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The expression of ecto-nucleotide pyrophosphatase/phosphodiesterase 1 (E-NPP1) is correlated with astrocytic tumor grade

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ABSTRACT

Objective: Astrocytic brain tumors are subdivided in four grades. The most aggressive and invasive one is grade IV or glioblastoma multiforme (GBM). Ecto-nucleotide pyrophosphatase/phosphodiesterase-1 (E-NPP1), a membrane-bound enzyme, is involved in many cellular processes such as modulation of purinergic signalling, nucleotide recycling, regulation of extracellular pyrophosphate levels and stimulation of cell motility. In this study, the use of anti-NPP1 antibody in the determination of astrocytic tumor grade is evaluated.
Materials and methods: Paraffin-embedded surgical specimens from 41 primary human astrocytic brain tumors (grade I = 2; grade II = 10; grade III = 9; grade IV = 20) and 5 control samples are immunostained against NPP1 and glial fibrillary acid protein an astrocytic marker.
Results: In this communication, we report the expression of NPP1 in human astrocytic brain tumors. No expression could be detected in control tissue. We observed a remarkable up regulated expression of NPP1 in GBM. Taking the latter as 100%, grade I has a relative NPP1 staining of 7%, whereas grade II and III have a similar NPP1 expression level of 53% and 47% respectively.
Conclusion: A correlation is found between the up-regulated expression of NPP1 and the grade of the astrocytic tumor. Further investigation of NPP1 expression, especially in GBM, is necessary to determine the role of NPP1 in astrocytic brain tumor progression.

1. Introduction

Astrocytic brain tumors or astrocytomas are the most common glioma, accounting for about half of all primary brain tumors. Astrocytomas are glial cell tumors, derived from star-shaped glial cells that can occur in most parts of the brain. Astrocytomas are of two main types: (i) high-grade and (ii) low-grade tumors [1]. The first group grows rapidly and can easily spread through the brain. They are aggressive and require very intensive therapy. The second group is usually localized and grow slowly over a long period of time. The World Health Organization (WHO) classification for astrocytomas is based on cellular origin and histological appearance. Astrocytomas are divided into four grades: (i) the low-grade tumors consist of WHO grade I (pilocytic astrocytoma) and WHO grade II (low-grade astrocytoma) and (ii) the high-grade tumors consist of WHO grade III (anaplastic astrocytoma) and WHO grade IV (glioblastoma multiforme (GBM)) [2–4]. Low-grade astrocytomas are one of the least common brain tumors, while high-grade astrocytomas are the most common malignant brain tumors in adults [2]. GBM is a highly aggressive tumor that originates from the astrocytic cells of the brain [5]. Primary brain tumors occur in all age groups, but the incidence increases in elderly patients [2]. The average survival time of patients with GBM is less than one year after diagnosis due to resistance of the tumor to therapeutic interventions [5]. However, GBM patients below the age of 50 years have a better prognosis than older individuals [6].

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In vitro, rat C6 glioma cells are often used as an experimental model system for the study of biochemical properties of astrocytes and glioblastoma growth and invasion. These cells originated from brain tumors induced in Wistar–Furth rats by exposure to *N*,*N*′-nitroso-methylurea [7]. The cell line is characterized by proliferative and invasive properties due to point mutations in PTEN resulting in a constitutively active PI 3K/PKB-signalling pathway [7,8]. We previously observed the expression of an ecto-nucleotide pyrophosphatase/phosphodiesterase (E-NPP), identified as NPP1, on the membrane of C6 cells. No other members of the E-NPP family were demonstrated in these cells [9]. Originally, Takahashi et al. [10] discovered NPP1 as the plasma cell differentiation antigen PC-1 on the surface of mouse B-lymphocytes. They also found that this glycoprotein was present in homogenates of liver, brain and kidney [11].

E-NPPs are membrane-bound ecto-enzymes that are involved in multiple physiological mechanisms such as nucleotide recycling, modulation of purinergic signalling, regulation of extracellular pyrophosphate levels and stimulation of cell motility [11]. These E-NPPs belong to a multigene family with seven members. NPP1 and NPP3 are type II transmembrane glycoproteins with a short intracellular amino-terminal domain, a single transmembrane domain and a large extracellular carboxy-terminal part. NPP2 is not a transmembrane protein but is synthesized as a pre-pro-enzyme, which is secreted after removal of the N-terminal signal peptide [12]. NPP1 and NPP3 have a broad substrate specificity and release nucleoside 5′-monophosphate from a variety of nucleotides and their derivates e.g. NPP1 converts ATP into AMP and PPi and can therefore affect purinergic signalling [11,13–15]. NPP2 is an extracellular lysophospholipase D [16]. The other E-NPP members, NPP4-7, are type I transmembrane glycoproteins with a short intracellular carboxy-terminus and a significant smaller extracellular domain as compared to NPP1-3 [11,13]. The functions of NPP4-5 are unknown while NPP6 and NPP7 hydrolyse choline phosphate esters [17,18]. Although molecular and structural characteristics of E-NPPs are intensively investigated, their functional role and distribution in the brain and other organs is still controversial.

In this study, we will exclusively investigate astrocytomas and GBM. We measured the expression of NPP1 in human astrocytic brain tumors and correlated its increased expression with the histological grade of astrocytomas.

2. Materials and methods

2.1. Tumor samples

The protocols and procedures were approved by the ethical committee of the University Hospital of Antwerp (A02-003). Surgical specimens of astrocytomas were obtained from patients treated between 1992 and 2003 in the department of Neurosurgery of Middelheim General Hospital (ZNA) of Antwerp, Belgium. Astrocytomas were classified according to the WHO classification system into low-grade astrocytoma (grades I–II), anaplastic astrocytoma (grade III), or glioblastoma multiforme (grade IV). Forty-one Bouin fixed specimens of male and female patients with an average age of 51.3 ± 2.6 years (range, 25–83 years; median, 53.5 years) were used in this study. The control material was autopsy brain samples from five patients with an average of 66.0 ± 11.6 years (range 25–88 years; median, 79 years) and without any history of cancer or brain damage. Controls were also fixed with Bouin's fixative.

2.2. Antibodies

Detection of NPP1 was performed by immunohistochemistry (IHC) with a rabbit polyclonal antibody (R1702). This anti-NPP1 antibody was raised against the cytoplasmatic tail of NPP1. The antibody and the antigen of this antibody were kindly provided by Dr. J. Goding from the Department of Pathology and Immunology, Monash University, Victoria, Australia. Detection of astrocytes was done with a rabbit polyclonal anti-GFAP antibody (DakoCytomation, Denmark).

2.3. Tissue fixation and slide preparation

All tumor brain specimens were immediately placed on ice after surgical removal. The tumor specimens were fixed with Bouin (1.5% (v/v) picric acid, 40% (v/v) formalin, 95% (v/v) glacial acetic acid) or 12% (v/v) formalin. Fixation was for approximately 24 h and was performed at room temperature (RT). Thereafter, specimens were subsequently washed each time for 2 h with 70% (v/v), 90% (v/v), and with 99% (v/v) ethanol. Fixed tissues were paraffin embedded and 5 μm thick sections were cut onto glass slides. Sections were routinely stained with haematoxylin–eosin (H&E) or with cresyl violet (CV) for diagnostic evaluation as described below. For immunohistochemistry, tissue sections were placed on 0.1% (w/v) poly-L-lysine covered glass slides. After drying, paraffin-embedded tissue sections were deparaffinated in four consecutive bathes of xylenes for 5 min each. Finally, Bouin fixative was washed out from the slides by placing them in decreasing concentrations of ethanol starting with a bath of 100% ethanol followed by a bath of 95% (v/v), 2 baths of 70% (v/v) followed by washing with distilled water. Each wash was for approximately 10 min. All chemicals were of analytic grade.

2.4. Tissue staining

For conventional H&E staining, the sections were treated with haematoxylin Carazzi for 3 min followed by rinsing with running tap water and distilled water for 5 min. Staining with haematoxylin was followed by immersion in eosin yellow solution for 30 s. Immediately thereafter, the sections were placed in running tap water. For cresyl violet staining, the sections were rehydrated as described above. Subsequently, sections were stained for 5–10 min in cresyl violet followed by a washing step with acetate buffer (0.1 M sodium acetate, 0.1 M glacial acetic acid; pH 4.6) and with 70% (v/v) ethanol.

After staining, all sections were rinsed in distilled water, followed by dehydration with increasing concentrations of ethanol starting with 70% (v/v) ethanol for a few seconds followed by 95% (v/v) and 100% ethanol for 3 min. Finally, sections were washed twice (10 min each) with xylene and were mounted in Eukitt (Labonord, Belgium). The histology of the tissue sections was evaluated without knowledge of the clinical diagnosis.

2.5. Immunohistochemistry

Immunohistochemical reactions were carried out using the sensitive method of StreptABComplex/HRP (DakoCytomation, Denmark). Sections were deparaffinated as described above. Specimens were placed in a plastic jug containing recovery buffer (10 mM citrate monohydrate; pH 6.0) to enhance antigen reactivity, and subsequently heated for 10 min in a microwave oven (750 W). Endogenous peroxidase activity was blocked with 0.5% (v/v) hydrogen peroxide in methanol for 30 min, followed by washing for 30 min with running tap water. Sections were left in Tris-buffered saline (TBS; 150 mM NaCl, 50 mM Tris/HCl; pH 7.6) at RT for 1 h, saturated with normal swine serum (1:25; diluted in TBS/1% (w/v) BSA) for 3 h and incubated overnight with the primary antibody at RT. The detection of NPP1 was performed with a rabbit polyclonal anti-NPP1 antibody (R1702) diluted 1:800 in TBS/1% (w/v) BSA. Astrocytes were detected with a rabbit polyclonal anti-GFAP antibody diluted 1:20 000 in TBS/1% (w/v) BSA. Biotinylated anti-goat