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Journal Article**Author(s):**

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Publication date:

2011

Permanent link:

<https://doi.org/10.3929/ethz-b-000034819>

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Originally published in:

JNCI: Journal of the National Cancer Institute 103(7), <https://doi.org/10.1093/jnci/djr036>

BRIEF COMMUNICATIONS

Differential Retinoic Acid Signaling in Tumors of Long- and Short-term Glioblastoma Survivors

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Manuscript received April 1, 2010; revised January 14, 2010; accepted January 19, 2011.

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Although the prognosis of most glioblastoma patients is poor, 3%–5% patients show long-term survival of 36 months or longer after diagnosis. To study the differences in activation of biochemical pathways, we performed mRNA and protein expression analyses of primary glioblastoma tissues from 11 long-term survivors (LTS; overall survival \geq 36 months) and 12 short-term survivors (STS; overall survival \leq 6 months). The mRNA expression ratio of the retinoic acid transporters fatty acid-binding protein 5 (FABP5) and cellular retinoic acid-binding protein 2 (CRABP2), which regulate the differential delivery of retinoic acid to either antioncogenic retinoic acid receptors or prooncogenic nuclear receptor peroxisome proliferator-activated receptor delta, was statistically significantly higher in the tumor tissues of STS than those of LTS (median ratio in STS tumors = 3.64, 10th–90th percentile = 1.43–4.54 vs median ratio in LTS tumors = 1.42, 10th–90th percentile = –0.98 to 2.59; $P < .001$). High FABP5 protein expression in STS tumors was associated with highly proliferating tumor cells and activation of 3-phosphoinositide-dependent protein kinase-1 and v-akt murine thymoma viral oncogene homolog. The data suggest that retinoic acid signaling activates different targets in glioblastomas from LTS and STS. All statistical tests were two-sided.

J Natl Cancer Inst 2011;103:598–601

Glioblastoma is the most common and most malignant primary brain tumor in adults (1). The prognosis is poor as indicated by a median survival of less than 12 months in a population-based study (2); however, a small fraction (3%–5%) of patients survive for 36 months or longer—these patients are known as long-term survivors (LTS) (3). These patients are usually younger than 50 years at the time of diagnosis, are in good clinical condition, and mostly have tumors with promoter hypermethylation of the DNA repair gene O-6-methylguanine-DNA methyltransferase (*MGMT*) (3,4). In addition, mutations in the isocitrate dehydrogenase 1 and 2 (*IDH1* and *IDH2*) genes have recently been reported in a subset of glioblastomas from younger patients and linked to a more favorable survival outcome (5–7). Nevertheless, the molecular and cellular mechanisms underlying the rare phenom-

enon of long-term survival in glioblastoma patients are not known.

To investigate the possible intrinsic differences in activation of different signaling pathways, in this study, we performed a comparative genome-wide expression profiling of patient tumors from 11 LTS (referred to as LTS tumors) and 12 short-term survivors (STS) (referred to as STS tumors) by microarray analysis (Supplementary Methods, available online). Written informed consent was obtained from the patients, and follow-up data were retrospectively determined and linked to molecular data in an anonymous manner as approved by the institutional review boards of the Medical Faculties of the Heinrich-Heine-University, Düsseldorf, Germany, and the University of Dresden, Dresden, Germany. The patient groups were balanced for Karnofsky performance score (8),

extent of tumor resection, and postoperative radiotherapy. However, the LTS patients were substantially younger in age (Supplementary Table 1, available online). Methylation-specific polymerase chain reaction analysis of CpGs within the *MGMT* promoter region revealed that methylation was observed in substantially more LTS tumors than STS tumors ($P = .181$, two-sided Fisher exact test). This finding is in agreement with previous data showing that *MGMT* promoter hypermethylation was nearly twice as common in glioblastomas from LTS compared with glioblastomas from patients with more typical average survival times of about 12 months (4). DNA sequencing analysis (Supplementary Methods, available online) of tumor series for mutations in *IDH1* codon 132 for arginine (R132) and *IDH2* codon 172 for arginine (R172) revealed mutations in nine of 11 (one R132C and eight R132H codon substitutions) LTS tumors, but in zero of 12 STS tumors ($P < .001$, two-sided Fisher's exact test). *IDH2* mutations were absent in both LTS and STS tumors. These data lend further support to the strong association of *IDH1* mutation with younger age and longer overall survival in primary glioblastoma patients (6,7,9).

Glioblastomas from LTS and STS were analyzed for mRNA expression using two different formats of the same oligonucleotide microarray platform, and two datasets were generated (Supplementary Methods, available online). To minimize experimental bias, a hypothesis was developed based on dataset 1 ($n = 7$ STS tumors and $n = 7$ LTS tumors) and validated using dataset 2 ($n = 5$ STS tumors and $n = 4$ LTS tumors), rather than pooling the raw data (Supplementary Methods, available online). Significance analysis of microarrays of dataset 1 identified 30 genes that were differentially expressed in LTS and STS tumors (Supplementary Table 2, available online); calculations were performed without applying a fold-change cutoff to identify genes with small but consistent changes. Three of the 30 genes have been described previously to participate in retinoic acid (RA) signaling—retinol binding protein 1 (*RBPI*), retinoic acid receptor responder 2 (*RARRES2*), and fatty acid-binding protein

5 (*FABP5*) (10). We investigated the mRNA expression of each of these three genes in dataset 1 and found that expression levels were higher in STS tumors ($n = 7$) compared with LTS tumors ($n = 7$) ($P < .001$ for *RBP1*, $P = .017$ for *RARRES2*, $P = .004$ for *FABP5*; two-sided Student *t* test) (Figure 1, A–C), suggesting that there was a difference in RA signaling between the two groups. Analysis of dataset 2 independently confirmed these findings (data not shown).

RBP1 stabilizes RA and transports it from the liver to the cells of the peripheral tissues (11). For nuclear import, RA is bound to cellular retinoic acid-binding protein 2 (CRABP2) and delivered to the nuclear retinoic acid receptors (RARs) (12). Activation of RARs typically leads to differentiation (13), cell cycle arrest (14), apo-

ptosis (15,16), and consequently to reduced cell proliferation (10). Recently, an alternative pathway of RA action was identified involving binding and transport of RA into the cell nucleus by FABP5, resulting in cell survival and proliferation via activation of peroxisome proliferator-activated receptor delta (PPAR δ) (10). Which of these diverse pathways is activated depends on the ratio of the respective transport proteins, FABP5 and CRABP2 (10). A low FABP5 to CRABP2 ratio leads to predominant RAR activation, whereas a high ratio results in preferential activation of PPAR δ .

In both datasets 1 and 2, CRABP2 mRNA expression did not differ between LTS and STS tumors (data not shown); however, because of consistent overexpression of FABP5 mRNA in STS tumors, statistically significantly elevated FABP5 to

CONTEXT AND CAVEATS

Prior knowledge

Approximately 3%–5% of glioblastoma patients survive 36 months or longer (long-term survivors [LTS]) compared with a median survival of less than 12 months (short-term survivors [STS]) for 95%–97% glioblastoma patients.

Study design

The intrinsic differences in activation of different signaling pathways between LTS and STS tumors were examined by microarray analysis of mRNA expression and in vitro immunohistochemical analysis of protein expression of 11 LTS and 12 STS tumors.

Contribution

The mRNA expression of three retinoic acid signaling genes was statistically significantly increased in STS tumors. Retinoic acid signaling via cellular retinoic acid-binding protein 2 (CRABP2) is involved in differentiation and reduced cell proliferation, whereas signaling via fatty acid-binding protein 5 (FABP5) is involved in cell survival and proliferation, which pathway gets activated depends on the FABP5/CRABP2 ratio. CRABP2 expression was similar in LTS and STS tumors, but a statistically significantly increased FABP5 expression was noted in STS tumors. The levels of the prosurvival proteins 3-phosphoinositide-dependent protein kinase-1 and v-akt murine thymoma viral oncogene homolog were also increased in STS tumors.

Implications

A differential retinoic acid signaling in LTS and STS tumors may be the reason for increased proliferation and reduced survival of STS tumors and could be used as a therapeutic target.

Limitations

The results are based on a small sample size and in vitro data.

From the Editors

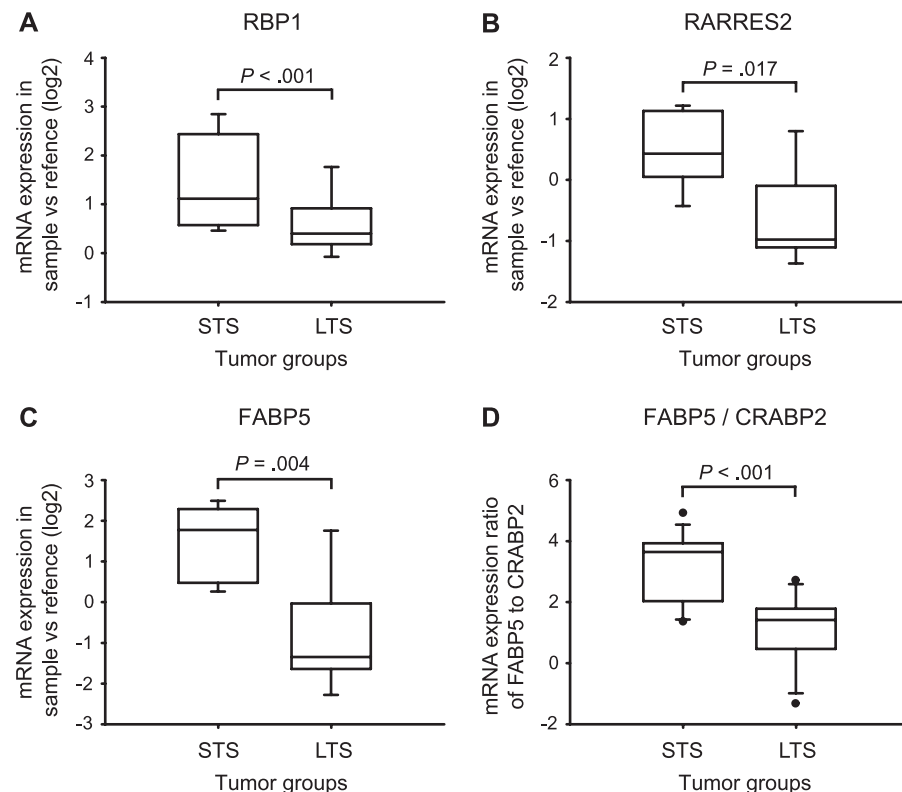


Figure 1. Analysis of mRNA expression of retinoic acid signaling genes in long-term survivors of glioblastoma (LTS) and short-term survivors of glioblastoma (STS) tumors. Two different formats of oligonucleotide microarrays were used to generate two different datasets. Microarray analysis was performed once per sample. **A**) Box plot showing the mRNA expression of retinol binding protein 1 (RBP1) in LTS tumors ($n = 7$) and STS tumors ($n = 7$) (dataset 1). **B**) Box plot showing the mRNA expression of retinoic acid receptor responder 2 (RARRES2) in LTS tumors ($n = 7$) and STS tumors ($n = 7$) (dataset 1). **C**) Box plot showing the mRNA expression of fatty acid-binding protein 5 (FABP5) in LTS tumors ($n = 7$) and STS tumors ($n = 7$) (dataset 1). **D**) Box plot showing the ratios of FABP5 to retinoic acid-binding protein 2 (CRABP2) mRNA expression in the combined datasets 1 and 2 of LTS tumors ($n = 11$) and STS tumors ($n = 12$). Medians, 25th to 75th percentiles and 10th to 90th percentiles are indicated by the horizontal lines, boxes, and error bars, respectively. In addition, the outliers are displayed as dots. *P* values were calculated using two-sided Student *t* test.

CRABP2 gene expression ratios were obtained in these tumors compared with LTS tumors (median ratio in STS tumors = 3.64, 10th–90th percentile = 1.43–4.54 vs median ratio in LTS tumors = 1.42, 10th–90th percentile = -0.98 to 2.59; $P < .001$; two-sided Student *t* test) (Figure 1, D). FABP5 to CRABP2 mRNA expression ratios did not correlate with age at diagnosis

in either group (for LTS, Spearman's rank correlation coefficient ρ [p] = -0.027, P = .924; for STS, ρ = 0.007, P = .974). Consistent with the proposed regulatory role of FABP5 to CRABP2 ratio in glioblastoma, the mRNA expression of the RAR target genes retinoic acid receptor beta (*RARB*) and cytochrome P450, family 26, subfamily A, polypeptide 1 (*CYP26A1*) was substantially higher in LTS tumors compared with STS tumors (for *RARB*, P = .005; for *CYP26A1*, P = .029; two-sided Student *t* test) (Supplementary Figure 1, available online). Increased FABP5 expression in STS tumors at the protein level was confirmed using immunohistochemistry (Figure 2, A and B). Costaining the tumor sections for the proliferation marker Ki67 protein showed that FABP5-positive cells

clustered exclusively in highly proliferative tumor regions from STS patients (Figure 2, A). FABP5 expression was generally low in LTS tumors (Figure 2, B). Double staining for glial fibrillary acidic protein, a marker of astrocytic cells, showed that FABP5 was expressed in STS tumor cells (Figure 1, C) but not in CD34-positive endothelial cells (Figure 1, D). One of the main transcriptional targets of PPAR δ is the 3-phosphoinositide-dependent protein kinase-1 (PDK1), which activates the v-akt murine thymoma viral oncogene homolog (AKT). AKT in turn promotes cell survival and inhibits apoptosis by a variety of signaling routes (17,18). Activation of the AKT signaling pathway in STS tumors was confirmed by immunohistochemical detection of high levels of phosphorylated PDK1 in

10 of 12 STS tumors, but none of 11 LTS tumors (Figure 2, E and F). This was further confirmed by immunoblot analysis (Supplementary Figure 2, available online), which showed higher ratios of phosphorylated AKT to total AKT level in STS tumors (mean ratio = 0.88; 95% confidence interval = 0.66 to 1.10) than in LTS tumors (mean ratio = 0.29; 95% confidence interval = 0.18 to 0.40).

Although RA exerts anticarcinogenic properties mainly by activating the nuclear RARs (retinoic acid receptor alpha, *RARB*, retinoic acid receptor gamma) via the fatty acid transporter CRABP2, its use in cancer therapy is rather limited because of acquisition of resistance to RA during pathogenesis (19). Indeed, in some cancers, RA is known to promote rather than reduce tumor growth (10,20). In malignant glioma, RA treatment revealed heterogeneous results (21,22). Our data indicate that the majority of glioblastomas may not be receptive to RA treatment because of the observed high FABP5 to CRABP2 ratios in these tumors. Moreover, in glioblastomas, endogenous RA may be sufficient to enforce malignant progression through activation of multiple antiapoptotic signaling cascades mediated by FABP5 to PPAR δ ratios, thereby possibly worsening the patient outcome. Therefore, pharmacological inhibition of FABP5, PPAR δ , or AKT signaling may allow bypassing of the adverse effects of RA treatment in glioblastoma patients.

Our findings provide evidence for a distinct molecular mechanism associated with long-term survival of glioblastoma, which could be exploited in the design of new treatment strategies for these still incurable tumors. However, this study has limitations such as small sample size and exclusive reliance on in vitro data. Further investigations using RA therapy in cell culture and in vivo models of LTS and STS glioblastoma are necessary to conclusively support our findings.

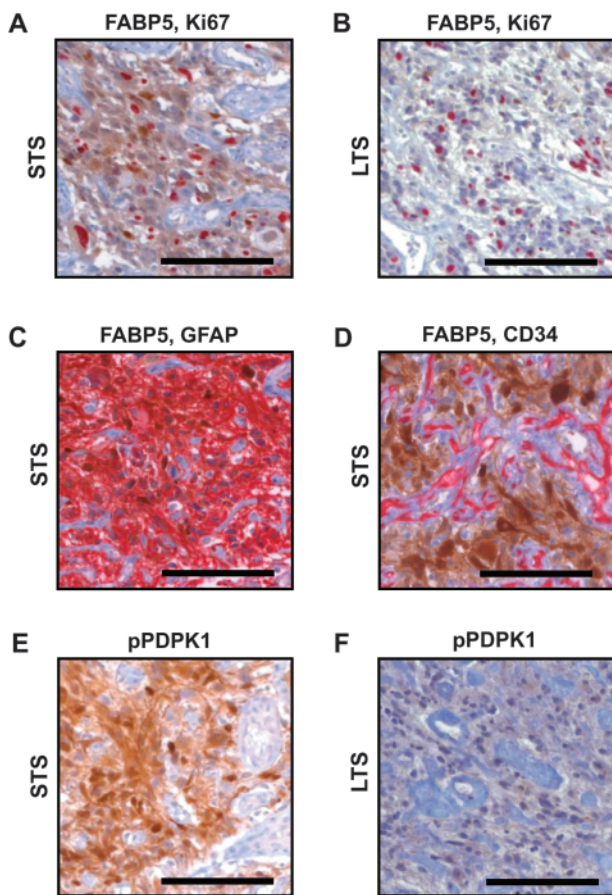


Figure 2. Association between retinoic acid signaling pathways and cell proliferation in long-term survivors of glioblastoma (LTS) and short-term survivors of glioblastoma (STS) tumors. Immunohistochemical (IHC) staining of formalin-fixed paraffin-embedded tissues was performed once per tumor per patient. **A)** IHC costaining for the cell proliferation marker Ki67 (**red**) and fatty acid-binding protein 5 (FABP5) (**brown**) in STS tumors and **B)** LTS tumors. **C)** IHC costaining for the astrocyte-specific marker glial fibrillary acidic protein (GFAP) (**red**) and FABP5 (**brown**) in STS tumors. **D)** IHC costaining for the endothelial cell marker CD34 (**red**) and FABP5 (**brown**) in STS tumors. **E)** IHC staining for phosphorylated 3-phosphoinositide-dependent protein kinase-1 (PDK1) (**brown**) in STS tumors and **F)** LTS tumors. Representative images of 12 STS tumors and 11 LTS tumors are shown. Magnification $\times 20$. Scale bar = 100 μm . p = phosphorylated.

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Funding

German Federal Ministry of Education and Research (BMBF) within the National Genome Research Network NGFN2 (01GS0460 to P.L., M.H. and B.R., 01GS0462 to G.R., 01GS0463 to C.H.) and NGFN plus (01GS0883 to P.L., M.H. and B.R., 01GS0884 to G.R.).

Notes

The authors are solely responsible for the design of the study, analysis or interpretation of the results; writing of the article; and decision to submit the article for publication. S. Barbus and B. Tews contributed equally to the manuscript. R. Martínez, G. Reifenberger, and P. Lichter share the senior authorship.

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