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Research report

# Regulation of myeloid leukemia factor-1 interacting protein (MLF1IP) expression in glioblastoma

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**Abstract**

The myelodysplasia/myeloid leukemia factor 1-interacting protein MLF1IP is a novel gene which encodes for a putative transcriptional repressor. It is localized to human chromosome 4q35.1 and is expressed in both the nuclei and cytoplasm of cells. Northern and Western blot analyses have revealed MLF1IP to be present at very low amounts in normal brain tissues, whereas a number of human and rat glioblastoma (GBM) cell lines demonstrated a high level expression of the MLF1IP protein. Immunohistochemical analysis of rat F98 and C6 GBM tumor models showed that MLF1IP was highly expressed in the tumor core where it was co-localized with MLF1 and nestin. Moreover, MLF1IP expression was elevated in the contralateral brain where no tumor cells were detected. These observations, together with previous data demonstrating a role for MLF1IP in erythroleukemias, suggest a possible function for this protein in glioma pathogenesis and potentially in other types of malignancies.


*Theme:* Disorders of the nervous system
*Topic:* Neuro-oncology

*Keywords:* Glioblastoma; MLF1-interacting protein; Tumor model; Overexpression

## 1. Introduction

High-grade gliomas are highly aggressive CNS tumors characterized by a rapid proliferation rate, invasiveness and angiogenesis. The most frequent and malignant type of these tumors is glioblastoma multiforme (GBM) [15,24]. These tumors are classically thought to originate from supporting glial cells of the CNS, and represent about 50% of all adult primary CNS tumors [25]. Although multiple genetic changes have been described in glioblastomas [13], and despite extensive research, no significant progress has been made in identifying specific genetic alterations within individual GBM tumors. In recent years, several genetic aberrations and gene expression changes have been shown to occur during malignant transformation, development, and progression of gliomas [16]. Some of the best-characterized aberrations include the loss of the tumor suppressor genes p53, p16, RB, and PTEN, as well as amplification of EGFR and CDK4 [20,23]. These genetic lesions result in altered expression not only of the affected genes themselves, but also of downstream targets. However, these genetic changes are not uniformly distributed in all glioma tumor samples due to the heterogeneous nature of the tumor. Thus, the identification of additional GBM-associated tumor markers is of utmost importance. In this study, we demonstrate that the novel gene MLF1IP (myelodysplasia/myeloid leukemia factor 1- interacting protein) is overexpressed in rat GBM tumor models, as well as in a number of GBM tumor cell lines, as compared with normal brain tissue. MLF1IP was first identified by our group as a novel protein that specifically associated with MLF1 (myelodysplasia/myeloid leukemia factor 1) in vitro and in vivo [9]. The MLF1IP

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cDNA encodes an ~47-kDa protein that contains several nuclear localization signals (NLS), two nuclear receptor binding motifs (LXXLL), two leucine zippers and a number of potential phosphorylation sites. The MLF1IP protein and transcripts are expressed in a variety of tissues, and MLF1IP appears to be a lineage-specific gene within the hematopoietic system, whose expression is confined exclusively to the CFU-E erythroid precursor cells [9]. Recently, a role for MLF1IP in transcriptional regulation was demonstrated by Pan et al. [19], suggesting that this protein may be a new member of cellular transcriptional repressors.

We report in this paper that MLF1IP is overexpressed in a variety of glioblastoma cell lines, as well as in two rat glioma tumor models where it co-localizes with the intermediate filament nestin. Nestin is a well-characterized marker for neural stem cells and neural progenitors in the CNS and has been implicated to play a role in the development of gliomas [4,10,12]. Furthermore, preliminary results from our laboratory have indicated that MLF1IP and nestin are also upregulated and co-localize in human GBM tumor specimens. These data imply that the enhanced neuropoiesis and upregulation of MLF1IP that accompany GBM tumor development may play an important role in the process of cellular transformation and in glioma pathogenesis.

## 2. Materials and methods

### 2.1. Cell culture

The F98, C6, and MOR86 glioma cell lines were obtained from ATCC (Manassas, VA). Cells were cultured as monolayers with medium supplemented with 10% FBS, penicillin, and streptomycin (Sigma-Aldrich, St. Louis, MO). The F98 and C6 cell lines were stably transduced with the pFB retrovirus (pFB-GFP) (Stratagene, La Jolla, CA) expressing GFP to allow for visual analysis of the cells. The cells were FACS-sorted to generate a homogeneous cell population uniformly expressing high levels of GFP (F98-GFP and C6-GFP).

### 2.2. Animals and tumor cell inoculation

Adult Fischer and Sprague–Dawley rats, 250–350 g in weight (Charles River Laboratories, Wilmington, MA), were used for all studies. All of the operative procedures and animal care were in conformity with the guidelines of the Animal Care and Use Committee at the University of Tennessee Health Science Center. Each rat was anesthetized (ketamine/xylazine, 8.7 mg/1.3 mg/100 g body weight) and prepared for craniotomy.

The tumor inoculation technique has been described elsewhere [2]. Briefly, an incision was made in the skin 3.5 mm to the right of the bregma. A 0.5-mm-diameter burr hole for implanting the tumor cells was drilled through the skull. PBS alone (vehicle-control) or $1 \times 10^5$ glioma cells resuspended in PBS in a volume of 10 μl were implanted over a period of 5 min at a depth of 5 mm from the brain surface using a Hamilton syringe (Hamilton, Reno, NV). The burr hole was sealed with bone wax, and the scalp was sutured thereafter. Rats were observed daily for signs of infection, alertness, feeding habits, and neurological deficits. The animals were sacrificed 14 days after tumor inoculation by transcardial perfusion with 0.9% saline/0.01% heparin, followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The brains were removed and fixed in 4% paraformaldehyde for 2 h at 4 °C, then transferred in a 30% sucrose solution overnight at 4 °C. The brain tissues were then frozen on dry ice for sectioning (20 μm) using a cryostat and mounted on glass slides for histology and immunohistochemical analyses.

### 2.3. Immunohistochemistry

Brain sections were incubated in 3% normal donkey serum for 1 h to block non-specific serum-binding sites. Immunohistochemistry was performed by incubating the specimens in primary antibody overnight at 4 °C, followed by incubation with secondary antibody for 2 h at room temperature (RT). The primary antibodies used were the following: MLF1 and MLF1IP-specific rabbit polyclonal antibodies (1:1000 dilution) were produced by us, as previously described [9], mouse anti-nestin and anti-β-tubulin III mAbs (1:200 dilution; Chemicon International, Inc., Temecula, CA), and mouse anti-GFAP mAb (1:400 dilution; Sigma-Aldrich). All primary antibodies were diluted in a 3% normal donkey serum/ PBS solution. The secondary antibodies used were Cy3-conjugated donkey anti-rabbit IgG and Cy2-conjugated donkey anti-mouse IgG, diluted 1:200 in PBS (Jackson Immunoresearch Laboratories, Inc., West Grove, PA). For negative controls, normal rabbit (NRS) or normal mouse (NMS) sera were used in place of the primary antibody. Finally, the slides were mounted and examined by confocal microscopy.

### 2.4. Western blot analysis

$1 \times 10^5$ cells of each glioma line were lysed in 4× SDS-sample buffer and subjected to SDS-PAGE under reducing conditions. The proteins were transferred onto a nitrocellulose membrane and subjected to immunoblotting using rabbit anti-MLF1IP, followed by goat anti-rabbit-HRP secondary antibody. The blots were developed using the ECL chemiluminescence reagent (Amersham Biosciences, Piscataway, NJ).

### 2.5. Northern blot analysis

Northern blot analysis was performed using total RNA extracted from human and rat glioma cell lines using TRIzol reagent, as described by the manufacturer (Invitrogen). 15