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Anna Maria Buccoliero [a,*], Chiara Caporalini [a], Mirko Scagnet [b], Federico Mussa [b], Flavio Giordano [b], Iacopo Sardi [c], Irene Migliastro [a], Selene Moscardi [a], Valerio Conti [d], Carmen Barba [e], Manila Antonelli [f], Francesca Gianno [f], Sabrina Rossi [g], Francesca Diomedi-Camassei [g], Marco Gessi [h], Vittoria Donofrio [i], Luca Bertero [j], Felice Giangaspero [f], Mariarita Santi [k], Eleonora Aronica [l], Lorenzo Genitori [b], Renzo Guerrini [d]

a Pathology Unit, Children's Hospital A. Meyer-University of Florence, Italy

b Department of Neurosurgery, Children's Hospital A. Meyer-University of Florence, Italy

c Neuro-oncology Unit, Children's Hospital A. Meyer-University of Florence, Italy

d Pediatric Neurology, Neurogenetics and Neurobiology Unit and Laboratories, Children's Hospital A. Meyer-University of Florence, Italy

e Pediatric Neurology, Neuroscience Department, Children's Hospital A. Meyer-University of Florence, Italy

f Department of Radiological, Oncological and Anatomo-Pathological Sciences, Sapienza University, Rome, Italy

g Pathology Department, Bambino Gesù Children's Hospital, IRCCS, Rome, Italy

h Institute of Pathology, Fondazione Policlinico Universitario 'Agostino Gemelli', Universita` Cattolica del Sacro Cuore, Rome, Italy

i Pathology Unit, Ospedale Santobono-Pausilipon, Naples, Italy

j Pathology Unit, Department of Medical Sciences, University of Turin, Italy

k Department of Pathology and Laboratory Medicine, Children's Hospital of Philadelphia, PA, USA

l Department of Neuropathology, AMC, University of Amsterdam, Amsterdam, the Netherlands

Abstract

Angiocentric glioma is a benign and very rare type of low-grade glioma. Its typical clinical presentation is with epileptic seizures, which are usually drug-resistant. The pathogenesis of tumor-associated seizures is poorly understood. Many possible mechanisms have been implicated, among which neurotoxic concentrations of the excitatory neurotransmitter glutamate. Glutamate transporters, pyruvate carboxylase and glutamine synthetase are enzymes involved in maintaining the physiological concentration of glutamate in the intersynaptic spaces. Specifically, glutamate transporters remove glutamate from the synaptic cleft, pyruvate carboxylase refund the oxidative glutamate loss and glutamine synthetase is involved in the transformation of glutamate into glutamine. We studied the immunohistochemical expression of EAAT2 (the most important glutamate transporter in the brain), pyruvate carboxylase and glutamine synthetase in a series of 19 angiocentric gliomas. EAAT2 was never expressed (0%) in the neoplastic cells in any of the cases studied. Glutamine synthetase was expressed in the cytoplasm of the neoplastic cells in 17/19 cases (89%). Pyruvate carboxylase was diffusely expressed in the cytoplasm of the neoplastic cells in 18/19 cases (95%). The net result of this enzymatic expression, in particular considering the loss of EAAT2, could be an increased glutamate concentration in the synaptic cleft. Elevated extracellular glutamate level might increase local network excitability initially involving intratumoral neurons. Despite further studies on larger series are needed, the observation that the angiocentric glioma-associated epilepsy might be determined from the EAAT2 deficiency open interesting therapeutic perspective.

Introduction

Angiocentric glioma is a benign and very rare type of low-grade glioma [1]. To date, less than 100 angiocentic gliomas have been reported. The initial reports date back to 2005 when two independent research groups described 18 tumors exhibiting clinic-pathological similarities [2, 3]. Angiocentric glioma was included in the 2007 World Health Organization (WHO) Classification of Tumors of the Central Nervous System (CNS) and classified as WHO grade I [4].

This tumor, which mainly affects children and young adults, develops superficially in the cerebral hemispheres most often in the frontal and temporal cortex and less frequently in mid-brain, amygdalae and hippocampus [5, 6, 7, 8]. On magnetic resonance imaging (MRI) angiocentric gliomas are hyperintense on FLAIR images, non-contrast-enhancing and at times show a band of hyperintensity on T1-weighed images. Calcifications are rare. The distinctive histopathological characteristics include perivascular arrangement of neoplastic bipolar cells and features of ependymal differentiation as evaluated by immunohistochemistry (dot-like positive staining for Epithelial Membrane Antigens -EMA- and negative staining for Oligodendrocyte transcription factor 2 -OLIG-2-) and by electron microscopy (micro lumens filled with microvilli and 'zipper-like' intermediated junctions).

An in-frame *MYB-QKI* gene fusion has been described in this tumor, which is believed to contributes to oncogenicity through three mechanisms which include the generation of oncogenic *MYB-QKI* fusion protein, enhancer translocation that establishes an auto regulatory feedback loop selectively driving *MYB-QKI* expression and partial loss of expression of *QKI* that is a tumor suppressor gene [1].

The typical clinical presentation is with epileptic seizures, which are usually drug-resistant [9]. It is estimated that more than 90% of patients with angiocentric glioma have long-standing epilepsy and the morbidity burden of these tumors is more often related to epilepsy than to their neoplastic nature. [10].

The pathogenesis of tumor-associated seizures is poorly understood. Many possible mechanisms have been implicated, among which neurotoxic concentrations of glutamate may play an important role. Glutamate, which is crucial for normal brain function, is the main excitatory neurotransmitter in the mammalian CNS [12]. Elevated concentrations of extracellular glutamate may determine neuronal hyper-excitability and seizures and have been implicated in several other neurological disorders including stroke, autism, intellectual disability, amyotrophic lateral sclerosis and Alzheimer's disease. Therefore, in view of the potential neurotoxicity of glutamate, its clearance is of great importance.

In glutamatergic synapses, glutamate is released by pre-synaptic neurons into the synaptic cleft and binds to postsynaptic glutamate receptors. Excess of glutamate is removed from the synaptic cleft by glutamate transporters mainly present on the astrocytic processes. Into astrocytes glutamate is converted into glutamine by glutamine synthetase that catalyzes the ATP-dependent condensation of ammonia and glutamate to form glutamine. Subsequently, glutamine is transported back to the neuronal presynaptic terminal where it is converted back into glutamate by glutaminase. Thus, glutamate-mediated neurotransmission is also based on recycling between the presynaptic axonal endings and the surrounding astrocytes. However, during the recycling process a fraction of the transmitter pool is lost in oxidative metabolism. This loss is replaced by *de novo* synthesis, which involves the action of astrocytic pyruvate carboxylase [14].

In order to better understand the pathogenesis of seizures associated with this rare tumor we studied the immunohistochemical expression of EAAT2 (the most important astrocytic glutamate transporter in the brain), glutamine synthetase and pyruvate carboxylase in a series of 19 angiocentric gliomas.

Patients and Methods

Tumor samples were obtained from 19 pediatric patients (age 2–18 years, mean 12 years; 16 boys, 3 girls) with a histological diagnosis of angiocentric glioma according to the 2016 WHO classification of CNS tumors. The commonest localizations were in the frontal, temporal and parietal lobe (6, 5 and 5 cases respectively). Two angiocentric gliomas occurred in the hippocampus, one in the thalamus and the localization was unknown in the last case. Data concerning symptomatology were known in 9 patients of whom 8 had epileptic seizures. The one patient having a thalamic localization of the tumor did not manifest epilepsy.

Tissue specimens were routinely fixed in 10% buffered neutral formalin, paraffin embedded and stained with hematoxilin-eosin (HE) for the morphological evaluation. Five μm sections of the most representative paraffin-embedded specimen of each case (specimens in which peritumoral nervous tissue was present were preferred) were mounted on electrostatic slides and used for immunohistochemistry. As primary antibodies we used Rabbit Polyclonal Anti Human anti-SLC1A2 [EAAT2] (1:100 dilution; Sigma-Aldrich), mouse monoclonal glutamine syntetase (ready to use; Ventana Medical System) and rabbit polyclonal anti human pyruvate carboxylase (1:50 dilution; Invitrogen) on a Ventana BenchMark ULTRA immunostainer (Ventana Medical Systems). The Ventana staining procedure included pretreatment with cell conditioner followed by incubation with antibody. The signal, for antibodies, was then developed with ultraView Universal DAB Detection Kit. After the staining run was completed, tissue sections were counterstained with hematoxylin. We also evaluated the EAAT2, glutamine synthetase and pyruvate carboxylase expression in peritumoral non neoplastic tissue, of at all present.

Results

Peritumoral tissue was included in 14 slides immunostained with EAAT2 and in 15 slides immunostained with glutamine syntetase and pyruvate carboxylase.

EAAT2 was never expressed (0%) in the neoplastic cells in any of the cases studied. Cytoplasmic immunostaining was present in the intralesional and perilesional astrocytes.

Pyruvate carboxylase was diffusely expressed in the cytoplasm in 18/19 cases (95%). A cytoplasmic immunostaining was present in the intralesional and perilesional astrocytes.

Glutamine synthetase was expressed in the cytoplasm of the neoplastic cells in 17/19 cases (89%; diffuse in 16 cases, focal in one). Cytoplasmic immunostaining was present in the intralesional and perilesional astrocytes.

Discussion

Glutamate is a non-essential aminoacid that acts as either a powerful excitatory neurotransmitter or potent signaling molecule playing an important role in synaptogenesis, cellular metabolism, migration, differentiation and death and intervening in higher order brain functions such as learning and memory. Excess of glutamate is associated with the initiation, spread and maintenance of seizure activity. High glutamate concentration is neurotoxic and consequently its homeostasis is important. Glutamate transporters, pyruvate carboxylase and glutamine synthetase are involved in maintaining the physiological concentration of glutamate in the intersynaptic spaces.

Glutamate transporters, present in both neurons and glial cells, are crucial to remove the glutamate from the synaptic cleft and maintaining low extracellular concentrations to prevent aberrant glutamate signaling. There are different families of glutamate transporters, among which the NA^{+}-dependent Excitatory Amino Acid Transporter (EAAT) which consists of five Na^{+}dependent high-affinity glutamate transporters progressively designated as EAAT1 to EAAT5. EAAT2 (also known as Glutamate Tansporter-1 -GLT1-) is a predominantly glial glutamate transporter and is responsible for 95% of total forebrain glutamate uptake activity. [17].

Pyruvate carboxylase is a biotin containing anaplerotic enzyme that converts pyruvate to oxaloacetate for entry into the tri-carboxylic acid (TCA) cycle. It is expressed in several tissues with highest activity in liver, kidney, adipose tissues, mammary gland, and pancreatic islets, moderate activity in brain, heart, and adrenal gland, and low activity in white blood cells and skin fibroblasts. Pyruvate carboxylase plays a crucial role in intermediary metabolism, controlling fuel partitioning toward gluconeogenesis, lipogenesis, glucose-induced insulin secretion and in the biosynthesis of neurotransmitters. Its role is particularly important for the *de novo* synthesis of glutamate which is crucial in compensating for the catabolism of glutamate. Indeed, pyruvate carboxylase refund the oxidative glutamate loss via oxaloacetate-TCA cycle-α-ketoglutarate. α-ketoglutarate can in turn be

converted to glutamate via glutamate dehydrogenase. In CNS it has been shown to be expressed in astrocytes.

Glutamine synthetase participates to several important metabolic pathways. In humans it is abundantly present in a limited set of hepatocytes surrounding the terminal hepatic veins and in skeletal muscle cells. It is also expressed in astrocytes, close to vascular structures and excitatory synapses. In the CNS glutamine synthetase participates in the detoxification of blood-derived and metabolically generated brain ammonia and in the synthesis and re-cyclization of neurotransmitters. In particular it is involved in the transformation of glutamate into glutamine (which in turn acts as a substrate for the new synthesis of glutamate through the neuronal enzyme glutaminase), but it is also the main precursor for the biosynthesis of the inhibitor neurotransmitter GABA.

Considering that loss of EAAT2 causes glutamate accumulation in the synaptic cleft, that pyruvate carboxylase activity results in glutamate synthesis, and that loss of glutamine synthetase causes a reduced conversion of glutamate in glutamine, it is conceivable that an altered expression of these enzyme could determine hyper-excitability and epilepsy in both non-neoplastic and neoplastic conditions. Several studies confirm this hypothesis.

Deletion of the *EAAT2* gene in mice causes nearly complete loss of glutamate uptake activity and seizures while at opposite transgenic mice overexpressing *EAAT2* are less prone to acute status epilecticus, exhibiting attenuated epileptogenesis and reduced seizures. Recent researches in the human have suggested that de novo mutations in *EAAT2* may determine early-onset epilepsy with multiple seizure types and that dysplastic cortical tissue has a reduced expression in EAAT2 protein. It has been demonstrated that gliomas associated with seizures have significantly higher glutamate levels and reduced expression of transporters (in particular EAAT2) both within the tumor and in peritumoral regions. The mechanisms whereby neoplastic astrocytes silence their *EAAT2* expression remain to be defined. A possible epigenetic regulation of *EAAT2* transcription based on the methylation of the corresponding promoter and the expression of aberrant *EAAT2*

mRNA have been postulated. However, whatever the cause for absent or reduced *EAAT2* expression in gliomas, it would seems that the consequent high glutamate concentration plays an important role in tumor-associated seizures. Consequently, an induced increased EAAT2 protein expression might represent a potential therapeutic approach for treating tumoral associated epilepsy.

Pyruvate carboxylase is implicated in several pathological conditions such as cancers including gliomas and pyruvate carboxylase deficiency disorders. In glioblastoma, up-regulation of pyruvate carboxylase enables glutamine-independent growth of wild-type isocitrate dehydrogenase 1 (IDH1) glioblastoma cells suggesting that this enzyme can serve as a metabolic alternative to glutamine. There are 3 type of pyruvate carboxylase deficiencies named A, B and C. In the more severe forms different CNS lesions have been described (i.e. subdural effusion, ischemia-like, periventricular hemorrhagic cysts, cerebral atrophy and delayed myelination) some of which are potentially epileptogenic. Furthermore, in affected patients epilepsy might be related to energy dysfunction secondary to defective Krebs cycle.

Glutamine synthetase is an astrocytic enzyme with putative antiepileptic activity as it catalyzes the conversion of glutamate and ammonia to glutamine. Glutamine synthetase is also one of the main sources of carbon and energy in many cancers and it has been noted that the administration of glutamine increases cell proliferation [25, 26]. In astrocytomas an inverse correlation between glutamine synthetase expression and epilepsy and adverse prognosis has been documented.

No studies specifically investigated the EAAT2, pyruvate carboxylase and glutamine synthetase expression in angiocentric gliomas in spite of a number of researches highlighted the role of a deregulated expression of these enzyme in the pathogenesis of tumor-associated epilepsy.

All angiocentric gliomas we studied lack EAAT2 expression while pyruvate carboxylase and glutamine synthetase expression are mostly preserved. Despite the preserved expression of glutamine synthetase in neoplastic cells, the net result could be an increased glutamate concentration in the synaptic cleft. Elevated extracellular glutamate level might increase local network excitability initially involving intratumoral neurons.

The hypothesis that the angiocentric glioma-associated epilepsy is determined from the EAAT2 deficiency might open interesting therapeutic perspective. Several studies emphasized the possibility to pharmacologically raise the EAAT2 levels.

In conclusion, our results show that angiocentric glioma cells loss EAAT expression and suggest that the main clinical consequence of this tumor might derive from deficient EAAT2 expression.

Further studies on larger series are necessary to confirm this hypothesis.

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