In search of druggable targets for GBM amino acid metabolism

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Abstract

Background: Amino acid (AA) pathways may contain druggable targets for glioblastoma (GBM). Literature reviews and GBM database (http://r2.amc.nl) analyses were carried out to screen for such targets among 95 AA related enzymes.

Methods: First, we identified the genes that were differentially expressed in GBMs (3 datasets) compared to non-GBM brain tissues (5 datasets), or were associated with survival differences. Further, protein expression for these enzymes was also analyzed in high grade gliomas (HGGs) (proteinatlas.org). Finally, AA enzyme and gene expression were compared among the 4 TCGA (The Cancer Genome Atlas) subtypes of GBMs.

Results: We detected differences in enzymes involved in glutamate and urea cycle metabolism in GBM. For example, expression levels of BCAT1 (branched chain amino acid transaminase 1) and ASL (argininosuccinate lyase) were high, but ASS1 (argininosuccinate synthase 1) was low in GBM. Proneural and neural TCGA subtypes had low expression of all three. High expression of all three correlated with worse outcome. ASL and ASS1 protein levels were mostly undetected in high grade gliomas, whereas BCAT1 was high. GSS (glutathione synthetase) was not differentially expressed, but higher levels were linked to poor progression free survival. ASPA (aspartoacylase) and GOT1 (glutamic-oxaloacetic transaminase 1) had lower expression in GBM (associated with poor outcomes). All three GABA related genes – glutamate decarboxylase 1 (GAD1) and 2 (GAD2) and 4-aminobutyrate aminotransferase (ABAT) – were lower in mesenchymal tumors, which in contrast showed higher IDO1 (indoleamine 2, 3-dioxygenase 1) and TDO2 (tryptophan 2, 3-dioxygenase). Expression of PRODH (proline dehydrogenase), a putative tumor suppressor, was lower in GBM. Higher levels predicted poor survival.

Conclusions: Several AA-metabolizing enzymes that are higher in GBM, are also linked to poor outcome (such as BCAT1), which makes them potential targets for therapeutic inhibition. Moreover, existing drugs that deplete asparagine and arginine may be effective against brain tumors, and should be studied in conjunction with chemotherapy. Last, AA metabolism is heterogeneous in TCGA subtypes of GBM (as well as medulloblastomas and other pediatric tumors), which may translate to variable responses to AA targeted therapies.

Keywords: Glioblastoma (GBM), Amino-acid (AA) metabolism, BCAT1 (branched chain amino acid transaminase 1), Asparagine (Asn), Glutamine (Gln)

Background

In addition to surgery and radiation, brain tumors are subject to systemic therapies, which circulate in the bloodstream and affect cancer cells all over the body. The systemic therapies for cancer can be grouped into 4 main categories: (1) DNA damaging and/or repair suppressing agents [1] (e.g., cytotoxic chemotherapy); (2) cell signaling inhibition [1–3] (e.g., blocking tumor angiogenesis and tyrosine kinases); (3) immunotherapy [4, 5]; and (4) metabolic strategies [6]. Metabolic approaches are based on assumed differences in metabolism in cancer cells compared to normal tissues [6, 7]. Antimetabolites largely act by diminishing synthesis of molecules essential for cancer cell survival, either by substrate depletion or by interfering with enzyme(s) [6]. Classic examples include asparaginase for acute leukemias [8] and the anti-folate drug, methotrexate, for a variety of tumors [9]. A major advantage of antimetabolites is the absence of direct DNA damage,
which leads to significant bone marrow toxicity [10], and may cause secondary malignancies [11]. Although signal-
ing inhibition and immunotherapy also lack myelosup-
pression, clinical efficacy of these "targeted" strategies has been limited to only certain types of cancer [3, 5].

The recent discovery of mutations in IDH (isocitrate dehydrogenase, a Krebs cycle enzyme) in some gliomas [12] has renewed interest in antimetabolic approaches in neuro-oncology [13]. In addition to the use of IDH1 and IDH2 inhibitors [12], targeting lipid [14] and carbohy-
drate (i.e., energy) metabolism has also been an area of research (e.g., use of metformin [15]). Moreover, the augmented amino acid metabolism in brain tumors has led to enhanced neuro-imaging with radiolabeled amino acids as a diagnostic tool [16, 17]. However, manipulation of amino acid metabolism remains an under-studied topic in current neuro-oncology research, and is therefore the topic of this investigation.

Methods

Publically available databases and published literature were used for this study. Our general hypotheses were:

(a) differential expression of genes related to amino-
acid (AA) metabolism and the corresponding enzymes can help to identify potential drug targets for glioblast-
oma treatment;
(b) correlations among certain genes (or enzymes) and patient survival may indicate clinical relevance; and (c) subtypes of brain tumors may show heterogeneity in AA metabolism.

First, we constructed a list of 95 genes that code for amino-acid metabolizing enzymes, based on known biochemical pathways (Table 1) [18]. Analyses of 22 AA KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways suggested by TCGA data were also used in developing the list. To assess potential differential expression, we used the "R2: Genomic Analysis and Visualization Platform" database (s) at http://r2.amc.nl [19]. R2 contains multiple datasets on various patho-
logical conditions from gene expression microarrays. Datasets generated on 2 Affymetrix chip types, both an-
alyzed by MAS5.0, were used in our study. In addition, certain datasets allowed patient survival analysis in re-
lation to gene expression levels. Selected glioblastoma
(GBM) datasets in R2 also allowed analysis based on TCGA subtypes.

Eight datasets, including 3 with GBM and 5 with non-GBM brain tissues, were used to review metabolic differences in GBM (Table 2). In order to minimize am-
biguity, we selected 5 non-GBM/control datasets con-
taining information on non-neoplastic brain tissues with or without concomitant conditions (such as mild cognitive impairment, agonal stress or Parkinson’s disease). Initially, we screened the entire pool of 95 genes in 3 of the largest GBM datasets, using R2 bar-graphing tools and Kaplan-Meier curves to identify potentially relevant candidates (representative graphs are shown in Results). Gene probes were selected based on higher expression and availability of the same probe across the datasets and for Kaplan-Meier analysis. About a third of the genes appeared to be either differentially expressed, or have significant association with clinical outcome (i.e., progression free survival and/or overall survival). A few genes were included in our analysis solely based on liter-

ature reports on relevance to GBM. For the 34 genes resulting from this initial analysis, we aimed to verify quantative expression in GBMs and compare these values to expression levels in non-GBM brain tissues.

Statistics for differential gene expression in GBM versus non-GBM

Datasets 1–5 from Table 2 were generated by Affymetrix Human Genome U133 Plus 2.0 arrays (u133p2), and datasets 6–8 by u133pa. To avoid possible misinterpre-

ation of results due to use of the two different arrays, the average gene expression levels were kept in two groups: Mean-A (for datasets 1, 2 and 3); and Mean-B (for datasets 6 and 7). Next, for each gene we calculated 3 ratios of expression, from 3 GBM datasets (using GBM/non-GBM from the same array):

1) Ratio 1 = Gene expression from dataset #4 over Mean-A,
2) Ratio 2 = Gene expression from dataset #5 over Mean-A, and
3) Ratio 3 = Gene expression from dataset #8 over Mean-B.

Last, averages (± standard errors) of ratios 1, 2 and 3 were calculated for each gene (Fig. 1). This procedure allowed us to evaluate differential expression more reli-
ably, and to eliminate a few genes that were proposed in the initial screen.

Protein expression of AA related enzymes in high grade gliomas

Gene expression levels may not always correlate with pro-
tein production. Therefore, further verification of our find-
ings at the protein level was considered. An online database (Proteinatlas.org) contains immunohistochemical
(IHC) data on most human proteins in a variety of tissues, including gliomas, as part of a cancer atlas project [20]. The database was used to evaluate protein expression for the panel of 34 genes with possible differential expression in high grade gliomas (HGGs). Each tested tumor has a semi-quantitative antibody staining score (i.e., high, medium, low or not detected; representative examples are shown in Fig. 2). The average number of high grade glioma specimens tested for each protein was 8 (range, 5–11).
| Pathways                                      | Gene/Enzyme                                                                 |
|----------------------------------------------|-----------------------------------------------------------------------------|
| Alanine, asparagine, aspartate, glutamine,   | 1. ABAT: 4-aminobutyrate aminotransferase                                    |
| & glutamate metabolism:                      | 2. ADSL: adenylsuccinate lyase                                               |
|                                              | 3. ADSS: adenylsuccinate synthetase                                          |
|                                              | 4. AGXT: alanine-glyoxylate aminotransferase                                |
|                                              | 5. DDO: D-aspartate oxidase                                                  |
|                                              | 6. ASNS: asparagine synthetase                                               |
|                                              | 7. ASPA: aspartoacylase                                                     |
|                                              | 8. GAD1: glutamate decarboxylase 1                                           |
|                                              | 9. GAD2: glutamate decarboxylase 2                                           |
|                                              | 10. GOT1: glutamic-oxaloacetic transaminase 1, soluble (i.e., AST: aspartate |
|                                              | transaminase, AspAT/ASAT/AAT or SGOT)                                      |
|                                              | 11. GOT2: glutamic-oxaloacetic transaminase 2, mitochondrial                |
|                                              | 12. GPT: glutamic-pyruvate transaminase (i.e. ALT: alanine aminotransferase) |
|                                              | 13. GLUD1: glutamate dehydrogenase 1                                         |
|                                              | 14. GLUD2: glutamate dehydrogenase 2                                         |
|                                              | 15. ALDH5A1: Aldehyde Dehydrogenase 5 Family, Member A1                     |
|                                              | 16. GLUL: glutamine synthetase (i.e., GS)                                   |
|                                              | 17. GFPT2: glutamine-fructose-6-phosphate transaminase 2                     |
|                                              | 18. MECP2: methyl CpG binding protein 2                                      |
|                                              | 19. GLS: glutaminase                                                        |
| Histidine metabolism:                        | 20. ALDH1B1: aldehyde dehydrogenase 1 family, member B1                     |
|                                              | 21. CNDP2: CNDP dipeptidase 2 (metallopeptidase M20 family)                 |
|                                              | 22. HDC: Histidine dioxoxygenase                                             |
|                                              | 23. HAL: histidine ammonia-lyase (i.e., Histidase: HIS or HSTD)              |
| Leucine, isoleucine, & valine metabolism:    | 24. BCAT1: branched chain amino-acid transaminase 1, cytosolic              |
|                                              | 25. BCAT2: branched chain amino-acid transaminase 2, mitochondrial          |
|                                              | 26. LRS: Leucyl-tRNA synthetase                                              |
|                                              | 27. BCKDHB: branched chain keto acid dehydrogenase E1, beta polypeptide      |
|                                              | 28. ILVBL: ilvB (bacterial acetolactate synthase)-like                       |
|                                              | 29. PCCB: propionyl CoA carboxylase, beta polypeptide                       |
| Lysine metabolism:                           | 30. AASDHPP1: L-aminoacidopsitate-semialdehyde dehydrogenase-phosphopantethe |
|                                              | 31. PIPOX: pipeolic acid oxidase                                             |
|                                              | 32. WHSC1L1: Wolf-Hirschhorn syndrome candidate 1-like 1                     |
| Phenylalanine metabolism:                   | 33. PAH: phenylalanine hydroxylase                                           |
|                                              | 34. FAH: fumarylacetoacetate hydrolase (fumarylacetoacetase)                |
| Serine, glycine, & threonine metabolism:    | 35. ALAS1: S'-aminolevulinate synthase 1                                    |
|                                              | 36. ALAS2: S'-aminolevulinate synthase 2                                    |
|                                              | 37. GCAT: glycine C-acetyltransferase                                        |
|                                              | 38. PHGDH: phosphoglycerate dehydrogenase                                   |
|                                              | 39. PSAT1: phosphoserine aminotransferase 1                                 |
|                                              | 40. PSHPH: phosphoserine phosphatase                                         |
|                                              | 41. SDS: serine dehydratase                                                  |
|                                              | 42. SHTMT1: serine hydroxymethyltransferase 1                               |
|                                              | 43. SHTMT2: serine hydroxymethyltransferase 2                               |
|                                              | 44. SPTLC1: serine palmitoyltransferase, long chain base subunit 1           |
|                                              | 45. SPTLC2: serine palmitoyltransferase, long chain base subunit 2           |
|                                              | 46. SPTLC3: serine palmitoyltransferase, long chain base subunit 3           |
|                                              | 47. PPP2R4: protein phosphatase 2A activator, regulatory subunit 4 (i.e.,  |
|                                              | 48. ALAD: Aminolevulinic dehydrogenase                                       |
| Tyrosine metabolism:                         | 49. PNMT: phenylethanolamine N-methyltransferase                            |
|                                              | 50. TH: tyrosine hydroxylase                                                |
|                                              | 51. TAT: tyrosine aminotransfer                                              |
|                                              | 52. DDC: DOPA decarboxylase (aromatic L-amino acid decarboxylase)           |
| Cysteine, methionine, & glutathione          | 53. CCBL1: cysteine conjugate-beta lyase, cytoplasmic                        |
| metabolism:                                  | 54. CCBL2: cysteine conjugate-beta lyase 2                                  |
|                                              | 55. LDHA: lactate dehydrogenase A                                            |
|                                              | 56. AHCY: adenosylhomocysteinase                                             |
|                                              | 57. MDH2: malate dehydrogenase 2, NAD (mitochondrial)                      |
|                                              | 58. TYMS: thymidylate synthase                                               |
|                                              | 59. CTH: cystathionine gamma-lyase                                           |
|                                              | 60. GCLC: glutamate-cysteine ligase, catalytic subunit                      |
|                                              | 61. GCLM: glutamate-cysteine ligase, modifier subunit                       |
|                                              | 62. GSS: Glutathione synthetase                                              |
|                                              | 63. MTR: 5-methyltetrahydrofolate-homocysteine methyltransferase            |
Table 1 Ninety-five genes for amino acid metabolism related enzymes that were subjected to initial screening (Continued)

|   |   |
|---|---|
| 64. | MAT2A: methionine adenosyltransferase II, alpha |
| 65. | OAT: ornithine aminotransferase |
| 66. | CKM: creatine kinase, muscle |
| 67. | LAP3: leucine aminopeptidase 3 |
| 68. | ASL: argininosuccinate lyase |
| 69. | ASS1: argininosuccinate synthetase 1 |
| 70. | ADC: arginine decarboxylase |
| 71. | DDAH2: dimethylarginine dimethylaminohydrolase 2 |
| 72. | GATM: glycine amidinotransferase (L-arginine:glycine amidinotransferase) |
| 73. | ARG1: arginase 1 |
| 74. | PADD2: peptide arginine deiminase, type II |
| 75. | PYCR1: pyrroline-5-carboxylate reductase 1 |
| 76. | PRODH: proline dehydrogenase (oxidase) 1 |

- **Arginine and proline metabolism:**
  - 64. MAT2A: methionine adenosyltransferase II, alpha
  - 65. OAT: ornithine aminotransferase
  - 66. CKM: creatine kinase, muscle
  - 67. LAP3: leucine aminopeptidase 3
  - 68. ASL: argininosuccinate lyase
  - 69. ASS1: argininosuccinate synthetase 1
  - 70. ADC: arginine decarboxylase
  - 71. DDAH2: dimethylarginine dimethylaminohydrolase 2
  - 72. GATM: glycine amidinotransferase (L-arginine:glycine amidinotransferase)

- **Tryptophan metabolism:**
  - 77. AANAT: aralkylamine N-acetyltransferase
  - 78. TDO2: tryptophan 2,3-dioxygenase
  - 79. TPH1: Tryptophan hydroxylase 1
  - 80. IDO1: indoleamine 2,3-dioxygenase 1

- **Selenocompound metabolism:**
  - 81. MARS: methionyl-tRNA synthetase
  - 82. SEPHS1: selenophosphate synthetase 1

- **Other:**
  - 83. AADAT: aminoadipate aminotransferase
  - 84. UIROS: Uroporphyrineogen synthase
  - 85. UIROD: uroporphyrinogen decarboxylase
  - 86. CPS1: carbamoyl-phosphatesynthase 1, mitochondrial
  - 87. OTC: ornithine carbamoyltransferase
  - 88. PDXP: pyridoxal (pyridoxine, vitamin B6) phosphatase
  - 89. PNPO: pyridoxamine 5′-phosphate oxidase

- **Amino acid transporters:**
  - 90. SLC3A2: solute carrier family 3 (amino acid transporter heavy chain), member 2 (i.e., 4F2hc)
  - 91. SLC7A11: solute carrier family 7 (anionic amino acid transporter light chain, xc- system), member 11 (i.e., xCT)
  - 92. SLC7A7: solute carrier family 7 (amino acid transporter light chain, y + L system), member 7 (i.e., LAT3)
  - 93. SLC7A5: solute carrier family 7 (amino acid transporter light chain, L system), member 5 (i.e., LAT1)
  - 94. SLC1A5: solute carrier family 1 (neutral amino acid transporter), member 5 (i.e., ASCT2)
  - 95. SLC6A14: solute carrier family 6 (amino acid transporter), member 14

Figure 2 shows the numbers of tumors with each of the 4 levels of antibody staining, for a given protein. IHC for a few proteins was done with more than one antibody. Selection was based on the most consistent staining pattern, for these proteins.

TCGA database in R2: subtypes and survival analyses
This enriched database contains 540 GBM samples and is the largest among the 3 tested. It allows detailed analysis of patient survival with the Kaplan-Meier method. Comparison of expression of various genes among the

| # | Name of dataset                        | Number of samples | Platform - Chiptype |
|---|---------------------------------------|-------------------|---------------------|
| 1 | Normal Brain regions - Berchtold      | 172               | u133p2              |
| 2 | Normal Brain PFC – Harris             | 44                | u133p2              |
| 3 | Disease Brain - Liang                 | 34                | u133p2              |
| 4 | Tumor Glioblastoma - Loeffler         | 70                | u133p2              |
| 5 | Tumor Glioblastoma - Hegi             | 84 (80 tumors)    | u133p2              |
| 6 | Normal Brain agonal stress - Li       | 1168              | u133a               |
| 7 | Disease Brain Parkinson - Moran       | 47                | u133a               |
| 8 | Tumor Glioblastoma - TCGA             | 540               | u133a               |
| 9 | Mixed Pediatric Brain (Normal-Tumor) – Donson | 130 (117 tumors) | u133p2              |
| 10| Tumor Medulloblastoma – Gilbertson    | 76 (73 tumors)    | u133p2              |

*Brain tissues are from individuals who had been diagnosed with mild cognitive impairment. Detailed description of each dataset is available at http://r2.amc.nl
4 TCGA subtypes is also possible (proneural, neural, classical and mesenchymal; 85 specimens). For Kaplan-Meier analysis, both progression-free survival (PFS) and overall survival (OS) were assessed for each of the genes with various cut-offs, aiming for \( P \) values <0.05 (which were considered significant). However, survival analysis in relation to gene expression levels within each subtype was not feasible, due to small sample sizes.

**Fig. 1** Differential expression of 34 genes in glioblastoma (GBM). The x-axis represents the logarithm of the ratio of gene expression in GBM over expression in normal brain tissue (calculations as described in Methods). Each horizontal bar with errors represents a gene, and ratios are shown as means ± standard errors. Genes listed starting from TDO2 and above are over-expressed genes. Genes listed starting from ASS1 and below are under-expressed genes. Refer to Table 1 for abbreviations. Log of Mean values = 1 indicates equal expression in GBMs and normal brain tissue.

**Gene expression “heat maps” for 34 genes**

Heat maps were constructed using 3 datasets from R2 (datasets 8, 9 and 10, Table 2). We aimed to display heterogeneity in the form of under- versus over-expression of 34 genes in the 4 GBM and 4 medulloblastoma subtypes (as defined in TCGA; Fig. 4 and Additional file 1: Figure S1, respectively), as well as in 4 types of pediatric brain tumors versus non-diseased brain (Additional file 2: Figure S2).
The heat maps were obtained by hierarchical clustering on samples within every defined subgroup of a data set separately, followed by clustering over the genes (complete cohort).

**Results**

**Differential expression of enzyme genes in GBM and proteins in HGG**

Differential expression was defined as a ≥40% difference (higher or lower) in gene expression for any gene, in GBM compared to non-GBM specimens. Fewer than 30 genes involved in AA metabolism met this criterion (Fig. 1). Protein detection by IHC reflected gene expression levels in roughly two-thirds of the 34 genes (Fig. 2). Specifically, over-expressed genes had a higher proportion of samples with medium to high IHC staining of the expressed protein. In contrast, under-expressed genes were associated with low or undetected protein staining. This observation was true for most, but not all, genes and enzymes analyzed.

**Survival in relation to gene expression**

Expression of some of the 34 genes correlated with progression free and/or overall survival (Fig. 3). For example, higher levels of some genes that are upregulated in GBM were associated with poor outcome, or versa. However, other genes showed the reverse (occasionally following predictions based on protein levels). Some genes did not play a role in patient outcome altogether (Table 3). Interestingly, we also identified a group of genes that may play a role in outcome, but were not differentially expressed. Overall, it appears that dramatic differences in expression are more likely to result in survival differences, especially when gene
expression correlates with protein production (Table 3). Genes that are over-expressed in GBM and also associated with poor survival at high expression levels may be the top candidates for therapeutic inhibition (dark gray shaded box in Table 3).

**TCGA subtypes demonstrate heterogeneity for genes involved in AA metabolism**

Thirty-four genes were tested in one of the datasets, where TCGA grouping was available for 85 samples (17 neural, 17 classical, 27 mesenchymal and 24 proneural). A complex pattern of heterogeneity was observed (Fig. 4).

Although further confirmation is needed, the results suggest distinct patterns of amino acid metabolism in the 4 TCGA subtypes, as measured by gene expression.

**Pediatric brain tumor types and medulloblastoma subtypes also may have distinct signatures of AA metabolism**

In addition to GBM, we analyzed the same 34 genes in two other datasets in R2 (#9 and #10 in Table 2). One contains pediatric brain tumor samples (15 pilocytic astrocytomas, 34 glioblastomas, 22 medulloblastomas and 46 ependymomas). The other is a medulloblastoma
dataset, grouped into 4 subtypes (10 SHH, 8 WNT, 16 G3, and 39 G4). As for GBM TCGA subtypes above (Fig. 4), we prepared gene expression heat maps reflecting over- and under-expression of genes in medulloblastoma subtypes and pediatric brain tumors (Additional file 1: Figure S1 and Additional file 2: Figure S2, respectively). In both cases, one can appreciate AA gene expression variability among the subtypes. There were no proteins or patient survival data available for analysis. However, these observations provide preliminary findings for further analysis and preclinical therapeutics development.

**Table 3** Relationship between expressions of 34 selected genes and Kaplan-Meier analysis

| Enzymes with higher expression in GBM | Enzymes with lower expression in GBM |
|--------------------------------------|--------------------------------------|
| BCAT1, ASL, LAP3, PIPOX, GFPT2, DDO  | PRODH, ASS1, GSS, GLUL, SEPHS1, ABAT, AGXT |
| | |

higher expression is linked to poor survival

lower expression is linked to poor survival

expression is not correlated with survival

Enzymes with expression as in normal brain

CNDP2, GSS, GLUL (GS), PHGDH, SEPHS1, ABAT, ALT (GPT), ASNS, MTR

Enzymes with lower expression in GBM

PRODH, ASS1, AST (GOT1), ASPA, GLUD1/GLUD2, GAD1/GAD2, FAH, TH

*Survival curves for footnoted genes are shown in Fig. 3. Genes in bold have concordant protein (by IHC) and mRNA expression (by microarray).*

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**Fig. 4** Heat map showing expression of 34 genes in GBM according to 4 TCGA subtypes. The colored bars at the tops of the heat maps indicate the GBM subtypes (from left to right): red – classical; purple – proneural; green – mesenchymal; blue – neural. Gene expression intensities are illustrated by shades of: green for lower levels of gene expression; black for a neutral level of gene expression; and red for higher levels of gene expression. Names of genes are abbreviated as in Table 1.
Findings on specific genes and enzymes are addressed in the Discussion section.

**Discussion**

Glioblastoma therapy continues to remain a major clinical challenge due to poor outcomes, with >90% of patients succumbing from their disease within 3 years of diagnosis [21]. Although immunotherapy and inhibition of cancer cell signaling hold promise, the “cornerstone” of current therapy against GBM remains DNA damaging strategies combined with surgery [22]. Targeting cancer metabolism by starving cancers of essential nutrients should be combinable with DNA damaging chemotherapy, due to lack of myelosuppression. Because lipid and energy metabolism is being investigated more intensively, this pilot study was designed to review brain tumor databases, to identify potentially druggable sites by interrogating amino acid-related metabolic pathways in GBM. Gene and protein expression patterns, in conjunction with survival data in GBM, were used as the main tools for searching for such targets. In addition, known amino acid depleting strategies, based on the available armamentarium and reported efficacy, are also considered in this discussion (Fig. 5). The analysis showed that 7 enzymes, namely, BCAT1, ASL, LAP3, PIPOX, GFPT2, DDO and FAH were upregulated variably in GBMs and were associated decreased survival. However, ASL and FAH upregulation did not translate into protein overproduction (Table 3 and Fig. 2). While it remains unclear how patient survival is affected by expression of these enzymes, a deeper follow-up metabolic exploration of brain cancers and other malignancies may be useful.

**BCAT1 (branched chain amino acid transaminase 1)**

The enzyme catalyzes the reversible transamination of branched-chain alpha-keto acids to branched-chain L-amino acids. BCAT1 has a well proven role in IDH\(^{WT}\) GBM reported in the literature [23]. In our study, there is higher expression of BCAT1 in GBM compared to non-GBM. Both PFS and OS are affected adversely by higher levels of expression in GBM, as well as by high levels of the protein (detected by IHC in HGGs). Taken together, these results suggest that development of BCAT1 inhibitors may have promising clinical potential. Neural and proneural tumors have lower BCAT1, making them less likely to respond to BCAA metabolism manipulation. The role of BCAT1 in other cancers may also be investigated.

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![Fig. 5 Summary of metabolic pathways in relation to selected potential targets for GBM therapy. The complex interplay among biochemical reactions in amino-acid metabolism in a metabolic network affects mitochondrial energy production and nitrogen utilization. Enzymes are in rounded boxes, and substrates are in squared boxes. A few black boxes highlight the most relevant targets. Abbreviations: ABAT, 4-aminobutyrate aminotransferase; αKG, alpha-keto-glutarate; ALT, alanine aminotransferase (also known as GPT); ARG, arginine; ASL, argininosuccinate lyase; ASN, asparagine; ASNS, asparagine synthetase; ASP, aspartate; ASPA, aspartoacylase; ASS1, argininosuccinate synthase 1; AST, aspartate aminotransferase (also known as GOT1); BCAAs, branched-chain amino acids; BCAT1, branched chain amino-acid aminotransferase 1; BCKA, branched chain ketoacids; CYS, cysteine; DDO, D-aspartate oxidase; GABA, gamma-amino butyric acid; GAD1, glutamate decarboxylase 1; GLN, glutamine; GLU, glutamate; GLS, glutaminase; GSS, glutathione synthetase; IC, isocitrate; IDH\(^{MUT}\), isocitrate dehydrogenase, mutated; 2HG, 2-hydroxoglutarate; NAA, N-acetyl-L-aspartic acid; OA, oxaloacetate; P5C, 1-pyrroline-5-carboxylate; PIPOX, pipecolic acid and sarcosine oxidase; PRO, proline; PRODH, proline dehydrogenase; SHMT1, serine hydroxymethyltransferase 1; TCA, tricarboxylic acid.](image-url)
Arginine metabolism
Higher expression of ASS1 (argininosuccinate synthase 1) and ASL (argininosuccinate lyase) genes are associated with poor PFS and/or OS. However, only the ASL gene is differentially over-expressed in GBMs. And at the protein level, both ASL and ASS1 enzymes are low or undetected in HGGs. In spite of this complex pattern, it has been shown recently that human recombinant arginase-induced arginine depletion is selectively cytotoxic to human glioblastoma cells [24]. Moreover, arginine deiminase is active against GBM in vitro and in vivo [25]. Low ASS1 and ASL proteins in HGGs support further testing of arginine-depletion against GBM. An alternative formulation to be considered is PEG-ADI, which was used in a phase 2 trial for hepatocellular carcinoma [26].

Amino-acid depleting enzymes, such as arginase or asparaginase are large molecules, which may not penetrate an intact blood–brain barrier (BBB). Nevertheless, it is well documented that CSF asparagine, for instance, decreases significantly after asparaginase administration to acute lymphoblastic leukemia patients [27]. Therefore, penetration of these enzymes into parenchyma may not be necessary for an anti-tumor effect, inasmuch as substrate depletion influences the extra-vascular microenvironment of the CNS. In addition, parts of the BBB may not be completely intact [28] – theoretically allowing direct entry of enzymes. Intracranial brain tumor mouse model testing will be the best next step to assess potential synergy of amino-acid depleting strategies with other therapies.

Methionine
MTR (5-methyltetrahydrofolate–homocysteine methyltransferase) was the main methionine related enzyme, whose gene expression levels were slightly elevated in GBM. However, expression levels did not meet our definition of differential expression. MTR was not associated with clinical outcome. Moreover, there was neither differential expression in TCGA subtypes, nor high protein levels. Nevertheless, clinical observations, such as great diagnostic yields from 11C-MET PET uptake testing [29], support recently suggested research on methionine-free diets in combination with temozolomide against GBM (https://clinicaltrials.gov/ct2/show/NCT00508456). This study was terminated due to low accrual. Yet, preclinical research continues to support methionine deprivation as a potential therapy for GBM [30].

Alanine and asparagine-glutamine networks
Some findings in these biochemical pathways can be summarized as differential under-expression of ASPA (aspartoacylase) and GOT1 (glutamic-oxaloacetic transaminase 1; previously known as AST, or aspartate aminotransferase) in GBM. Both are associated with poor outcome at lower gene levels, as is lower GPT (glutamic-pyruvic transaminase; previously known as ALT, or alanine aminotransferase). The neural group had higher GOT1 and ASPA gene expression, but lower GPT. Protein counterparts of GPT and GOT1 are overall more detectable in HGGs, compared to normal tissue, whereas ASPA protein is less detectable. ASPA catalyzes conversion of N-acetyl-L-aspartic acid (NAA) to acetate and is mutated in patients with Canavan disease. Detection of elevated NAA by magnetic resonance spectroscopy (MRS) is indicative of GBM progression. Some investigators have suggested that acetate supplementation (used for Canavan disease) may serve as an adjuvant therapy against GBM [31]. Acetate use against GBM may be supported by our findings of under-expression of the ASPA gene in GBM and the ASPA protein in HGGs. Acetate use is also supported by a strong signal from another over-expressed gene in our study – PIPOX (pipecolic acid and sarcosine oxidase). PIPOX also shows high protein levels in HGGs, and high PIPOX is associated with poor outcome in GBM. PIPOX converts sarcosine to glycine (used by GSS, or glutathione synthetase) and can be inhibited by acetate [32].

The only individual, key-enzyme gene effect observed for glutamine metabolism in our study was for GLUL (glutamate-ammonia ligase; previously known as GS, or glutamine synthetase). Low GLUL levels correlated with better OS (Table 3). Nevertheless, a large body of literature suggests that the asparagine-glutamine node of amino acid metabolism may contain a credible potential target against GBM metabolism [33]. The combined effect of increased ASNS (asparagine synthetase), GLUL, and/or BCAT1 expression was shown in one of our recent studies to have a detrimental effect on patient outcomes [34]. Therefore, we consider and propose asparaginase/glutaminase as another potential adjuvant strategy against GBM. Differential expression of ASNS in ependymomas and certain types of medulloblastomas also supports asparaginase testing against these pediatric brain tumors.

GABA metabolism
Mixed gene expression for GABA related enzymes indicated that decreased production and possibly increased catabolism may be linked to poor outcome. Gabapentin, a GABA analog, inhibits substance P-induced NF-κB activation in rat gliomas and may play role in regulating inflammation-related intracellular signaling [35]. However, the hypothesis of a significant antitumor effect of GABA against GBM remains unexplored, because its analogue, gabapentin (widely used in clinical practice without major anti-GBM effects), has no direct effect on GABA binding, uptake or degradation.
Glutathione synthetase (GSS)
Interestingly, overexpression of the rate-limiting enzyme in glutathione synthesis (GCLM, or glutamate-cysteine ligase modifier subunit) was not detected in these analyses. Likewise, GSS levels were not much altered at baseline. One may predict that a potential role of GSS inhibition by the available agent, buthionine sulfoximine (BSO), may be limited to chemotherapy-induced, GSS-up-regulation cases. This has been a subject of significant research for other cancers, but not GBM [36]. A study to assess GSS upregulation after chemotherapy in GBM may be useful. Analysis of enzymatic and non-enzymatic components of antioxidant pathways – apart from amino-acid metabolism – is another valid topic for study.

Tryptophan
IDO1 (indoleamine 2, 3-dioxygenase 1) catalyzes tryptophan breakdown. Its inhibitors are aimed at suppressing tryptophan catabolism-induced cancer immunotolerance and are in clinical trials (https://www.clinicaltrials.gov/show/NCT02052648). No survival link or differential expression was observed in our analysis for GBM versus non-GBM brain tissues for IDO1 or TDO2 (tryptophan 2, 3-dioxygenase, also involved in tryptophan catabolism). However, our findings showed higher TDO2 and IDO1 in GBM, and particularly in the mesenchymal subtype, which may show better responses to immunotherapy [37]. These reports further support a potential role for manipulating tryptophan metabolism for cancer immunomodulation effects [30, 38].

Other genes
Potential targets can be expanded to a few other important genes based on our results, including: GFPT2 (glutamine-fructose-6-phosphate transaminase 2; previously reported to be high in GBM [39]); LAP3 (leucine aminopeptidase); DDO (D-aspartate oxidase); and PRODH (proline dehydrogenase, a putative tumor suppressor). Retrospective studies and preclinical validations are needed, because gene and protein databases used in this study are not the same. Also, no protein data were available on pediatric tumors and medulloblastoma. Furthermore, changes may occur in response to chemotherapy, and the tumors may harbor unknown mutations in some of these pathways (a possible subject of future studies).

Conclusions
Brain tumors have distinct gene expression patterns for certain amino acid-metabolizing enzymes. These enzymes may provide valid targets for therapeutics development. Although drugs used clinically, such as asparaginase and arginase, are readily available for preclinical testing, inhibitors have yet to be developed against other promising targets, such as BCAT1 or PIPOX. Heterogeneity is evident in various types (and subtypes) of brain tumors, which indicates the possible need for tailored manipulation of amino acid metabolism to achieve enhanced therapeutic effects and less toxicity than encountered with conventional chemotherapy.

Additional files

Additional file 1: Figure S1. Heat map of expression of 34 genes in 4 subtypes of medulloblastoma – WNT, SHH, Group 4 and Group 3. Names of genes are abbreviated as in Table 1. (DOX 92 kb)

Additional file 2: Figure S2. Heat map of expression of 34 genes in 4 types of pediatric brain tumors and non-diseased (nd) brain tissue (from left to right: glioblastomas, nd, ependymomas, medulloblastomas and pilocytic astrocytomas). Names of genes are abbreviated as in Table 1. (DOX 111 kb)

Abbreviations
ZHGC: 2-hydroxylglutarate; AA: Amino-acids; ABAT: 4-aminobutyrate aminotransferase; ARG: Arginine; ASNS: Asparagine synthetase; ASL: Argininosuccinate lyase; ASP: Asparaginase; ASPA: Aspartoacylase; ASS1: Argininosuccinate synthase 1; BBB: Blood-brain barrier; BCAs: Branched-chain amino acids; BCAT1: Branched chain amino acid transaminase 1; BCKA: Branched chain ketoacids; DDO: D-aspartate oxidase; GABA: Gamma-amino butyric acid; GAD: Glutamate decarboxylase 1; GAD2: Glutamate decarboxylase 2; GCLM: Glutamate-ammonia ligase; GCL: Glutamine; GLS: Glutaminase; GLU: Glutamate; GLUL: Glutamate-ammonia ligase; GOT1: Glutamicoxaloacetic transaminase 1; GPT: Glutamicoxaloacetic transaminase; GSS: Glutathione synthetase; IC: Isocitrate; IDH: 2HG; Isocitrate dehydrogenase, mutated; IDHWT: Isocitrate dehydrogenase, wild type; IDO1: Indoleamine 2,3-dioxygenase 1; KEGG: Kyoto Encyclopedia of Genes and Genomes; LAP3: Leucine aminopeptidase; MRS: Magnetic resonance spectroscopy; MTR: 5-methyltetrahydrofolate-homocysteine methyltransferase; NAA: N-acetyl-L-aspartic acid; NFkB: Transcription factor complex nuclear factor-kappa-B; OA: Oxaloacetate; OS: Overall survival; PFS: Progression-free survival; PIPOX: Pipecolic acid and sarcosine oxidase; PRO: Proline; PRODH: Proline dehydrogenase; PRDH: Proline dehydrogenase; TCGA: The Cancer Genome Atlas; TDO2: Tryptophan 2,3-dioxygenase

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Availability of data and materials
Publically available databases from R2: Genomics Analysis and Visualization Platform (http://r2.amc.nl) [19], and from The Human Protein Atlas (http://www.proteinatlas.org/cancer)[20] were used as materials for this study. Literature review was conducted using PubMed.

Authors’ contributions
All authors have read and approved the manuscript. EP study design, data acquisition and analysis, manuscript drafting and writing. HL contributions to conception and design, analytical discussions, manuscript writing. JK contributions to data acquisition and analysis, manuscript writing. JL contributions to conception and design, analytical discussions, manuscript writing.

Competing interests
The authors declare that they have no competing interests.

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