

Relative Expression of mRNAs Coding for Glutaminase Isoforms in CNS Tissues and CNS Tumors

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Abstract Glutaminase (GA) in mammalian tissues occurs in three isoforms: LGA (liver-type), KGA (kidney-type) and GAC (a KGA variant). Our previous study showed that human malignant gliomas (WHO grades III and IV) lack expression of LGA mRNA but are enriched in GAC mRNA relative to KGA mRNA. Here we analyzed the expression of mRNAs coding for the three isoforms in the biopsy material derived from other central nervous system tumors of WHO grades I–III. Non-neoplastic resective epileptic surgery samples served as control, as did cultured rat astrocytes and neurons. The GAC mRNA/KGA mRNA expression ratio was as a rule higher in the neoplastic than in control tissues, irrespective of the cell type dominating in the tumor or tumor malignancy. LGA mRNA expression was relatively very low in cultured astrocytes, and very low to absent in astrocytoma pilocyticum, ependymoma and subependymal giant cell astrocytoma (SEGA), tumors of astrocytic origin. LGA mRNA expression was almost as high as that of KGA and

GAC mRNA in cultured neurons and epileptic surgery samples which were enriched in neurons. LGA mRNA was also relatively high in ganglioglioma which contains a discernable proportion of neuronal cells, and in oligodendroglioma. The results show that low expression of LGA mRNA is a feature common to normal astrocytes and astroglia-derived tumor cells or ependymomas and can be considered as a cell-type, rather than a malignancy marker.

Keywords Astrocytes · Neurons · Human brain · Brain tumors · Liver-type glutaminase (LGA) · Kidney-type glutaminase (KGA) · GAC · RT-PCR

Introduction

Glutamine plays a crucial role in the metabolism of growing and proliferating cells, including neoplastic cells [1–3]. For this reason, the expression and activity of the glutamine-catabolizing enzyme glutaminase (GA, EC 3.5.1.2) has been expected to play a significant role in tumor metabolism and growth. In mammals, there are at least three isoforms of glutaminase: the liver-type (LGA), kidney-type (KGA) and an alternatively spliced variant of KGA named GAC [4]. LGA has been shown to be expressed in liver, brain and pancreas [5]. KGA is expressed in all the tissues except liver, particularly strongly in kidney and brain [6], whereas GAC is expressed in human kidney [4], rat kidney and pig renal cells [7].

A few studies have reported altered GA expression or activity in different neoplastic tissues. Originally, increased activity of GA as a whole has been noted by Matsuno and Goto [8] in hepatocellular carcinomas. Aledo et al. [9] reported increased GA activity and mRNA expression in Ehrlich ascites carcinoma cells. Later studies have

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investigated the expression of GA variants, but their relative role in different tumors could not be derived from these descriptions. Breast cancer cells have been reported to specifically overexpress GAC [4], but LGA has been shown to be a predominant isoform in a breast cancer cell line [5]. Colorectal tumor cell lines express all GA transcripts, with KGA being the dominating isoform [10], while LGA mRNA expression was found to be higher in a slow-growing adenoma cell line than in carcinoma cells [10]. Precursor malignant blast cells, as well as mature non-proliferating lymphocytes, exhibit much higher LGA than KGA mRNA levels [11]. Inhibition of KGA expression in tumor cells decreases their proliferation rate and reverts their transformed phenotype [12].

Our previous study revealed high KGA and GAC expression but lack of LGA mRNA expression in human malignant gliomas grade III and IV; however, LGA mRNA was expressed in control brain tissue and metastases from different organs [13]. In this study we extended the analysis of GA expression patterns to other brain tumors, with the specific aim to find out whether the expression of the particular isoforms relates to tumor malignancy (grade) or to the cellular composition (neuron-, astroglia- or oligodendroglia-derived). The latter question appeared of relevance in view of the recently identified abundance of LGA in neuron-rich regions of the rat cerebral cortex (14); (for an exhaustive review of the LGA issue see Ref. [15]). The study comprised human biopsy material derived from nervous system tumors of different malignancies, cellular composition and localization and for comparison, neuron-rich non-neoplastic tissue from resective epileptic surgery, and rat astrocytes or neurons grown in culture.

Experimental procedure

Biopsy material

The studies were performed on frozen tissue samples from biopsy material of pediatric brain tumors collected at the time of surgery (approved by the Ethics Committee): six astrocytomas pilocyticum (WHO grade I), six ependymomas (WHO grades II–III), six gangliogliomas (WHO grades I–III), two subependymal giant cell astrocytomas (SEGA, WHO grade I) and one oligodendroglioma (WHO grade II). The tumors were diagnosed according to the WHO classification of CNS tumours in the Department of Pathology of the Children's Memorial Health Institute, Warsaw. Non-neoplastic adult human brain tissues were obtained from three patients undergoing temporal lobe resection due to medically intractable epilepsy at the Department of Neurosurgery, Medical University of Warsaw.

Samples for isolation of nucleic acids were stored at -70°C . Histopathological analysis of tissue samples was repeated to confirm the original diagnosis and to reject non-representative material.

Cultured rat primary astrocytes and cortical neurons

Rat cerebral cortical astrocytes were cultured as described by Hertz et al. [16] Rat cortical neurons prepared as described by Hertz et al. [17] were kindly provided by Słomka (Department of Neurochemistry, Medical Research Center, PAS, Poland).

RNA isolation and RT-PCR

Total RNA from frozen tissues and from cultured astrocytes and cortical neurons was extracted using a guanidinium-thiocyanate-based commercial kit (TRI-Reagent, Sigma). 2 μg of RNA was digested with DNaseI (Invitrogen) and reverse-transcribed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's protocol. A volume of 2 μl of cDNA was used as a substrate for PCR. Cycling conditions included an initial denaturation step at 94°C for 5 min, followed by 40 cycles of 30 s at 94°C , 1 min at 58°C (LGA), 59°C (KGA and GAC), 62°C (β -actin) and 1 min at 72°C , and a final extension step of 10 min at 72°C . The PCR was carried out in a 25 μl reaction mixture with 1 unit of *Taq* DNA polymerase (Fermentas) in 1 \times PCR buffer (Fermentas), using 0.25 mM dNTPs, and 0.5 μM of each forward and reverse primer. The sequences of primers and the lengths of the products are outlined in Table 1. The primers were obtained from the Laboratory of DNA Sequencing, Institute of Biochemistry and Biophysics, Polish Academy of Science, Warsaw. PCR products were resolved on 1.5% agarose gel. Electrophoresis of PCR products for β -actin and each of the 3 GA's were always run simultaneously. A negative control containing 2 μl of RT-omitted reaction mixture instead of cDNA did not yield any product (results not shown). Each PCR was repeated at least three times on at least two independent cDNAs.

Statistical analysis

Relative intensities of the PCR products corresponding to LGA, GAC and KGA mRNAs versus β -actin were compared and statistical significance of the differences was analyzed with the unpaired *t*-Student's test.

Table 1 Primer sequences used for RT-PCR.

Name	Sequence (5'→3')	Position	Size (bp)	Genbank number
hLGAF	TCCAGCTGTGTCTGTGGAG	1322–1341	224	NM_013267
hLGAR	GGAGGATGGCTCCTGATACA	1546–1527		
hKGAF	GGTCTCCTCTCTGGATAAGATGG	1732–1755	464	NM_014905
hKGAR	CCCGTTGTCAGAAATCTCCTTGAGG	2195–2172		
hGACF	GGTCTCCTCTCTGGATAAGATGG	1745–1769	248	AF158555
hGACR	GATGTCCTCATTTGACTCAGGTGAC	1993–1969		
hACTBF	TCCTTCTGGGCATGGAGT	866–884	418	NM_001101
hACTBR	AAAGCCATGCCAATCTCATC	1284–1265		
rLGAF	GAGGGGCATCCTCAGGCT	67–84	209	NM_138904
rLGAR	GCATCTTGCTCATGCAGTCC	276–257		
rKGAF	GGTCTCCTCTCTGGATAAGATGG	1575–1598	457	NM_012569
rKGAR	CCCGTTGTCAGAAATCTCCTTGAGG	2032–2015		
rGACF	GGTCTCCTCTCTGGATAAGATGG	623–646	245	AY083459
rGACR	GATGTCCTCATTTGACTCAGGTGAC	68–846		
rACTBF	TCCTTCTGGGTATGGAAT	874–893	393	NM_031144
rACTBR	AAAGCCATGCCAATCTCATC	1267–1248		

Results

Expression of GA isoforms in cultured rat astrocytes and neurons

A ~3 times lower LGA mRNA expression was noted in astrocytes than in neurons. There was no significant difference between the levels of KGA and GAC transcripts in either cell type. LGA mRNA expression in neurons amounted to ~90% of the expression of the remaining two isoforms (Fig. 1).

Expression of GA isoforms in non-neoplastic brain tissues

In the three human neuron-rich fragments of cerebral cortex obtained from epileptic surgery (Fig. 2), KGA and

GAC transcripts showed similar expression levels, while LGA mRNA was 70% that of the other two (Fig. 3).

Expression of GA isoforms in tumor samples

We have examined expression patterns of GA isoforms in human cerebral tumors of different origin. The RT-PCR analysis revealed a low expression of LGA mRNA in 4 of 6 cases and lack of LGA mRNA expression in 2 other cases of astrocytoma pilocyticum. On average, pilocytic tumors showed ~2 times higher expression of GAC mRNA relative to KGA mRNA, while the expression of LGA mRNA was about 15% that of GAC mRNA (Fig. 4). A similar pattern of expression was found in ependymomas: expression of GAC mRNA exceeded on average ~2.3-times the expression of KGA mRNA and occurred in all cases examined. LGA mRNA was expressed in small

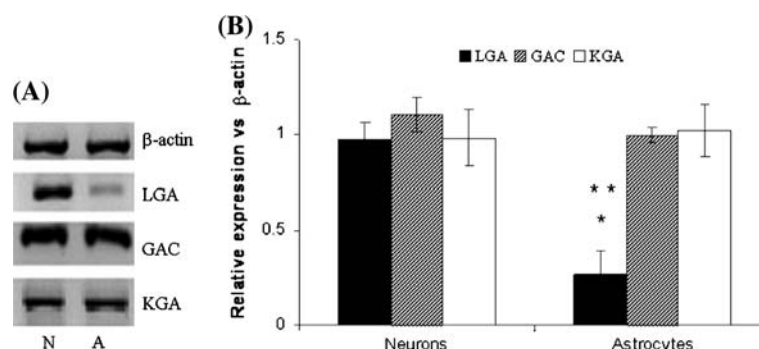


Fig. 1 Expression of mRNAs coding for GA isoforms in cultured rat cerebral cortical neurons and astrocytes. (A) Representative mRNA separation profiles for neurons (N) and astrocytes. (B) Relative

expression versus β -actin mRNA. Results are mean values \pm S.D. for three measurements. * $p < 0.05$ versus other GA isoforms; ** $p < 0.05$ versus LGA mRNA in neurons

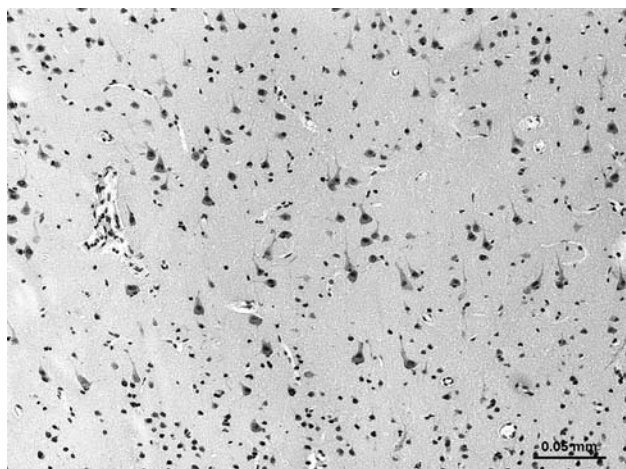


Fig. 2 Fragment of cerebral cortex from epileptic surgery. Hematoxylin & Eosin

amounts in 3 of 6 cases and not expressed at all in the remaining 3 cases (Fig. 5), which on the average amounted to 10% of the GAC mRNA expression. In the 2 cases of SEGA, GAC mRNA was likewise overexpressed as compared to KGA mRNA. LGA mRNA was undetectable in one case, and present in a very low amount in the other case (Fig. 6).

Fig. 3 Expression of mRNAs coding for GA isoforms in cerebral cortex from resective epilepsy surgery. (A) mRNA separation profiles for individual cases. (B) Relative expression versus β -actin mRNA. Results are mean values \pm S.D. for three cases. * $p < 0.05$ versus other GA isoforms

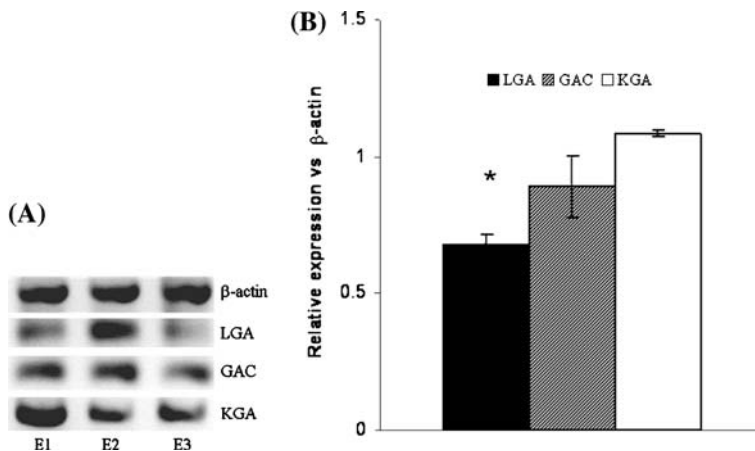
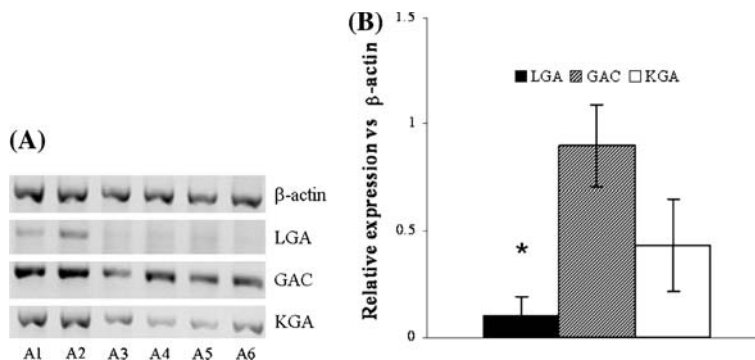


Fig. 4 Expression of mRNAs coding for GA isoforms in astrocytoma pilocyticum. (A) mRNA separation profiles for individual cases. (B) Relative expression versus β -actin mRNA. Results are mean values \pm S.D. for six cases. * $p < 0.05$ versus other GA isoforms



A different pattern of expression of PAG transcripts was observed in the material containing distinct populations of non-astrocytic cells. LGA mRNA was expressed along with KGA mRNA and GAC mRNA in all 6 cases of gangliogliomas examined: GAC mRNA showed an ~ 1.8 times higher expression than KGA mRNA, whereas LGA mRNA expression was $\sim 20\%$ that of GAC mRNA (Fig. 7). Similarly, all three GA isoforms were found in a single case of oligodendroglioma. GAC mRNA was moderately overexpressed as compared to KGA mRNA, while LGA mRNA expression was at the level of $\sim 60\%$ of GAC mRNA (Fig. 8).

Discussion

The primary goal of this study was to assess whether expression of different GA isoforms in brain tumors is related to tumor malignancy, cellular origin or both. The question was derived from a previous study from this laboratory which revealed a surprising lack of expression of a liver-type isoform (LGA) mRNA in highly malignant gliomas [13]. The results of the current study allow to draw two major general conclusions: (1) the lack of, or low expression of LGA is a feature common to astrocytes and

Fig. 5 Expression of mRNAs coding for GA isoforms in ependymoma. (A) mRNA separation profiles for individual cases. (B) Relative expression versus β -actin mRNA. Results are mean values \pm S.D. for six cases. * $p < 0.05$ versus other GA isoforms

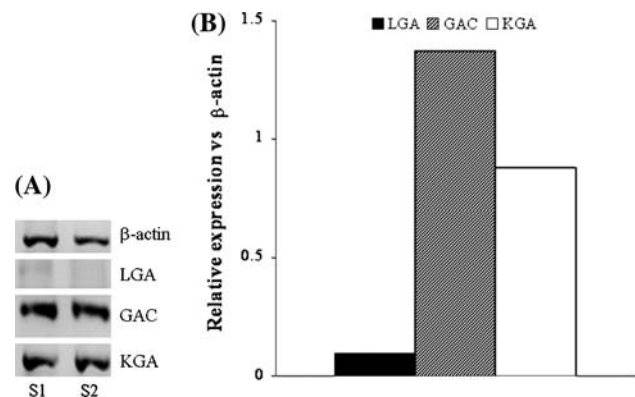
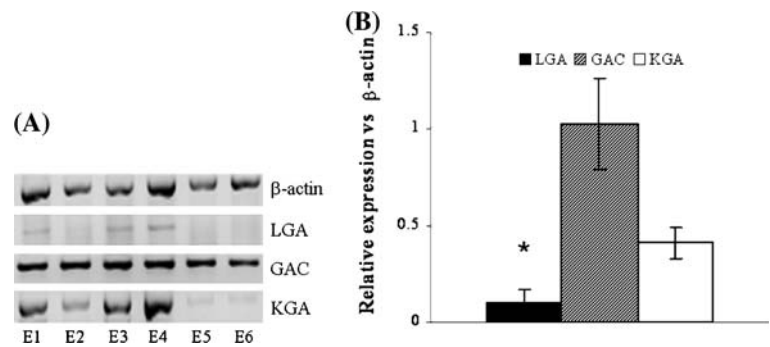


Fig. 6 Expression of mRNAs coding for GA isoforms in subependymal giant cell astrocytoma. (A) mRNA separation profiles for individual cases. (B) Relative expression versus β -actin mRNA. Results are mean values for 2 cases: LGA (0.18; 0), GAC (1; 1.7), KGA (0.7; 1.1)

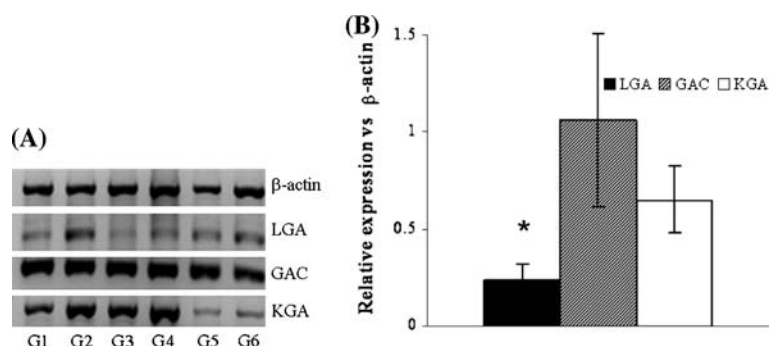
astroglia-derived tumors irrespective of their malignancy; (2) regardless of the tumor grade or cellular composition, all cerebral tumors express more strongly GAC than the other two isoforms. Taken together, the results exclude the possibility to consider expression of mRNAs coding for the GA variants as a clinically valuable diagnostic marker.

Conclusion (1) was prompted by observations made on non-neoplastic tissues which served as control. Non-neoplastic, cerebral cortex samples from epileptic surgery which contain a significant number of neuronal cells exhibit LGA mRNA levels similar to KGA and GAC transcripts. Among the two cell cultures derived from rat brain, cultured neurons

exhibit a 3-times higher relative LGA mRNA expression than cultured astrocytes. Significant LGA expression was also evidenced in all cases of gangliogliomas—tumors with mixed neuronal and glial components, both of which participate in the neoplastic process. The lack or only a trace of LGA mRNA expression in tumors of pure astrocytic or ependymal origin, and the relatively higher amounts of this transcript in tumors containing a discernable proportion of neoplastic neuronal cells, further bespeak the absence of LGA mRNA as a feature of astroglial cells, irrespective of their degree of differentiation and malignant transformation. Of note, Kvamme et al. [18] reported low immunoreactivity of cultured mouse astrocytes with regard to all the GA isoforms, and Laake et al. [19] found virtually no GA immunostaining of astrocytes, but a high GA immunoreactivity in neurons in rat hippocampus in situ. Also, a direct comparison revealed that phosphate-activated glutaminase activity is much higher in cultured mouse brain neurons than astrocytes [20]. Hence, relatively low LGA mRNA expression may contribute to the relative impoverishment of glia in glutaminase immunostaining or activity previously noted in CNS cell cultures [18] and in brain in situ [19].

The biological significance of the relatively high expression of LGA mRNA in neurons and its virtual absence in astrocytes and astroglia-derived tissues is unknown. The prerequisite step to address this problem: Western blot analysis of the amount of LGA protein content and its activity has only recently been undertaken in this laboratory with no conclusive results in so far. However, reasonable hypotheses may be put forward on the

Fig. 7 Expression of mRNAs coding for GA isoforms in ganglioglioma. (A) mRNA separation profiles for individual cases. (B) Relative expression versus β -actin mRNA. Results are mean values \pm S.D. for six cases. * $p < 0.05$ versus other GA isoforms



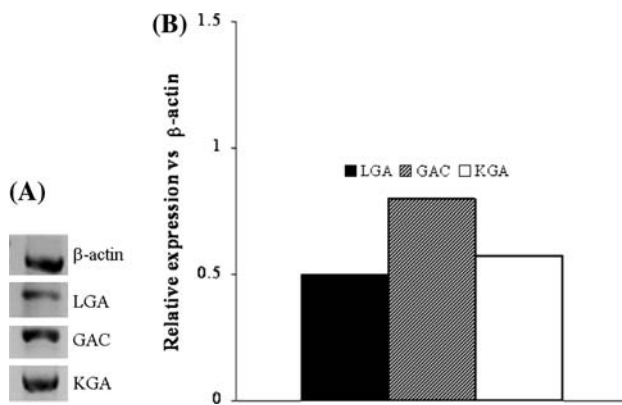


Fig. 8 Expression of mRNAs coding for GA isoforms in a single case of oligodendroglioma. (A) Representative electrophoretic separation. (B) Relative expression versus β -actin mRNA

basis of available literature data. Olalla et al. [19] described the expression of the LGA transcript in different regions of human brain and found the strongest signal in the cerebral cortex. Furthermore, these authors reported nuclear localization of LGA protein in monkey and rat brain and postulated the role of this protein in the regulation of transcription. The present observations in conjunction with the data provided by Ollala et al. [19] would suggest that LGA resides in the nuclei of cerebral cortical neurons, not astrocytes, and may therefore modulate aspects of transcription that are specifically involved in the cerebral cortical function. Whether or not the absence of LGA affects in any specific way the metabolism or viability of astroglia-derived tumors will be assessed by comparing the relevant events in glioma cell lines depleted off, and transfected with LGA.

Relatively higher expression of GAC than KGA in the brain tumors confirms and extends previous observations made in some, but not all peripheral classes of tumors. Increased expression of GAC was noted in breast cancer cells [4] and in highly malignant gliomas [13], but not in a lymphoma cell line, where no GAC signal could be detected [11]. Therefore, no reasonable predictions can be made at present as to the specific role of the altered GAC mRNA/KGA mRNA ratio in the metabolism of cerebral tumors, albeit this issue deserves further evaluation.

In conclusion, this study revealed low expression of LGA mRNA to be a feature of both normal and transformed astrocytes, while relatively high expression of GAC mRNA versus KGA mRNA characterizes all the types of CNS tumors so far studied. However, neither of the two characteristics appears to be directly related of tumor malignancy.

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