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Differences in methylation profiles between long-term survivors and short-term survivors of IDH-wild-type glioblastoma

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Abstract

Background. Patients with glioblastoma (GBM) have a median overall survival (OS) of approximately 16 months. However, approximately 5% of patients survive >5 years. This study examines the differences in methylation profiles between long-term survivors (>5 years, LTS) and short-term survivors (<1 year, STS) with isocitrate dehydrogenase (IDH)-wild-type GBMs.

Methods. In a multicenter retrospective analysis, we identified 25 LTS with a histologically confirmed GBM. They were age- and sex-matched to an STS. The methylation profiles of all 50 samples were analyzed with EPIC 850k, classified according to the DKFZ methylation classifier, and the methylation profiles of LTS versus STS were compared.

Results. After methylation profiling, 16/25 LTS and 23/25 STS were confirmed to be IDH-wild-type GBMs, all with +7/–10 signature. LTS had significantly increased O6-methylguanine methyltransferase (MGMT) promoter methylation and higher prevalence of FGFR3-TACC3 fusion ($P = .03$). STS were more likely to exhibit CDKN2A/B loss ($P = .01$) and higher frequency of NF1 ($P = .02$) mutation. There were no significant CpGs identified between LTS versus STS at an adjusted P-value of .05. Unadjusted analyses identified key pathways involved in both LTS and STS. The most common pathways were the Hippo signaling pathway and the Wnt pathway in LTS, and GPCR ligand binding and cell–cell signaling in STS.

Conclusions. A small group of patients with IDH-wild-type GBM survive more than 5 years. While there are few differences in the global methylation profiles of LTS compared to STS, our study highlights potential pathways involved in GBMs with a good or poor prognosis.

Key Points

- A small subset of patients with an isocitrate dehydrogenase wild-type glioblastoma survive >5 years.
- While the global methylation patterns between short-term and long-term survivors are similar, our study highlights genes and pathways that can explain these survival differences.

Importance of the Study

Glioblastoma (GBM) is the most common primary malignant brain tumor in adults. The vast majority have a very poor prognosis. A small population of isocitrate dehydrogenase (IDH)-wild-type GBMs are long-term survivors (LTS), defined as >3 years in previous studies. Genetically, GBMs are very heterogeneous. Since methylation has become a very accurate tool in the diagnosis and classification of central nervous system tumors, we studied the methylation profiles of LTS, defined in this study as >5 years, compared to short-term survivors (STS; <1 year). We showed that methylation is very useful in identifying true IDH-wild-type GBM, since some LTS had a mismatch in their methylation classification diagnosis compared to their histological diagnosis and were another entity. Moreover, methylation analysis identified differences in key genes between LTS and STS, and different activated pathways in LTS, compared to STS. This analysis leads to a better understanding of what characterizes a favorable or poor prognosis in IDH-wild-type GBM.

Glioblastomas (GBMs) are the most common primary malignant brain tumors in adults.[1] The median overall survival (OS) of patients with a GBM is approximately 16 months.[2–5] A small population of glioblastoma patients survive for more than 5 years after their initial diagnosis. They are considered long-term survivors (LTS) and make up about 5% of GBM patients.[6,7]

Several clinical factors, such as younger age at diagnosis, high-performance status, maximal tumor resection, and treatment with radiation and chemotherapy, are associated with better outcomes in patients with GBM.[8–10]

Over the last decades, molecular features have become more important in understanding the pathology of glioma. Isocitrate dehydrogenase (IDH) mutation has been associated with long-term survival (>36 months),[11] and a group of alterations has been associated with a worse prognosis: epidermal growth factor receptor (EGFR) amplification, gain of chromosome 7 and loss of chromosome 10 (+7/–10), and telomerase reverse transcriptase promotor (*TERTp*) mutations. These findings have been incorporated in the 2021 World Health Organization (WHO CNS5) classification: an IDH-wild-type(wt) diffuse glioma with 1 or more of these alterations defines a GBM regardless of histological grade.[12] Moreover, the WHO CNS5 is the first CNS classification that included methylation profiling, which can be done using the DKFZ classifier, and is highly accurate in diagnosing and subtyping CNS tumors.[13] To date, some molecular features within IDHwt glioma in adult patients (ie, GBM) seem to have a prognostic role, including O6-methylguanine methyltransferase (MGMT), cyclin-dependent kinase inhibitor 2A/B (CDKN2A/B), and TERT.[14–16]

Among these prognostic molecular makers in IDH-wild-type GBM, MGMT promoter methylation is the most well established. MGMT is a gene that encodes a DNA repair protein responsible for removing alkyl groups from guanine residues. Epigenetic silencing by promoter methylation has been associated with improved survival in patients with GBM treated with alkylated agents such as temozolomide and lomustine in trials.[17,18] Furthermore, in large cohort studies, MGMT hypermethylation was associated with better survival[7,19]

In previous studies, LTS was defined as surviving more than 3 years after initial diagnosis.[20–23] Those studies that defined LTS as surviving more than 5 years included IDH-mutant GBMs in their cohort,[24,25] and as discussed above, being IDH-wild-type is the hallmark of a GBM.

While next-generation sequencing and methylation analysis have given us a greater understanding of molecular features that may lead to a better survival in GBMs, we still do not have a complete understanding of why certain GBM patients survive significantly longer than others. The identification of a precise gene signature has been challenging given the genetic heterogeneity of GBM and the limited number of patients with long-term survival. The purpose of this study was to characterize the methylation profile of GBMs in patients with long-term survival (defined as an OS of >5 years) compared to short-term survivors (STS; defined as <1 year). We used methylation profiling to select "true" IDH-wild-type GBM, excluding other diagnoses that can mimic a GBM histologically (eg, pleomorphic xantroastrocytoma [PXA] and astrocytoma, IDH mutant, WHO grade 4). In addition, following methylation analysis, we aimed to determine which pathways were upregulated in long-term and short-term GBM survivors.

Methods

Patients

We selected adult (≥18 years old) patients with a histologically confirmed GBM, diagnosed between January 1995 and December 2010. Patients were diagnosed at St. Michael's Hospital or at University Health Network, Toronto, Ontario, Canada. Radiation and chemotherapy treatment were given at Princess Margaret Cancer Centre, Toronto, Ontario, Canada. Those who survived >5 years after surgery were matched, based on sex and age (±3 years) at the time of diagnosis, to GBM patients who survived <1 year. Patients were excluded if samples for methylation profiling were unavailable and/or if follow-up data were missing. All samples were obtained during the first surgical procedure. For all patients, age at diagnosis, sex, tumor location, extent of resection, primary treatment, and survival were collected.

Methylation Profiling

DNA from tumor samples was extracted from formalin-fixed paraffin-embedded (FFPE) tissue (QIAamp DNA

FFPE Tissue Kit, Qiagen). Subsequently, between 200 and 550 ng of DNA was then bisulfite converted (EZ DNA methylation Kit, Zymo Research) and hybridized on the Infinium MethylationEPIC BeadChip array (Illumina, San Diego, CA).[26] The methylation results were classified according to the DKFZ methylation classifier, v11b4 (https://www.molecularneuropathology.org/mnp/).[13] The methylation classifier provides a list of methylation classes and calibrated methylation scores between 0 and 1 for each methylation class, with a higher score representing a higher confidence in the associated methylation class. Each of the 3 GBM subtypes (classical, mesenchymal, and proneural) has a distinct methylation class and calibrated methylation score. Only tumors identified as IDH-wild-type GBM in all 3 of the top methylation classifier results, independent of GBM subtype, were included for further analyses. All tumors identified as non-GBM methylation classes in the top 3 methylation classifier results were removed, regardless of calibrated score. In this manner, heterogeneous primary IDH-wild-type GBM samples with low calibrated scores for 1 specific subtype were still included, but samples that may not be GBM were removed.

Tumor Purity

The leukocyte unmethylation for purity (LUMP) score was calculated for all samples with DNA methylation data, as previously described, to estimate tumor purity/cellularity.[27] In short, the LUMP score is calculated by taking the average methylation levels of 44 CpG sites, which have been shown to be unmethylated in immune cells and methylated in tumors in a pan-cancer analysis, and dividing the number by 0.85. LUMP estimates were compared between prognostic subtypes.

MGMT Status

MGMT methylation status was obtained directly from the DKFZ classifier v11b4 reports, which uses the MGMT-STP27 algorithm and validated cutoff as previously described by Bady et al.[28]

CNV Analyses

Methylation data were analyzed for copy-number variations using the *conumee* package in R by comparing the 29 CNS-relevant genes used by default in the DKFZ methylation classifier[29] between the long-term and short-term survival groups.

Methylation Data Processing

Raw methylation data within.idat files were processed in R v4.0.3 (R Foundation for Statistical Computing) using the *minfi* package and data was normalized using the single-sample Noob approach. CpG sites with low-quality data for 1 or more samples (CpG detection $P > .01$) were removed from further analysis as well as those located on X and Y chromosomes, overlapping with single-nucleotide polymorphisms, or designated as cross-reactive.

Differential CpG Analysis

Differential CpG analysis was performed on LTS versus STS samples. At an unadjusted level, statistically significant *P*-values were defined as those with $P < .05$ and delta Beta (dB) of 0.1 or greater. The number of differentially methylated CpGs (hypermethylated and hypomethylated) after correction for multiple testing was measured using a range of adjusted *P*-values and dB values.

Pathway Analysis

All CpGs from the above differential analysis were mapped to areas of the genome and only CpGs within the gene promoter region were selected for analysis. In this manner, genes with promoter hypomethylation were considered to be upregulated, and genes with promoter hypermethylation were considered to be downregulated.[30] While previous research has suggested that gene promoter methylation is associated with gene expression,[30] it is important to note that not all differentially methylated genes may correspond to significant changes in gene expression.

The G:Profiler package in R was used for functional enrichment analysis, using the hypermethylated genes in LTS vs STS (downregulated in LTS and conversely upregulated in STS) and hypomethylated genes (upregulated in LTS and conversely downregulated in STS). The end results included biological processes and pathway bar plots generated.

Statistical Analysis

Differences between the LTS and STS regarding baseline characteristics, MGMT status, LUMP score, GBM subtype, and gene CNV were tested using a chi-square test for categorical data and a Mann–Whitney *U* for continuous data, respectively.

Ethics Statement

This study was approved by the Institutional Review Board (IRB) of University Health Network, University of Toronto, Ontario, Canada. The IRB waived the requirement for written informed consent for this retrospective observational study.

Results

Patients

From all patients treated for a histologically proven GBM in Princess Margaret Cancer Centre between 1995 and 2010, 36 patients survived >5 years. Of these patients, only 25 had adequate FFPE tumor samples available for analysis. These 25 patients had a median age of 53 at diagnosis

Table 1. Baseline characteristics

	LTS, *n* = 25	STS, *n* = 25
Sex (*n* female)	15	14
Age (median, IQR)	53 years, 46–56	52 years, 46–55
Tumor location (*n*)		
Frontal	6	8
Parietal	2	5
Temporal	15	7
Occipital	1	2
Unknown	1	3
Extent of resection (n)		
Biopsy	1	3
Subtotal resection	19	19
Gross total resection	5	2
Unknown		1
Primary treatment (n)		
Radiation	25	22
Temozolomide	24	22
Vital status	8 alive 17 dead	25 dead
Overall survival (median, IQR)	6.8 years, 5.8–9.3	0.9 years, 0.6–1.0

IQR = interquartile range; LTS = long-term survivors; *n* = number of patients; STS = short-term survivors.

(interquartile range 46–56 years old), and 15 (60%) were women. The median OS in this LTS group was 6.8 years. These patients were sex and age (±3 years) matched to STS. No differences were found regarding the extent of resection and primary treatment between the LTS and STS groups (Table 1).

Methylation Profile

Methylation profiling yielded results in all 50 cases, with no samples failing due to low tumor cell content or returning a result of "no match" on the methylation classifier. Overall, 16 of the 25 LTS and 23 of 25 STS were classified as an IDH-wild-type GBM (Table 2). Nine out of the 25 LTS were classified with an alternative diagnosis, with the Heidelberg classifier: polymorphous low-grade neuroepithelial tumor of the young ($n = 2$), high-grade astrocytoma, IDH mutant ($n = 3$), PXA ($n = 2$), high-grade astrocytoma with piloid features ($n = 1$), and inflammatory environment ($n = 1$). In the STS, only 2 out of the 25 patients were not classified as an IDH-wild-type GBM: 1 was classified as a PXA, and the other as control tissue from the cerebral hemisphere. Only those with samples classified as glioblastoma, IDH-wild-type were included for further analysis.

Tumor Purity

There was no difference in tumor purity (LUMP score) between LTS and STS, and no differences in purity between GBM subtypes. Although there were no significant differences in GBM subtype between LTS and STS, there was a trend in increased proneural subtype in STS (Table 3).

MGMT Status and CNV Analyses

The MGMT promoter was methylated in 13 of the 16 LTS (81%), compared to 8 of the 23 STS (35%), $P = .01$ (Table 3). All included GBMs had a complete gain of chromosome 7 and a complete loss of chromosome 10 (+7/–10). Interestingly, LTS had a significantly higher frequency of fibroblast growth factor receptor–transforming acidic coiled-coil (FGFR3-TACC3) fusions ($P = .03$), compared to STS. In STS, CDKN2A/B homozygous deletion was significantly more often present ($P = .01$). A higher prevalence of Neurofibromin 1 (NF1) mutation was found in STS ($P = .01$), compared to LTS.

Methylation Data Processing and Differential CpG Analysis

All samples passed quality control assessment (sample detection $P < .05$). At an unadjusted level, this resulted in a total of 5863 hypermethylated CpGs and 2240 hypomethylated CpGs between LTS versus STS samples (Supplementary Table 1). However, after correction for multiple testing, there were no significantly differentiated CpGs between LTS and STS, even at a less-stringent adjusted *P*-value of ≤.2.

Pathway Analysis

Following the CpG analysis, we included 1514 hypermethylated CpGs and 559 hypomethylated CpGs, corresponding to a total of 1210 hypermethylated genes and 437 hypomethylated genes in LTS, compared to STS, from the unadjusted analysis in Supplementary Table 1. These genes are involved in several pathways. Using the Kyoto Encyclopedia of Genes and Genomes, we identified multiple upregulated pathways (Figure 1A and 1C) and biological processes (Figure 1B and 1D) in LTS (Figure 1A and 1B) and STS (Figure 1C and 1D). The Hippo pathway was the most upregulated pathway in LTS and the G-protein-coupled receptors (GPCR) pathway was the most common in STS.

Discussion

In this multicenter retrospective case–control study, we showed that a small group of IDH-wild-type GBM patients survived >5 years. Compared to STS (<1 year), there are a few differences in the global methylation profiles. We identified that LTS were more likely to have MGMT methylation and were enriched for Hippo and Wnt-signaling pathways. STS were more likely to demonstrate homozygous deletion of CDKN2A/B, NF1, and were enriched for cell–cell signaling and GPCR-related pathways.

Most other studies,[20–22] but not all,[9,23] also showed a significant increase in MGMT methylation in LTS. However, in contrast to our study, these studies defined LTS as a survival of >36 months, or in some cases, just >24 months. Of note, studies that did not find an increase of MGMT in LTS were smaller studies (both $n = 16$).

Table 2. Methylation classification, diagnosis, and score for the long-term survivors (LTS) and short-term survivors (STS)

ID	Group	Top methylation Class	Diagnosis	In-cluded
1	LTS	GBM_MES_TYP	MC Glioblastoma, IDH-wild-type, mesenchymal subtype	Yes
2	LTS	GBM_MES_TYP	MC Glioblastoma, IDH-wild-type, mesenchymal subtype	Yes
3	LTS	GBM_MES_TYP	MC Glioblastoma, IDH-wild-type, mesenchymal subtype	Yes
4	LTS	GBM_MES_TYP	MC Glioblastoma, IDH-wild-type, mesenchymal subtype	Yes
5	LTS	GBM_MES_TYP	MC Glioblastoma, IDH-wild-type, mesenchymal subtype	Yes
6	LTS	GBM_MES_TYP	MC Glioblastoma, IDH-wild-type, mesenchymal subtype	Yes
7	LTS	GBM_MES_TYP	MC Glioblastoma, IDH-wild-type, mesenchymal subtype	Yes
8	LTS	GBM_MES_TYP	MC Glioblastoma, IDH-wild-type, mesenchymal subtype	Yes
9	LTS	GBM_RTK1	MC Glioblastoma, IDH-wild-type, RTK1 subtype	Yes
10	LTS	GBM_RTK2	MC Glioblastoma, IDH-wild-type, RTK2 subtype	Yes
11	LTS	GBM_RTK2	MC Glioblastoma, IDH-wild-type, RTK2 subtype	Yes
12	LTS	GBM_RTK2	MC Glioblastoma, IDH-wild-type, RTK2 subtype	Yes
13	LTS	GBM_RTK2	MC Glioblastoma, IDH-wild-type, RTK2 subtype	Yes
14	LTS	GBM_RTK2	MC Glioblastoma, IDH-wild-type, RTK2 subtype	Yes
15	LTS	HGG_E	MC Adult-type diffuse high-grade glioma, IDH-wild-type, subtype E (novel)	Yes
16	LTS	pedHGG_A	MC Diffuse pediatric-type high-grade glioma, H3 wild-type and IDH-wild-type, subtype A (novel)	Yes
17	LTS	A_IDH_HG	MC Astrocytoma, IDH mutant; high-grade	No
18	LTS	A_IDH_HG	MC Astrocytoma, IDH mutant; high-grade	No
19	LTS	A_IDH_HG	MC Astrocytoma, IDH mutant; high-grade	No
20	LTS	HGAP	MC High-grade astrocytoma with piloid features	No
21	LTS	INFLAM_ENV	MC Inflammatory microevironment	No
22	LTS	PLNTY	MC Polymorphous low-grade neuroepithelial tumor of the young	No
23	LTS	PLNTY	MC Polymorphous low-grade neuroepithelial tumor of the young	No
24	LTS	PXA	MC Pleomorphic xanthoastrocytoma	No
25	LTS	PXA	MC Pleomorphic xanthoastrocytoma	No
26	STS	GBM_MES_TYP	MC Glioblastoma, IDH-wild-type, mesenchymal subtype	Yes
27	STS	GBM_MES_TYP	MC Glioblastoma, IDH-wild-type, mesenchymal subtype	Yes
28	STS	GBM_MES_TYP	MC Glioblastoma, IDH-wild-type, mesenchymal subtype	Yes
29	STS	GBM_MES_TYP	MC Glioblastoma, IDH-wild-type, mesenchymal subtype	Yes
30	STS	GBM_MES_TYP	MC Glioblastoma, IDH-wild-type, mesenchymal subtype	Yes
31	STS	GBM_MES_TYP	MC Glioblastoma, IDH-wild-type, mesenchymal subtype	Yes
32	STS	GBM_MES_TYP	MC Glioblastoma, IDH-wild-type, mesenchymal subtype	Yes
33	STS	GBM_MES_TYP	MC Glioblastoma, IDH-wild-type, mesenchymal subtype	Yes
34	STS	GBM_RTK1	MC Glioblastoma, IDH-wild-type, RTK1 subtype	Yes
35	STS	GBM_RTK1	MC Glioblastoma, IDH-wild-type, RTK1 subtype	Yes
36	STS	GBM_RTK1	MC Glioblastoma, IDH-wild-type, RTK1 subtype	Yes
37	STS	GBM_RTK1	MC Glioblastoma, IDH-wild-type, RTK1 subtype	Yes
38	STS	GBM_RTK1	MC Glioblastoma, IDH-wild-type, RTK1 subtype	Yes
39	STS	GBM_RTK1	MC Glioblastoma, IDH-wild-type, RTK1 subtype	Yes
40	STS	GBM_RTK1	MC Glioblastoma, IDH-wild-type, RTK1 subtype	Yes
41	STS	GBM_RTK1	MC Glioblastoma, IDH-wild-type, RTK1 subtype	Yes
42	STS	GBM_RTK2	MC Glioblastoma, IDH-wild-type, RTK2 subtype	Yes
43	STS	GBM_RTK2	MC Glioblastoma, IDH-wild-type, RTK2 subtype	Yes
44	STS	GBM_RTK2	MC Glioblastoma, IDH-wild-type, RTK2 subtype	Yes
45	STS	GBM_RTK2	MC Glioblastoma, IDH-wild-type, RTK2 subtype	Yes
46	STS	GBM_RTK2	MC Glioblastoma, IDH-wild-type, RTK2 subtype	Yes
47	STS	pedHGG_RTK1A	MC Diffuse pediatric-type high-grade glioma, RTK1 subtype, subclass A (novel)	Yes
48	STS	pedHGG_RTK1C	MC Diffuse pediatric-type high-grade glioma, RTK1 subtype, subclass C (novel)	Yes
49	STS	CTRL_HEMI	MC Control tissue, cerebral hemisphere	No
50	STS	PXA	MC Pleomorphic xanthoastrocytoma	No

The last column shows whether the patients was included in the analysis, based on the methylation classification. H3, histone 3; ID, patient study number; IDH, isocitrate dehydrogenase; MC, methylation class; RTK, receptor tyrosine kinase.

Table 3. Glioblastoma (GBM) subtype, methylation status for O6-methylguanine methyltransferase (MGMT), and leukocytes unmethylation for purity (LUMP) score for long-term survivors (LTS) and short-term survivors (STS) with a confirmed IDH-wild-type GBM

ID	Group	GBM subtype	MGMT status	LUMP score
1	LTS	Mesenchymal	Methylated	0,49301
2	LTS	Mesenchymal	Methylated	0,55363
3	LTS	Mesenchymal	Methylated	0,58812
4	LTS	Mesenchymal	Methylated	0,65676
5	LTS	Mesenchymal	Unmethylated	0,31907
6	LTS	Mesenchymal	Methylated	0,46122
7	LTS	Mesenchymal	Methylated	0,4415
8	LTS	Mesenchymal	Methylated	0,4897
9	LTS	Proneural	Methylated	0,72869
10	LTS	Classical	Methylated	0,80401
11	LTS	Classical	Methylated	0,80194
12	LTS	Classical	Methylated	0,63695
13	LTS	Classical	Unmethylated	0,67929
14	LTS	Classical	Methylated	0,82088
15	LTS	NA	Unmethylated	0,67598
16	LTS	NA	Methylated	0,7426
26	STS	Mesenchymal	Unmethylated	0,64247
27	STS	Mesenchymal	Unmethylated	0,47079
28	STS	Mesenchymal	Unmethylated	0,55096
29	STS	Mesenchymal	Unmethylated	0,62976
30	STS	Mesenchymal	Unmethylated	0,48075
31	STS	Mesenchymal	Methylated	0,42674
32	STS	Mesenchymal	Methylated	0,54891
33	STS	Mesenchymal	Unmethylated	0,34751
34	STS	Proneural	Methylated	0,73158
35	STS	Proneural	Methylated	0,48358
36	STS	Proneural	Methylated	0,61091
37	STS	Proneural	Unmethylated	0,53849
38	STS	Proneural	Unmethylated	0,5556
39	STS	Proneural	Methylated	0,61741
40	STS	Proneural	Unmethylated	0,63249
41	STS	Proneural	Unmethylated	0,58583
42	STS	Classical	Unmethylated	0,68724
43	STS	Classical	Unmethylated	0,68781
44	STS	Classical	Methylated	0,82667
45	STS	Classical	Unmethylated	0,60219
46	STS	Classical	Unmethylated	0,67339
47	STS	Proneural	Unmethylated	0,80295
48	STS	Proneural	Methylated	0,63177

ID, patient study number; NA, not available.

Some other studies ($n = 18–55$) found that some genes are more common in LTS, but did not reach the level of being statistically significant: EGFR,[20] TP53,[20,21] and PTEN.[21] In our study, the homozygous deletion of CDKN2A/B was significantly more present in the STS group, which is in line with CDKN2A/B loss that has been described as a prognostic factor for a worse outcome in IDH-mutant glioma[31] and in IDH-wild-type GBM.[14,15]

Following our CpG analysis, we identified differentially methylated genes between LTS versus STS. Subsequently,

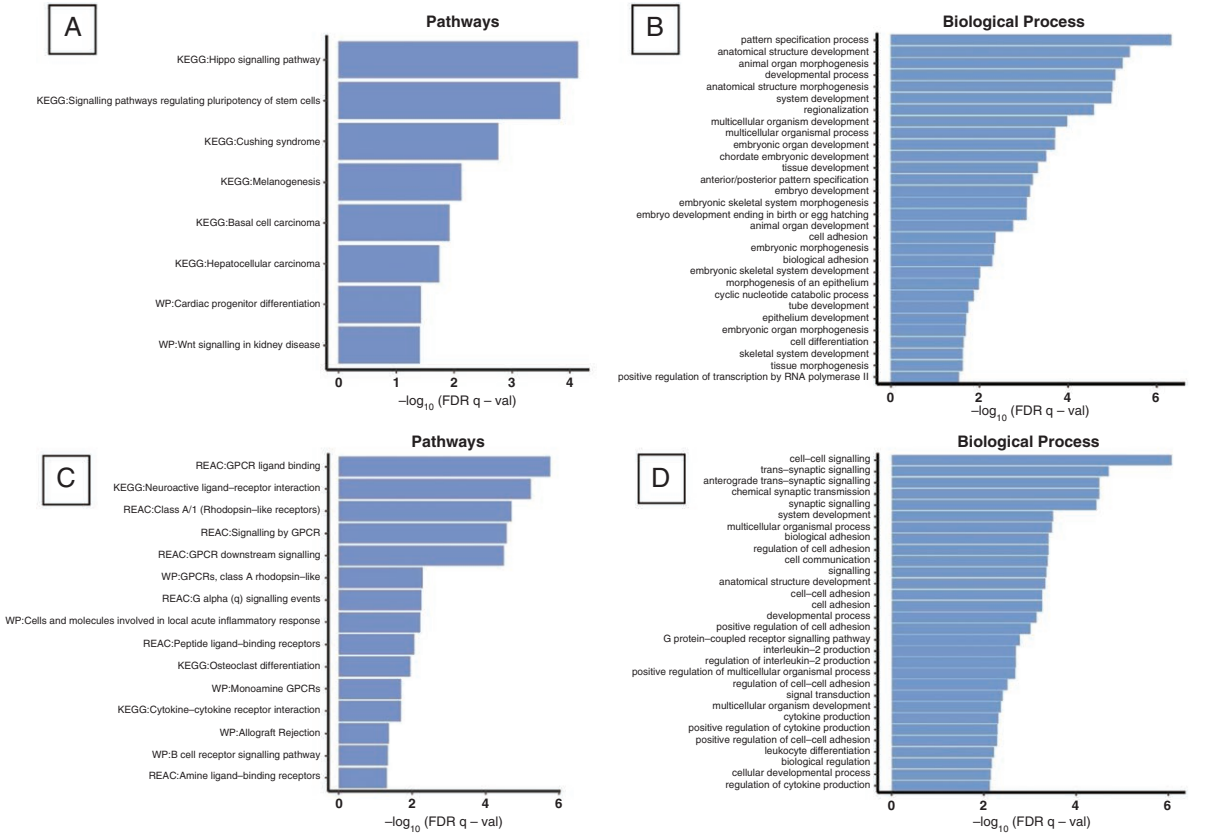

Figure 1. Upregulated pathways in long-term survivors (A) and in short-term survivors (C), and involved biological processes in long-term survivors (B) and short-term survivors (D).

we identified upregulated pathways in LTS and in STS. The most common upregulated pathway in LTS is the Hippo signaling pathway. This pathway is involved in many different cancers, such as renal cell carcinoma, non–small cell lung cancer, and breast cancer. In GBM, this pathway is involved in cell proliferation, migration, invasiveness, and chemotherapy resistance.[32] Most genes involved in the Hippo pathway are tumor suppressor genes, which explains why it is upregulated in our LTS group. In more detail, when this pathway is downregulated, the next step in the cascade Yes-Associated Protein/Transcriptional Co-activator with PDZ-binding motif (YAP/TAZ) is active and promotes cell growth and inhibits apoptosis. Conversely, when the Hippo pathway is upregulated, YAP/TAZ remains inactive.[33] Moreover, the Hippo pathway has many interactions with other pathways, including the Wnt pathway (another upregulated pathway in our LTS group), which, in addition, to its own role in stem cell maintenance, tumor growth, and invasion, stimulates the Hippo signaling pathway. The Wnt pathway was also found in LTS of a recent trial in GBM in the elderly (Nordic phase 3 trial).[34] It is unclear in humans, however, why the Hippo and Wnt pathways were upregulated in LTS. In a recent mouse model, a decreased activity of the Wnt pathway was associated with an increased delivery of temozolomide.[35]

In our STS group, G-protein-coupled receptors (GPCR) were the most upregulated pathway. GPCRs represent a large family of cell-surface molecules involved in signal transduction. In various cancers, including breast cancer, small cell lung cancer, and ovarian cancer, many GPCRs are upregulated.[36] This upregulation leads to stimulated cell growth, proliferation, and angiogenesis. In GBM, a decreased expression of *ING4*, a candidate tumor suppressor gene, leads to upregulation of interleukin 8 (IL8), which stimulates angiogenesis, and subsequently tumor progression via GPCR pathways.[37]

The strength of this study derives from a relatively large cohort of sex- and age-matched patients with GBM who survived more than 5 years. Additionally, patients were collected from 2 different centers and comprehensive patient and treatment data were complete. Moreover, comprehensive methylation data including methylation-based diagnostic classification, CNV analysis, and gene analysis were performed. However, due to the retrospective nature of this study, there are several limitations. Most importantly, some FFPE samples of LTS were no longer available because samples were destroyed after the legal duration for storage has passed. As a result, some clinical information (eg, performance status at baseline, recurrences, etc.) was missing in some LTS. Although we describe a relatively large group of LTS, the sample size is modest and comparable

to previously reported studies.[9,20,21,23] Our findings have not been validated in an external cohort. The methylation classifier rendered nontumor diagnoses, based on the analyzed FFPE sample. This discrepancy with the histological diagnosis could be explained due to the analyzed sample which contained more nontumor cells than tumor cells. Moreover, some samples date back to the era before temozolomide was available and/or IDH testing was possible. Lastly, we excluded these samples in order to have a homogeneous group in the analysis of methylation differences. Larger, prospective studies are needed to confirm our results and to expand our knowledge of genetic and epigenetic changes in long-term GBM survivors. These efforts may also shed further insight into the pathogenesis of GBM and identify new molecular targets for therapeutic intervention. Notably, an ongoing large, international prognostic study of long-term (ie, >5 years) GBM patients ($n = 189$), the ETERNITY study, addresses these questions.[7]

In conclusion, few patients with an IDH-wild-type GBM survive more than 5 years, and some clinical LTS GBM patients can be shown to have an alternative diagnosis upon methylation profiling. This study highlights differences in a cohort of methylation-confirmed GBM and describes some differences in methylation patterns of gene expression and pathways between long-term and short-term GBM survivors.

Supplementary material

Supplementary material is available online at *Neuro-Oncology* (https://academic.oup.com/neuro-oncology).

Keywords

glioblastoma | long-term survivors | methylation | pathway analysis

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Conflict of interest statement

None declared.

Authorship statement

Collection of the data: R.C.R., S.A.C., N.K., R.O., K.A., P.D., and D.M.; analyzing the data: M.v.d.M., M.R.V., V.P., Q.W., and O.S.; interpretation of the data: all authors; first draft of the manuscript: M.v.d.M. and M.R.V.; revision of the manuscript: all authors; supervision of the study: G.Z. and W.P.M.

Data availability

All data have been collected and stored at University Health Network and are available anonymously upon request (https://www.zadehlab.com/biobank/).

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