Cancer Letters 356 (2015) 929–936

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Original Articles

# EZH2 is a negative prognostic factor and exhibits pro-oncogenic activity in glioblastoma

Junxia Zhang [a,b,c,1], Luyue Chen [a,c,1], Lei Han [a,c], Zhendong Shi [a,c], Jianning Zhang [a], Peiyu Pu [a,c], Chunsheng Kang [a,c,*]

[a] Department of Neurosurgery, Tianjin Medical University General Hospital, Laboratory of Neuro-Oncology, Tianjin Neurological Institute, Key Laboratory of Post-trauma Neuro-repair and Regeneration in Central Nervous System, Ministry of Education, Tianjin Key Laboratory of Injuries, Variations and Regeneration of Nervous System, Tianjin, China
[b] Department of Neurosurgery, The First Affiliated Hospital of Nanjing Medical University, Nanjing, China
[c] Chinese Glioma Cooperative Group (CGCG), China

ARTICLE INFO

*Article history:*
Received 4 August 2014
Received in revised form 2 November 2014
Accepted 4 November 2014

*Keywords:*
Glioblastoma
EZH2
Prognostic biomarker
Tumor progression

ABSTRACT

The identification of single or less genes based on mRNA expression as clinical diagnostic markers for glioblastoma (GBM) remains a challenge. Recent data have shown the potential oncogenic role and prognostic significance of EZH2 in several human cancers. However, the clinical signature and further mechanisms of EZH2 function in gliomagenesis are still poorly understood. In this study, we found that increased EZH2 expression was associated with tumor grade. High expression of EZH2 in GBM was determined to be a strong and independent predictor of short overall survival. Further, we screened EZH2 targets and associated genes in GBM. Repression of EZH2 induced cell cycle arrest and inhibited tumor growth in vivo. This event represents a positive feedback loop with β-catenin/TCF4 and STAT3 signaling. Taken together, EZH2 could be an independent prognostic factor and potential therapeutic target for GBM.



## Introduction

Gene mRNA expression profiling has provided better approaches for screening meaningful prognostic marker for human cancer. Numerous prognostic gene expression signatures have been identified in human cancers and some are now being tested in prospective randomized clinical trials for use in predicting patient outcome and guiding treatment decisions in breast and colorectal cancer [1,2]. However, in glioblastoma multiforme (GBM), the identification of single or less genes based on mRNA expression for clinical diagnostic markers remains a challenge.

Polycomb group (PcG) proteins are believed to be epigenetic regulators that form polycomb repressor complexes (PRC), such as PRC1 and PRC2, which modify chromatin and repress gene expression. PRC2 has three core components: enhancer of zeste homolog 2 (EZH2), suppressor of zeste 12 (SUZ12), and embryonic ectoderm development (EED). EZH2 has histone methyltransferase activity for the trimethylation of histone 3 on lysine 27 (H3K27me3), thereby epigenetically silencing the genes [3,4]. Further, EZH2 recruits DNA methyltransferases to their target promoters [5–7]. Therefore, DNA methylation and histone methylation are involved in EZH2 mediated epigenetic silencing. Consistent EZH2 overexpression has been observed in a variety of different cancers [8–10], including gliomas [11]. Recent data have shown that EZH2 is upregulated in gliomas and involved in miR-101, inducing the inhibition of proliferation, migration, and angiogenesis [12]. Moreover, EZH2 depletion strongly impairs GBM cancer stem cell (CSC) self-renewal in vitro and tumor-initiating capacity in vivo [13]. However, the clinical signature and further mechanisms of EZH2 in gliomagenesis remain poorly understood.

In the current study, we analyzed the association of EZH2 expression with clinicopathologic variables in glioma samples. We observed that positive and increased EZH2 expression is associated with tumor grade and shorter overall survival (OS). In vitro and in vivo experiments consistently demonstrated that EZH2 reduction inhibited tumor growth which was accompanied by β-catenin/TCF4 and STAT3 signaling feedback regulation. These data demonstrate that EZH2 drives malignant behavior of gliomas, and its expression is a novel independent prognostic biomarker in GBM.

## Materials and methods

### *Patients and samples*

A total of three large gene expression profiling cohorts of gliomas were used in this study. Gene expression data from the TCGA (173 core GBMs) and the

* Corresponding author. Tel.: +86 22 60817499; fax: +86 22 60817500.
*E-mail address:* kang79061@gmail.com (C. Kang).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.canlet.2014.11.003
0304-3835/

validation data set (260 GBMs) were downloaded from the TCGA database (http://tcga-data.nci.nih.gov/docs/publications/gbm_exp/). Additionally, we performed gene expression profiling on 220 glioma samples collected from the Chinese Glioma Genome Atlas (CGGA, http://www.cgcg.org.cn/), which included 58 astrocytomas (A), 18 oligodendrogliomas (O), 21 oligoastrocytomas (OA), 8 anaplastic astrocytomas (AA), 11 anaplastic oligodendrogliomas (AO), 15 anaplastic oligoastrocytomas (AOA), 4 secondary GBMs and 85 primary GBMs. 5 normal brain tissue samples (N) were added after informed consent was obtained from patients with severe traumatic brain injury who needed post-trauma surgery and patients who had undergone surgery for primary epilepsy. All of the 220 Chinese glioma patients underwent surgical resection between January 2006 and December 2009, and subsequently received radiation therapy and/or alkylating agent-based chemotherapy. This study was approved by the institutional review boards of all hospitals involved in the study, and written informed consent was obtained from all the patients who were selected.

*RNA extraction and whole genome gene profiling*

After the surgery, all tissue samples were immediately snap-frozen in liquid nitrogen. A hematoxylin and eosin stained frozen section was prepared from each sample to assess the percentage of tumor cells before RNA extraction. Total RNA was extracted from the frozen tumor samples and the RNA concentration and quality were measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies).

Microarray analysis was performed using the Whole Human Genome Array (Agilent) according to the manufacturer's instructions. The integrity of the total RNA was checked using an Agilent 2100 Bioanalyzer. cDNA and biotinylated cRNA were synthesized and hybridized to the array. Data were acquired using the Agilent G2565BA Microarray Scanner System and Agilent Feature Extraction Software (v9.1). Probe intensities were normalized using GeneSpring GX v. 11.0 (Agilent).

*Pyrosequencing for IDH1 mutation and MGMT promoter methylation*

For IDH1 mutation analysis, genomic DNA was isolated from frozen tumor tissues using the QIAamp DNA Mini Kit (Qiagen). The primers used were forward 5′-GCTTGTGAGTGGATGGGTAAAAC-3′ and reverse 5′- TTGCCAACATGACTTACTTGATC-3′. For MGMT promoter methylation analysis, bisulfite modification of the DNA was performed using the EpiTect Kit (Qiagen). The primers used were forward 5′-GTTTYGGATATGTTGGGATA-3′ and reverse: 5′-biotin-ACCCAAACACTCACCAAATC-3′. Pyrosequencing analysis of IDH1 mutation and MGMT promoter methylation was performed by Gene Tech (Shanghai, China).

*Immunohistochemistry*

Briefly, specimens were fixed in formalin, routinely processed and paraffin embedded. Five-micron-thick sections were prepared, and immunohistochemical staining with streptavidin-biotin immunoperoxidase assay was performed using antibodies against MGMT, EGFR, Ki-67 (1:100, Santa Cruz Biotechnology), EZH2, β-catenin, and STAT3 (1:100, Cell Signaling Technology). The staining intensity was scored by two experienced pathologists without knowledge of clinical information on a scale of 0 to 3 (0, negative; 1, slight positive; 2, moderate positive; 3, intense positive). A score of 0 and 1 or 2 and 3 indicated low or high expression, respectively. Controls without primary antibody and positive control tissues were included in all experiments to ensure the quality of staining. In case of a discrepancy, the 2 observers simultaneously reviewed the slides to achieve a consensus.

*Cell culture and treatment*

Human glioblastoma cells (U251 and U87) were obtained from the Chinese Academia Sinica Cell Repository (Shanghai, China). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum, and incubated at 37 °C with 5% $CO_2$. Oligonucleotides were chemically synthesized and purified by high-performance liquid chromatography (GenePharma, Shanghai, China). The sequences are: EZH2 siRNA1, 5′- GAGGGAAAGTGTATGATAATT -3′, and siRNA2, 5′- TTCATGCAACACCC AACACT -3′. EZH2 siRNAs were transfected using Lipofectamine 2000 (Invitrogen). Cells transfected with nonsense siRNA oligonucleotides (scramble) were used as control. The β-catenin/TCF inhibitor FH535 and STAT3 inhibitor WP1066 were purchased from Merck and was added with the final concentration of 10 μmol/l.

*Cell cycle analysis*

For cell cycle analysis by FCM (flow cytometry), cells treated with EZH2 siRNA1 for 48 h were harvested, washed with PBS, fixed with 75% ethanol overnight at 4 °C, and then incubated with RNase at 37 °C for 30 min. The cell nuclei were stained with propidium iodide for an additional 30 min. A total of $10^4$ nucleuses were examined in a FACS Calibur flow-cytometer (Becton Dickinson, USA). The results are presented as the percentage of cells in each phase.

*Cell growth assay*

Cells were plated at $10^4$ cells per well in 96-well plates with six replicate wells. After transfection as described previously, 20 μl of MTT (5 g/L, Sigma, USA) was added into each well at each day of consecutive 4 days after treatment and the cells were incubated for additional 4 h; the supernatant was then discarded. 200 μl of DMSO was added to each well to dissolve the precipitate. Optical density (OD) was measured at wavelength of 550 nm. The data are presented as the mean ± SD, which are derived from triplicate samples of at least three independent experiments.

*Western blot analysis*

Equal amounts of protein per lane were separated by 8% SDS-polyacrylamide gel and transferred to PVDF membrane. The membrane was blocked in 5% skim milk for 1 h and then incubated with a specific antibody for 2 h. The antibodies used in this study were: EZH2, β-catenin, and STAT3 (Cell Signaling Technology, USA). The antibody against GAPDH (Santa Cruz, USA) was used as a control. The specific protein was detected by using a SuperSignal protein detection kit (Pierce, USA). The band densities of specific proteins were quantified after normalization with the density of GAPDH.

*Real-time PCR*

Real-time PCR was performed according to the manufacturer's instructions. Human EZH2 (forward)/(reverse): 5′ -TTGTTGGCGGAAGCGTGTAAAATC -3′; 5′ -TCCCTAGTCCCGCGCAATGAGC -3′. STAT3 (forward)/(reverse): 5′ -ACCTGCAGCAATACCATTGAC -3′; 5′ -AAGGTGAGGGACTCAAACTGC -3′. β-cantenin (forward)/(reverse): 5′ -GATTTGATGGAGTTGGACATGG -3′; 5′ -TGTTCTTGAGTGAAGGACTGAG -3′. All experiments were performed using biological triplicates and experimental duplicates. The relative expression was calculated via the 2–ΔΔCt method.

*Nude mouse tumor xenograft model and treatment*

U251 glioma cells were subcutaneously injected into 5-week-old female nude mice (Cancer Institute of the Chinese Academy of Medical Science). When the tumor volume reached 50 $mm^3$, the mice were randomly divided into four groups (six mice per group). Each group was treated with EZH2 siRNA1 or nonsense siRNA through local injection of the xenograft tumor at multiple sites. The treatment was performed once every 3 days for 15 days. The tumor volume was measured with a caliper every 3 days, using the formula: volume = length × $width^2/2$.

*Statistical analysis*

A t-test was used to analyze differences in each two-group comparison, while one-way ANOVA was used to determine the difference among at least three groups. Kaplan–Meier analysis was employed to assess the survival rate of patients. $P < 0.05$ was considered to be a statistically significant difference.

## Results

### *Increased EZH2 expression correlates with glioma grade*

Because EZH2, SUZ12 and EED are the core components of PRC2, we first explored their expression profile in 220 glioma and 5 normal tissues of the CGGA (Fig. 1A, B, C and D). EZH2 and SUZ12 were overexpressed in tumors compared to normal tissue (Fig. 1B and C). In addition, the level of EZH2 mRNA increased markedly in higher grade gliomas in comparison to lower grade gliomas (Fig. 1B). Among the core components of PRC2, EZH2 expression was most significantly associated with glioma grade. Thus, we employed two independent glioma gene expression datasets (GSE16011 and GSE4290) to examine the association of EZH2 levels with glioma grade. EZH2 was significantly upregulated in high grade gliomas compared to low grade gliomas and normal tissues (Fig. 1E and F). These findings suggest that EZH2 may play an important role in glioma development.

### *EZH2 overexpression confers a poor prognosis for GBM patients*

Next we investigated the correlation between EZH2 expression and overall survival using Kaplan–Meier survival curve analysis with a log-rank comparison. From the CGGA data, we chose 83 primary