, excluding the patients included in this study) were removed as these are likely population variants specific to the Dutch population. SAGE accounted for false positive calls or poor sensitivity by recalibrating the empirical base quality score provided by the sequencer. The ad-hoc cut-off based on these recalibrated Phred-scaled quality scores was determined using a density plot of the recalibrated Phred quality of all obtained variants for the included patients and set at 235.6 for variants to be included in further analyses (*Supplementary Figure 3*).

Structural variants and copy number variations calling
By default, structural variants (SVs) and copy number variations (CNVs) were called genome-wide by GRIDDS2 in the Hartwig pipeline.[42] After processing, this data was annotated and filtered by GRIPPS germline and stored in a SQL database (pipeline release v5.33). All SVs and CNVs within the regions defined in the BED file were obtained from the SQL database. Because gnomAD does not provide population

frequencies for SVs, the data was filtered based on the variant frequency within the Hartwig database (excluding the patients included in this study). All obtained SVs occurring in ≥5% of all other patients in the Hartwig database were excluded. Since the Hartwig databases contained 5,778 patients next to the patients included in this study, SVs occurring in ≥289 patients were discarded.

The interpretability of copy number gains is low as there is no international consensus on the significance of differences between the exact number of copies, e.g. three versus more than three copies. Moreover, most genes are tumor suppressor genes. Therefore, we assessed only copy number losses and no copy number gains.

Clinical significance

The clinical significance of variants was based on their annotation in ClinVar, a public archive of human genetic variants and interpretations of their significance to disease.[43] The main conclusions in our study were based on 'pathogenic' and 'likely pathogenic' variants. Variants of unknown significance (VUS) were not studied. To direct the potential effect of these variants on the functional protein, the Ensembl Variant Effect Predictor (VEP) was used.[44] All shortlisted variants were manually reviewed by a clinical laboratory geneticist (RK) to determine pathogenicity according to routine diagnostic procedures, all likely-pathogenic (class 4) and pathogenic variants (class 5) were considered as pathogenic germline variants (PGVs). As a second step to assess the clinical significance of PGVs, tumor type-specific manual curation and tumor genome analysis was performed. The following subdivision was used: category 1 were causal events (gene associated with glioblastoma + matching tumor findings), category 2 were known predisposition genes but without demonstrated causality (gene associated with glioblastoma without matching tumor findings, or gene not associated but having matching tumor findings), and category 3 contained variants less likely to contribute to glioblastoma.

Tumor sample analysis

For tumor suppressor genes, the common model for pathogenicity is that both alleles of the gene become inactivated in the tumor. In case of germline predisposition, the second allele is typically inactivated by a second mutation or loss of heterozygosity (LOH, although epi-genetic inactivation through methylation is also possible). Therefore, we assessed all candidate genes for somatic events, and, in case of LOH, determined if the normal or mutated germline allele was lost. In addition, we explored if any of the candidate genes was also a common somatic driver in glioblastoma patients, i.e. inactivated bi-allelic by somatic events. Finally,

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mutational signatures were studied for DNA repair genes. In case of splice site variants, RNA sequencing data (which was available for approximately 80% of the patients) was used to validate the impact of the variant at transcript levels. A graphic overview of the methods for identification of predisposition can be found in *Figure 1*.

Statistics

Sociodemographic characteristics were compared by using chi-square test for categorical variables and t-test for continuous variables. In case of violation of the normality assumption, a non-parametric test was used for the continuous variables.

Figure 1. Flowchart methods.

Identification of genetic predisposition

Patient inclusion
Patients identified from:
Hartwig database (*n* = **98**)

Genes
Manually curated list of genes
(*n* = **170**)

Calling & annotation
Small variant calling, using:
Somatic Alterations in Genome
(**SAGE**) algorithm

Structural variant and CNA
calling, using:
GRIDDS2

Small variant annotation, using:
Prediction and Annotation of
Variant Effects (**PAVE**) germline

Structural variant and CNA
annotation, using:
GRIPPS germline

Filtering
Small variant filtering, based on:
Population frequency >1%
(**gnomAD**), recalibrated **quality
score** 235.6, **frequency in
database** ≥5%
(*n* = **418**)

Structural variant filtering, based
on:
Frequency in database ≥5%
(*n* = **5**)

Clinical significance
Filter for (likely) pathogenic and
unknown variants in **ClinVar**
(*n* = **59**)

Tumor type-specific **manual
curation** by Clinical Laboratory
Specialist
(*n* = **37**)

Tumor sample
Tumor driver likelihood analysis, assessing:
Second hit mutation, loss of heterozygosity (**LOH**), **bi-
allelic inactivation**
In case of LOH: normal or mutated allele analysis
Mutational signatures for DNA repair genes
*Category 1, causal = **8** (8%)*
*Category 1+2, causality uncertain = **11** (11%)*

RESULTS

Patient characteristics

A total of 98 patients met the inclusion criterium of 'adult glioma WHO grade 4' and were included in this study for germline predisposition analysis. Of these, 70.4% (69/98) were male and the median age for males and females was 61 years. Most of the patients had a primary, isocitrate dehydrogenase (*IDH*) wildtype glioblastoma (93.9%, 92/98) while 6.1% (6/98) of the tumors had a somatic *IDH* mutation (classifying them as astrocytoma WHO grade 4). The family history, particularly regarding the occurrence of malignant neoplasms, was unidentified.

Germline findings in an average glioblastoma population

After filtering for canonical coding effects, gnomAD population frequency and quality score, a total of 418 small variants and five structural variants (SVs) were detected in 107 of the 170 different genes. Filtering for variants that were annotated as '(likely) pathogenic' or 'unknown' in ClinVar following manual curation, resulted in a total of 30 (including three SVs) PGVs in 18 different genes in 25 unique patients (25.5% of all patients). Of these 30 PGVs, 11 were observed in genes with an explicitly recessive inheritance and 19 in genes having dominant inheritance. All 11 PGVs in recessive genes were monoallelic and, therefore, excluded from overall prevalence, because only biallelic or compound heterozygous germline variants in such genes are considered as having associated hereditary risks (*Table 1*).

The 19 dominant inheritance PGVs were present in 11 different genes in 16 unique patients (16% of all patients). Six of these PGVs were in cancer predisposition genes (*ATR*, *CHEK2* (3x), *SDHA* and *MITF*) without an established association with familial glioblastoma. Interestingly, the majority, 13 PGVs in 11 patients, were in established cancer predisposition genes with a strong association with familial glioblastoma (*MSH6* (3x), *PMS2* (5x), *MSH2*, *TP53*, *NF1* and *BRCA1*) or with medulloblastoma (*SUFU*). Thus, the prevalence of known genetic predisposition to glioblastoma was 11% (11/98) in our unselected cohort, with additional candidates in another 5.1% of patients (5/98).

Genetic predisposition driving glioblastoma oncogenesis

As most predisposition genes involve tumor suppressors, all candidate causal events were assessed for second hit (somatic) events in the tumor data. PGVs with a second (somatic) event are considered causal for glioblastoma oncogenesis. For all six PGVs (*ATR*, *CHEK2* (3x), *SDHA* and *MITF*) without an established association with familial glioblastoma and for 10 out of 11 PGVs in recessive genes (*BLM* (4x),

ERCC3, *MUTYH* (2x), *FANCF*, *SBDS* and *WRN*), no second (somatic) event (small variant or structural variant resulting in LOH) or matching mutational signature was detected in the tumor. Thus, those variants, except for possibly *BUB1B*, were unlikely to contribute to the development of glioblastoma in our cohort (category 3 – see *Table 1*). Additionally, for three patients with PGVs in genes with a strong association with familial glioblastoma (*NF1*, *MSH6* and *MSH2*) also no second (somatic) event or expected matching mutational signature was detected, indicating that for these variants the causality for tumorigenesis in these patients remains unclear (category 2 – see *Table 1*).

Importantly, for the remaining 10 PGVs that were identified in genes with a strong association with familial glioblastoma or medulloblastoma (*SUFU*, *MSH6* (2x), *PMS2* (5x), *TP53* and *BRCA1*), a second (somatic) event and/or a matching mutational signature was identified in the tumor. These variants were present in eight different patients, resulting in a proven germline predisposition rate of 73% in the patients with relevant PGVs (8/11). Of interest, two of these patients most likely have constitutional mismatch repair deficiency (CMMRD) syndrome, since they each harbored two PGVs in *PMS2* and both were microsatellite instable with a high tumor mutational burden (*Table 1*).

### DNA damage response – significant role for mismatch repair (MMR) in glioblastoma

The known pathogenic predisposition variants in 11 patients could be divided in two main mechanisms. First, two patients had PGVs in genes involved in cell proliferation/survival (Ras/mitogen-activated protein kinase pathway; NF1 & Shh signaling pathway; *SUFU*). Second, nine patients had 11 PGVs in genes involved in the DNA damage response or cell cycle pathway (*TP53*, *BRCA1*, *PMS2*, *MSH6* and *MSH2*). These included a patient showing LOH for *TP53* and another patient showing LOH for *BRCA1* along with a homologous recombination deficiency (HRD) footprint. The tumor in this patient underwent whole genome duplication after LOH (*Supplement Figure 4*).

The majority of patients was thus found to harbor a PGV in one of the mismatch repair (MMR: MSH2, PMS2, MLH1, MSH6) genes (7 out of 11). By measuring microsatellite instability (MSI) based on GS, we observed that, within the total cohort, seven patients had > 1.3 microsatellite Indels Per Mb (overall average 1.3, median 0.12) and six of seven patients had ≥ 4 microsatellite Indels Per Mb (diagnostic cutoff of WGS handled by Hartwig Medical Foundation – see *Figure 2A*). For one of the seven patients with MSI no evidence for either germline or somatic

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mutations in any of the four MMR genes was found. For the remaining six patients with MSI, one patient with somatic loss of function of *MLH1* and five patients with germline loss of function of *MSH6* (2x) or *PMS2* (3x) matched with MSI (*Figure 2A*).

The percentage of patients with PGVs in MMR genes within this unselected glioblastoma cohort were compared to the percentage of patients with PGVs in MMR genes within other unselected cancer cohorts[20, 36, 45, 46] and the gnomAD v2.11 (non-cancer) cohort[41]. Although numbers remain small, a higher than expected frequency of patients with glioblastoma carrying a PGV in MMR genes was seen, with the biggest difference for *MSH6* and *PMS2* (*Figure 2B*).

Figure 2. Significant role for mismatch repair (MMR) in glioblastoma.
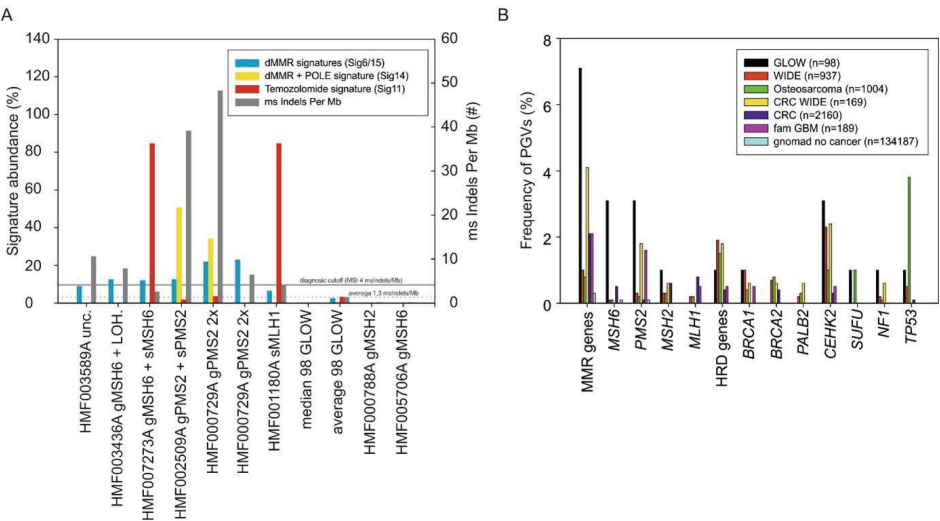
(A) Cosmic single base substitution (SBS) signatures for dMMR (Sig 6+15) dMMR+POLE (Sig14) and Temozolomide (Sig11) and number of ms Indels per Mb are depicted for seven patients within the total cohort having > 1.3 microsatellite Indels Per Mb. (B) Frequency of pathogenic germline variants (PGVs) in the genes as described in the GLOW study versus other cohorts.

CRC: colorectal cancer[46], CRC WIDE: subgroup WIDE colorectal cancer patients[36], fam GBM: familial glioblastoma cohort[20], GLOW: current composite cohort, gnomad: non-cancer reference cohort[41], HRD genes: homologous recombination deficiency genes (BRCA1/2 & PALB2), dMMR genes: deficient mismatch repair genes (MSH6, PMS2, MSH2 & MLH1), MSI: microsatellite instability, osteosarcoma[45], WIDE: metastatic cancer[36]

Table 1. Findings after manual curation of possibly interesting variants.

Patient	Age	Gene	c.HGVS[1]	p.HGVS[2]	Classification	Inheritance	Mechanism	Tumor status	Category	TMB	MSI	IDH1/2
HMF002821A	55	SUFU	c.436C>T	p.Arg146*	P	dominant	LOF	LOH	1	2,1	0,1	WT
HMF003436A	72	MSH6	c.467C>G	p.Ser156*	P	dominant	LOF	MSI & second hit(s) c.1444C>T, p.Arg482*, c.3254delC, p.Phe1088fs	1	37.9	**7.8**	WT
HMF002509A	45	PMS2	c.1882C>T	p.Arg628*	P	dominant	LOF	MSI & second hit(s) c.943C>T, p.Arg315*, c.1732C>T, p.Arg578Cys	1	339	**39**	IDH2 p.A370T
HMF003795A	53	BRCA1 SDHA	c.2210delC c.91C>T	p.Thr737fs p.Arg31*	P P	dominant dominant	LOF LOF	LOH, HRD signature no second hit	1 3	8,6	0,2	WT
HMF001910A	19	PMS2	c.137G>T c.736_741delins TGTGTGGAAG	p.Ser46Ile p.Pro246fs	LP P[5]	dominant	LOF	MSI	1	11,7	**6,4**	IDH2::IDH2 fusion
HMF000729A	33	PMS2	c.325dupG c.825A>G	p.Glu109fs p.Gly275= (splice)[5]	P P[5]	dominant	LOF	MSI	1	279	**48,2**	IDH1 p.T325M
HMF007273A	50	MSH6	c.742delC	p.Arg248fs	P	dominant	LOF	MSI, LOH	1	89	**2,6**	WT
HMF006786A	N/A	TP53	c.711G>A	p.Met237Ile	P	dominant	LOF	biallelic	1	3,1	0,1	IDH1 p.R132C
HMF006763A	55	NF1	complex event[8] (see below)	unbalanced	P	dominant	LOF	no second hit[#]	2	3,5	0,1	WT
HMF005706A	N/A	MSH6	c.3514dupA	p.Arg1172fs	P	dominant	LOF	MSS, no second hit	2	3,2	0,1	WT
HMF000788A	51	MSH2	c.942+2delT	p.? (splice?)[6]	LP[6]	dominant	LOF	MSS, no second hit variant lost in tumor	2	3	0,1	WT
HMF000649A	73	BUB1B	c.2210T>G	p.Leu737*	P	recessive	LOF	second hit, large deletion including BUB1B	3	3,7	0,1	WT
HMF000655A	57	ATR	inversion (see below)	Inversion exon 42-47 + XRN1	P	dominant	LOF	no second hit	3	4,4	0,2	WT

Table 1. Continued

Patient	Age	Gene	c.HGVS[1]	p.HGVS[2]	Classification	Inheritance	Mechanism	Tumor status	Category	TMB	MSI	IDH1/2
HMF000925A	71	CHEK2	c.1229delC	p.Thr410fs	P	dominant±	LOF	no second hit	3	3,5	0,1	WT
HMF000842A	54	CHEK2	c.1229delC	p.Thr410fs	P	dominant±	LOF	no second hit	3	2,7	0,1	WT
HMF007010A	77	CHEK2	c.1229delC	p.Thr410fs	P	dominant±	LOF	no second hit	3	4	0,1	WT
HMF001235A	47	ERCC3	c.760C>T	p.Gln254*	P	recessive	LOF	no second hit	3	2,5	0,1	WT
HMF001434A	68	BLM	deletion (see below)	deletion exon 2-22	P	recessive	LOF	no second hit	3	3	0,1	WT
HMF003406A	39	BLM	c.3558+1G>T	p.? (splice)	P	recessive	LOF	no second hit	3	2,9	0,1	WT
HMF001701A	54	BLM	c.1642C>T	p.Gln548*	P	recessive	LOF	no second hit	3	1,7	0	WT
HMF001787A	55	MUTYH	c.1178G>A	p.Gly393Asp	P	recessive	LOF	no MUTYH signature, no second hit	3	1,5	0,1	WT
HMF007027A	73	SBDS	c.258+2T>C	p.? (splice)	P	recessive	LOF	no second hit	3	4,1	0,2	WT
HMF006506A	65	FANCF	c.484_485delCT	p.Leu162fs	P	recessive	LOF	no second hit	3	2,8	0,1	WT
HMF000329A	55	BLM / MITF / WRN	c.1933C>T / c.1255G>A / c.1105C>T	p.Gln645* / p.Glu419Lys / p.Arg369*	P / P / P	recessive / dominant + / recessive	LOF / GOF / LOF	no second hit	3	2,6	0,1	WT
HMF006895A	72	HERC2 / MUTYH	c.8002G>C / c.1138delC	p.Val2668Leu / p.Ala382fs	VUS / P	dominant / recessive	LOF / LOF	no second hit / no MUTYH signature, no second hit	3	4	0,1	WT
HMF003589A	55							MSI	n/a	216	11	WT
HMF001180A	73							MSI, somatic MLH1	n/a	80	4	WT

[1]Coding reference sequence, [2]Protein reference sequence, [&]14:102878068-17:29458864, G: gain of function, HGVS: Human Genome Variation Society, LOF: loss of function, WT: wildtype

Classifications: LP: likely pathogenic, P: pathogenic, VUS: variant of uncertain significance

Inheritance: ±Risk factor for breast cancer, +Risk factor for melanoma, -Associated with glioblastoma

[5]Out-of-frame skipping of the first 22 nucleotides of exon 8[67, 68] and observed with RNAseq, see supplemental Figure 5

[6]Splicing could not be proved with RNAseq data. This could be because the variant is lost in the tumor. The expert panel InSiGHT consideres this variant as Likely pathogenic

Tumor status: #Somatic gain 17:29421945-29709134; complete gene is amplified in tumor but amplification involved a large part of the chromosome (non-focal), HRD/HRP: homologous recombination deficient/proficient, MSI/MSS: microsatellite instability/stability

Category: 1: causal event, 2: known predisposition gene but causality not demonstrated, 3: less likely to contribute to glioblastoma

NC_000014.8:g.pter_102878068delins[NC_000017.10:g.29458864_qter] / p.? (deletion of chr14pter_1st exon NF1)

NC_000003.11:g.142091960_142182304inv / p.? (inv exon 42-47 ATR + XRN1)

NM_001287246.2(BLM):c.-4-1619_*16491del / p.?

DISCUSSION

This study showed the germline predisposition in a cohort of 98 adult glioblastoma patients. In 11% of the patients, pathogenic germline variants (PGVs) were observed in genes previously associated with familial glioblastoma; thus these PGVs likely contributed to the oncogenesis of these unselected glioblastoma patients. PGVs were found in the following genes: *BRCA1*, *MSH6*, *PMS2, TP53, NF1* and *SUFU*. Furthermore, for ten PGVs in *SUFU, MSH6* (2x), *PMS2* (5x), *TP53* and *BRCA1*, in eight different patients causality was proven, since second (somatic) events and/ or matching mutational signature were detected. Several of these PGVs were in predisposition genes that are increasingly important for (targeted) therapy selection and for all findings counseling by a clinical geneticist is indicated. Mismatch repair deficiency formed the main mechanism of the unselected cohort, with 7.1% of the patients harboring a PGV in one of the mismatch repair (MMR) genes, including five patients with microsatellite instability.

The results of this study are unique in several aspects. First, no preselection based on personal and/or family history of malignant neoplasms was applied to the study cohort. Second, the pairing of both blood and tumor tissue samples allowed for verification of the causality of potentially interesting events. Third, since all patients underwent paired GS testing combined with RNA sequencing (~80%), we were able to not only study point mutations (which is a limitation in most of the cancer predisposition research) but also copy number variations, structural variants, splice site variants (*supplementary Figure 5*) and mutational signatures.

 We detected a number of PGVs in dominant and recessive genes without proven causality for glioblastoma, since the tumor sample analyses did not show second hit mutations in almost all of these cases. In the Netherlands, observed putative PGVs in dominant genes that do not match the tumor type (*ATR*, *CHEK2* (3x), *SDHA* and *MITF*) are normally not reported back to the patient, except if there is a matching personal and/or familial history. Unfortunately, in the current retrospective study design, we were not able to identify the pedigrees of the patients with PGVs, making further details of the inheritance pattern and possible consequences for family members impossible. In recessive genes, all 11 PGVs were monoallelic and considered low/no risk for cancer predisposition. Still, these variants potentially modified the genesis of the tumor as risk loci associated with susceptibility to glioblastoma. Unfortunately, our study lacked sufficient power to study these monoallelic PGVs in recessive genes in a statistically sound manner. Interestingly, in one patient with a PGV in *BUB1B*, the remaining wildtype allele was somatically

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lost due to a large deletion. The causality of the PGV in this recessive gene could not been demonstrated, although there is evidence for the role of *BUB1B* as a (pan-)cancer predisposing gene[47], including glioblastoma.[48] When variants like these are identified, they are normally not reported back to the patient. Because these variants do not have any relevance for the patient nor for the patient's family except if there is consanguinity in the family, no genetic counselling and testing is recommended.

Currently, the international guideline of the European Association of Neuro-Oncology (EANO) on the diagnosis and treatment of diffuse gliomas of adulthood recommend genetic counselling in patients with 'relevant germline variants or suspected hereditary cancer syndromes.'[49] This recommendation is based on low level evidence (i.e. class IV, level C evidence) and did not specify which germline variants are considered relevant. The familial tumor syndromes associated with gliomagenesis named in this EANO guideline include neurofibromatosis type I, tuberous sclerosis, Turcot syndrome, Li-Fraumeni syndrome and Lynch syndrome. Other international guidelines of neuro-oncology or medical oncology societies lack recommendations on germline testing and genetic counseling of gliomas in adults.[50, 51] However, the more recent EANO guideline on molecular testing of gliomas in adults recommend genetic counseling prior to germline testing, as for instance specific attention is paid to MMR gene deficiencies.[52] Yet, most of the PGVs found in our study are currently not tested for in most of the Dutch laboratories.[53]

As the use of comprehensive tumor genetic and genomic diagnostic tests continues to grow, the detection of PGVs is occurring more frequently than previously expected.[36, 48, 54, 55] In our unselected cohort, many PGVs are identified in genes such as *BRCA1*, *MSH6*, *PMS2*, and *NF1*, which are crucial not only for germline follow-up but also for selecting appropriate therapies, particularly immune-based or targeted treatments, as observed in other tumor types. For example, melanoma, MMR deficient colorectal cancer, and other non-colorectal MMR deficient tumors have shown remarkable responses to immunotherapy.[13-16, 56] While some glioblastoma patients exhibit long-term responses to immunotherapy, this treatment has shown limited efficacy in over 90% of unselected glioblastoma cases.[57-60] Among those who responded (partially or fully), most likely were patients with hypermutated tumors, possibly due to MMR deficiency or MMR deficiency + *POLE* defects.[57, 61-65] Our findings indicate that most of these hypermutated tumors harbor a PGV in one of the MMR genes. Thus, comprehensive tumor genetic and genomic profiling for glioblastoma patients requires an integrated approach that facilitates appropriate referral to clinical geneticists.

This study has some limitations that have to be considered. First, this type of research cannot be done without making assumptions. Assumptions were not only made when defining the pathogenicity of variants[38], but essentially every single step in our methods, e.g. variant calling, annotation, filtering, curation involved choices based on assumptions. Although these are based on generally accepted international standards, changes over time based on progressive insights may impact outcomes. A second limitation is the relatively small sample size, which hampered statistically powered analyses of the PGVs. Third, our cohort contained six patients (6.1%) with a somatic *IDH* mutation, which might be extra relevant, in terms of prognostic relevance, in the context of MMR deficiency.[66] Lastly, due to consent and privacy regulation limitations, we were not able to assess the pedigrees of the patients with PGVs, making assessment of the inheritance pattern and possible consequences for family members impossible.

To conclude, this study investigated the germline predisposition to glioblastoma in an average adult glioblastoma population. 11% of these patients had a pathogenic germline variant that (likely) predisposed to the development of the glioblastoma, with potential associated therapy options. The results could guide clinicians who have to inform patients about broad molecular tests for personalized medicine and its associated putative germline findings, once current gene panels are adapted to these findings.

AUTHOR CONTRIBUTIONS

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Study concept and design: MPvO, DRvV, MLDB, EC. Material preparation, data collection, and analysis: MPvO, DRvV, JdL, EC, RK. Writing and revision of manuscript: MPvO, DRvV, JdL, FYFdV, MLDB, EC, RK.

FUNDING

None

AVAILABILITY OF DATA AND MATERIALS

The underlying research are partly facilitated by Hartwig Medical Foundation and the Center for Personalized Cancer Treatment (CPCT) which have generated,

analysed and made available data for this research. Data can be requested via https://www.hartwigmedicalfoundation.nl/en/data/data-access-request/. Hartwig Medical Foundation is willing to share with external qualified researchers access to patient-level data and supporting clinical documents. These requests are reviewed and approved by an independent review committee on the basis of scientific merit. All data provided are anonymized to respect the privacy of patients who have participated in the study, in line with applicable laws and regulations.

CONFLICT OF INTEREST STATEMENT

None of the authors declare a conflict of interest.

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SUPPLEMENTARY

Supplementary Figure 1. Example of command-line arguments that were used to run SAGE in default germline mode: see https://github.com/MvOglow/germlineGBM/blob/66c02a570c85c630038f3c5644346cbaf25385ac/Supp%20Figure%201.png

Supplementary Figure 2. Example of command line arguments that were used to run PAVE in default germline mode: see https://github.com/MvOglow/germlineGBM/blob/621feeac129493396680c14c6bd848b306df926f/Supp%20Figure%202.png

Supplementary Figure 3. Quality density plot of small variant scores**:** see https://github.com/MvOglow/germlineGBM/blob/57955240ee192e8239c7c00da93dee9195834570/Supp%20Figure%203.png

Supplementary Figure 4. Circos plot of HMF006786A: see https://github.com/MvOglow/germlineGBM/blob/080f00ba444cd6e6f8b817b08cdf7147beba2ac8/Supp%20Figure%204.png

Supplementary Figure 5. In silico predictions and RNAseq analysis of *PMS2* exon 1-12 for HMF000729A: see https://github.com/MvOglow/germlineGBM/blob/c36c7c4d636c9d681eec358cca0cd8308fed1ff0/Supp%20Figure%205.png

Supplementary Table 1. Overview of the genes included in the gene panel that was used: see https://github.com/MvOglow/germlineGBM/blob/b97de47f93218de719e216bc7ce8e38a3b42d78b/Supp%20Table%201.docx