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The long pentraxin PTX3 as a correlate of cancer-related inflammation and prognosis of malignancy in gliomas

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ABSTRACT

Inflammation is a component of glioma microenvironment. PTX3 is a component of the humoral arm of innate immunity and a candidate marker of inflammation.
In the present study we assessed the expression of PTX3 in gliomas by immunohistochemistry. PTX3 expression differed across low and high-grade tumors based on histopathological diagnosis and clinical severity, positively correlating with tumor grade and severity. In a multivariate logistic regression model, only the PTX3 score was significantly associated with the presence of a high-grade tumor. Thus, PTX3 may represent a new marker of cancer-related inflammation and glioma malignancy.

1. Introduction

Neuroepithelial tumors are the most common primary histologically heterogeneous tumors of the central nervous system (CNS). Among these, gliomas are the most common and are classified, according to the pathological characteristics, as tumors of astrocytic, oligodendroglial and ependymal (very rare) origin. The current WHO classification of CNS astrocytic and oligodendroglial tumors (Louis et al., 2007) recognizes four separate tumor grades (I–IV), which can be grouped into low-grade (I and II) or high-grade (III and IV) categories depending on the presence or absence of high-grade features, such as cytological atypia, increased cellularity, microvascular proliferation and necrosis. Glioblastomas are grade IV astrocytic tumors and are considered highly malignant (Wen and Kesari, 2008; Schomas et al., 2009). Indeed, in spite of optimal treatment, patients with glioblastoma survive less than one year and prognosis has not changed in the last two decades.

Inflammation is a key component of tumor microenvironment (Balkwill et al., 2005; Mantovani et al., 2008). Glioblastomas are characterized by a highly aggressive and angiogenic phenotype and by extensive areas of necrosis, which are surrounded by highly anaplastic cells. Necrotic cell death and hypoxia associated to tumor progression trigger release of inflammatory mediators and leukocyte recruitment (Kuppner et al., 1988; Desbaillets et al., 1997; Du et al., 2008; Murat et al., 2009; Tafani et al., 2011; Yeung et al., 2012). In the brain, neuroinflammatory cytokines affect the growth and differentiation of both normal and malignant glial cells, with interleukin-1 (IL-1) shown to be secreted by the majority of glioblastoma cells (Lu et al., 2007; Paugh et al., 2009), as well as by inflammatory cells. Indeed, a high number of activated microglial cells expressing CD11b and macrophage migration inhibitory factor (MIF) accumulate at the tumor border forming an inflammatory tumor microenvironment (Desbaillets et al., 1997; Engelhorn et al., 2009). Accordingly, the expression of inflammatory cytokines, chemokines (IL-6, CXCL8/IL-8, CX3CL1) and chemokine receptors (CXCR3, CXCR4, CXCL8 receptors and CX3CR1), as well as angiogenic mediators (vascular endothelial growth factor

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[VEGF], and cyclooxygenase [COX]-2) has been associated to prognosis of gliomas (Brat et al., 2005; Perdiki et al., 2007; Marchesi et al., 2008; Samaras et al., 2009; Erreni et al., 2010; Locatelli et al., 2010). Proinflammatory cytokines have also been implicated in glioblastoma invasiveness through the induction of metalloproteases (Sarkar and Yong, 2009), or through the modulation of the plasminogen activator system (Bellail et al., 2004; Bryan et al., 2008). A key role in this deleterious inflammatory process is played by the transcription factor NF-κB, which is constitutively activated by the prolyl isomerase Pin1 in glioblastoma, and regulates cell proliferation, survival and migration, inflammation and apoptosis (Raychaudhuri et al., 2007; Atkinson et al., 2009; Sen, 2011). In addition, the activation state of NF-κB is controlled by pro-inflammatory mediators produced by neighboring inflammatory cells.

Hypoxia contributes to the generation of an inflammatory milieu by inducing the expression of genes regulating the glycolytic pathway and angiogenesis, and genes encoding chemotactic molecules such as CCL2, CXCL8 and VEGF, responsible of the recruitment of macrophages along a hypoxia-mediated chemotactic gradient. Macrophages recruited to hypoxic sites exert a tumor-promoting effect through the expression of genes with mitogenic, angiogenic, and migration/invasion stimulating properties (Du et al., 2008; Murat et al., 2009).

Pentraxin 3 (PTX3), like C reactive protein (CRP), is a member of the highly conserved pentraxin superfamily (Agrawal, 2005; Garlanda et al., 2005). In particular, PTX3 is the prototypic member of the long pentraxin subfamily that differs from CRP for the presence of an unrelated long N-terminal domain coupled to the C-terminal domain, as well as for gene organization, cellular source and tissue source, inducing stimuli and ligand recognition (Garlanda et al., 2005). PTX3 expression is rapidly induced in a variety of cell types by several stimuli, such as cytokines (e.g. IL-1β, TNF-α) and microbial moieties (Jaillon et al., 2007; Bottazzi et al., 2010). In the case of microglia, PTX3 was identified among the proteins of mixed glia secretome, and was most highly induced by LPS/IFN-γ stimulation (Jeon et al., 2010). Glia-derived PTX3 was also found to modulate phagocytic functions of microglia inhibiting uptake of apoptotic cells (Jeon et al., 2010). PTX3 contributes in regulating inflammation and complement activation, and participates in tissue remodeling and angiogenesis (Bottazzi et al., 2010; Deban et al., 2010).

PTX3 is a candidate new marker of inflammation (Muller et al., 2001; Norata et al., 2010), including cancer-related inflammation (Willeke et al., 2006; Ravenna et al., 2009; Germano et al., 2010; Diamandis et al., 2011), better reflecting inflammatory processes in the tissue microenvironment.

The present study was designed to assess the presence and significance of PTX3 in human gliomas. Here we report that PTX3 is a component of the glioma microenvironment, being produced by tumor cells and infiltrating CD68 positive macrophages and local PTX3 levels correlated with grade and malignancy. These results suggest that PTX3 is a candidate novel marker of the severity of the disease, possibly reflecting the inflammatory process associated to gliomas.

2. Material and methods

2.1. Patients

Tumor specimens of sixty-three patients with primary CNS tumors who attended the Neurosurgery Division of IRCCS Ospedale Maggiore Policlinico, Mangiagalli and Regina Elena, Milan, Italy, between 2005 and 2007 have been assessed for the immunohistochemical PTX3 score. Patients were 35 males and 28 females with a median age of 44.9 ± 12.9 years (Standard Deviation). Tumor specimens were pathologically diagnosed according to the last 2007 WHO classification (Louis et al., 2007): low-grade oligodendrogliomas (n = 30); anaplastic oligodendrogliomas (n = 8); low-grade astrocytomas (n = 6); anaplastic astrocytomas and glioblastomas (n = 19). Mixed tumors were excluded. Informed consent was obtained from all patients.

2.2. Immunohistochemical analysis

All original hematoxylin-eosin slides of all cases under study were reviewed by one of the Authors (SF) to confirm the original diagnosis made according to the last 2007 WHO recommendation. For each case, a representative paraffin block was selected in order to meet these major criteria: a good morphology representative of the entire tumor lesion avoiding necrotic or degenerative non-specific areas. From each block, three consecutive sections, 4 μm in thickness were cut and dewaxed in xylene and used for immunohistochemistry. Antigen retrieval for PTX3 immunostaining was performed with a sodium citrate buffer solution (pH 6.0) in a microwave oven, three times for 5 min; an affinity-purified polyclonal rabbit IgG against human PTX3 was used (250 ng/ml, 1:8000 dilution; raised in our laboratory). Reactions were revealed using peroxidase Novolink Polymer Detection System (Leica Microsystems, Newcastle upon Tyne, UK) and 3-3′ diaminobenzidine substrate as chromogen (DAB, brown staining; Liquid DAB + Substrate Chromogen System, DakoCytomation), according to manufacturer's instructions. Sections were counterstained with Mayer's hematoxylin (Diapath, Martinengo, Italy).

We evaluated the percentage of PTX3 positive cells and the intensity of the staining. Scores of the percentage of positive cells were assessed as follows: Score 0: no immunoreactive cells, score 1: <10% of immunoreactive cells, and score 2: immunoreactivity between 10 and 50%, score 3: >50% immunoreactive cells. Staining intensity was scored as follows: 0: no immunostaining, 1: faint staining, 2: moderate and 3: strong immunostaining. The final score of the presence of PTX3 in brain surgical specimens was calculated as the product of the scores of percentage and intensity of immunostaining.

To characterize PTX3 expressing cells, double immunohistochemical staining was performed by using the following antibodies: monoclonal mouse anti-human CD68 (clone PGM1; DakoCytomation, Glostrup, Denmark; 1:500 dilution; the fixative resistant CD68 epitope recognized by the clone PGM1 is expressed by phagocytic macrophages of microglial and monocytic origin and by "activated" microglia) and polyclonal rabbit anti human glial fibrillary acidic protein (GFAP, DakoCytomation, 1:6000 dilution, to detect astrocytes or small gemistocytes). Specific secondary antibodies conjugated with peroxidase and alkaline phosphatase were chosen.

In a selected group of grade II astrocytomas, grade III anaplastic astrocytomas and grade IV glioblastoma, double immunostained for PTX3 (DAB, brown staining) and CD68 (aminoethyl carbazole, red staining), we counted PTX3- and CD68-positive cells in three representative high power fields (original magnification 40×).

2.3. Loss of heterozygosity at chromosomes 1p and 19q

Tumor DNA was extracted from paraffin-embedded tissues using the DNeasy Tissue Kit (QIAGEN, Inc. Milano, Italy) according to the manufacturer's protocol. Following DNA extraction, all tumor samples were subjected to control gene (PGK) amplification to assess DNA integrity. Constitutional DNA from peripheral blood leukocytes was isolated using the standard phenol/chloroform extraction method with ethanol precipitation. Constitutional DNA/tumor DNA pairs were evaluated by standard PCR-based LOH assays, as described (Barbashina et al., 2005).

2.4. Methylation of O^6-methylguanine-DNA methyltransferase (MGMT) promoter

The methylation status of the MGMT promoter gene was determined by performing methylation-specific PCR. Tumor DNA from paraffin embedded tissues (10 μm sections) was modified by sodium bisulfite which converts unmethylated, but not methylated, cytosine to uracil, as described (Palmisano et al., 2000; Hegi et al., 2005). Modified DNA was submitted to methylation specific polymerase chain