Increased expression of the histone H3 lysine 4 methyltransferase MLL4 and the histone H3 lysine 27 demethylase UTX prolonging the overall survival of patients with glioblastoma and a methylated *MGMT* promoter

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OBJECTIVE The purpose of the present study was to investigate the epigenetic and prognostic roles of an H3K4 methyltransferase (mixed lineage leukemia 4 [MLL4]) and H3K27 demethylase (ubiquitously transcribed tetratricopeptide repeat gene on X chromosome [UTX]) in progression-free survival (PFS) and overall survival (OS) of patients with glioblastoma (GBM) who were treated with radiotherapy, chemotherapy, or both after resection. In addition, the authors examined methylation at the promoter of the O-6-methylguanine-DNA methyltransferase (*MGMT*) gene and other prognostic factors predicting length of PFS and OS in these patients.

METHODS The medical records of 76 patients having a new diagnosis of histologically ascertained GBM in the period of January 2002 to December 2013 at the authors' institution were retrospectively reviewed. Immunohistochemical staining for MLL4 and UTX was performed on archived paraffin-embedded tissues obtained by biopsy or resection. The methylation status of the *MGMT* promoter in these tissues was determined by methylation-specific PCR analysis.

RESULTS During the follow-up period (mean length 18.1 months, range 4.1–43.5 months), 68 (89.5%) of the patients died. The *MGMT* promoter was methylated in 49 patients (64.5%) and unmethylated in 27 (35.5%). The immunoreactivity pattern of UTX was identical to that of MLL4; increased expression of these 2 proteins was observed in samples from 34 patients (44.7%) and decreased expression in 42 patients (55.3%). The mean length of PFS was 9.2 months (95% CI 6.8–11.6 months). Extent of surgery, recursive partitioning analysis (RPA) class, and methylation status of the *MGMT* promoter were all associated with increased PFS in the multivariate analysis of factors predicting PFS. The mean length of OS was 18.6 months (95% CI 14.3–22.9 months). Patient age ($p = 0.004$), WHO performance status score ($p = 0.019$), extent of surgery ($p = 0.007$), RPA class ($p = 0.036$), methylation status of the *MGMT* promoter ($p = 0.010$), and increased expression of UTX-MLL4 ($p = 0.001$) were significantly associated with increased OS in multivariate analysis. Interestingly, in patients with an unmethylated *MGMT* promoter, immunoreactivity of UTX-MLL4 was not associated with changes in OS ($p = 0.350$). However, in the patients with a methylated *MGMT* promoter, increased UTX-MLL4 expression was strongly associated with increased OS ($p < 0.001$).

CONCLUSIONS The results of this study suggest that increased expression of UTX-MLL4 positively influences the outcome of patients with GBM having a methylated *MGMT* promoter. Therefore, UTX-MLL4 immunoreactivity could be a useful predictor of the response to conventional treatment with radiotherapy or chemotherapy among GBM patients whose tumors have a methylated *MGMT* promoter.

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KEY WORDS glioblastoma; epigenome; O-6-methyl guanine methyltransferase; survival; UTX–MLL4 complex; prognosis; oncology

ABBREVIATIONS CpG = cytosine-phosphate-guanine; FFPE = formalin-fixed, paraffin-embedded; GBM = glioblastoma; *MGMT* = O-6-methylguanine DNA methyltransferase; MLL4 = mixed lineage leukemia 4; MSP = methylation-specific PCR; OS = overall survival; PFS = progression-free survival; PSQ = pyrosequencing; ROC = receiver operating characteristic; RPA = recursive partitioning analysis; UTX = ubiquitously transcribed tetratricopeptide repeat gene on X chromosome.

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GLIOMASTOMA (GBM) is the most common malignant brain tumor, accounting for approximately 12%–15% of all primary intracranial neoplasms and for 60%–75% of glial tumors.^{10,14} Typically, if untreated, patients with a diagnosis of GBM die within a few months, and even if treated with radiotherapy, chemotherapy, or both, patients with GBM often do not survive for 1 year.²³ In 2004, a randomized Phase III trial with GBM patients showed that concomitant and adjuvant temozolomide in addition to standard postoperative radiotherapy improved length of survival relative to postoperative radiotherapy alone.⁴ To date, this therapeutic approach involving temozolomide has been considered the standard treatment for GBM and increases the median length of survival to 12–15 months, although the disease typically progresses within 6–9 months, and the 2-year survival rate is less than 25%.²⁵ Overall, the outcomes for patients with GBM is still dismal, with a median length of survival of only 14.6 months and a 2-year survival rate of 26% at best. Research studies have attempted to identify the biological characteristics of GBM that could be manipulated to prolong the survival of patients with this disease.

In oncological research, the use of cancer epigenetics, which is defined as those stable changes in gene expression that occur without alteration of DNA sequences, has become popular in the study of several cancers. Biochemical modification of histones is one of the major epigenetic mechanisms that regulate gene expression and includes methylation, acetylation, ubiquitylation, and phosphorylation of histone proteins. Histone methylation occurs at the side chains of arginine and lysine residues. Histone H3 is primarily methylated at the 4 lysine residues within the N-terminal region (i.e., at K4, K9, K27, and K36) of H3. Specifically, methylation of H3K4 and H3K36 activates expression of genes interacting with H3, and methylation of H3K9 and H3K27 suppresses gene expression. The pathological and clinical significance of several enzymes associated with methylation or demethylation of histone H3 lysine residues has been reported in other cancers.^{8,13,16,19,30,32} Ubiquitously transcribed tetratricopeptide repeat gene on X chromosome (UTX) is an H3K27 demethylase that forms a complex with mixed lineage leukemia 4 (MLL4), an H3K4 methyltransferase,¹¹ and both are unique enzymes that are being fairly comprehensively studied in cancer. Therefore, we investigated the expression of these 2 enzymes and determined their clinical roles in patients with GBM.

In this study, we immunohistochemically analyzed tumor samples obtained by resection to determine the prognostic value of expression of UTX (the H3K27 demethylase) and MLL4 (the H3K4 methyltransferase) for predicting progression-free survival (PFS) and overall survival (OS) of patients with newly diagnosed GBM. To verify our results, we also examined other factors already known to be prognostic markers of OS and also markers of conventional treatment that predict outcomes among patients with GBM.

Methods

Sample Collections

The study protocol was approved by the institutional

review boards of Samsung Changwon Hospital, which is the sole regional university hospital serving a population of 1,500,000 people. All patients or their families provided written informed consent. We conducted a retrospective case study and clinical review of patients with GBM who had been surgically treated at Samsung Changwon Hospital from January 2002 to December 2013. All patients included in this study had a newly diagnosed GBM and were treated and followed up at our institution until death. The available histological samples obtained from the Department of Pathology archives of Samsung Changwon Hospital included formalin-fixed, paraffin-embedded (FFPE) tissue specimens ($n = 81$ patients), all of which were newly diagnosed as GBM. All patients had undergone resection or biopsy sampling of their tumors. In most cases, adjuvant conventional radiotherapy, chemotherapy, or both were performed after the GBM diagnosis. All H & E-stained slides were reviewed by 2 pathologists using the WHO classification of 2007 and who were blinded to the clinical and pathological parameters. Five samples were excluded because the tissue was almost entirely necrotized, or the tumor contribution to the section was less than 80%.⁶ In total, data from 76 patients were included in this study.

Clinical and Radiological Data

Epidemiological characteristics (including sex, age at the time of surgery, WHO performance status score, and recursive partitioning analysis [RPA] class), type of postoperative treatment, type of salvage treatment after tumor recurrence, length of follow-up, and time of death were retrospectively reviewed in the medical record of each patient. The radiation dosage, type of radiotherapy administered, and the regimen and timing of chemotherapy were also examined.

A radiological evaluation of the extent of tumor resection and response to treatment was performed by 2 different neuroradiologists who were blinded to clinical and pathological parameters. The extent of resection was estimated from MRI scans acquired within 48 hours of surgery. A subtotal resection was defined as removal of more than 90% of the Gd-enhancing lesion on enhanced T1-weighted MRI scans, and gross-total resection was defined as lack of a detectable Gd-enhancing lesion. In terms of treatment response, tumor measurements for determining treatment responses according to the Macdonald criteria were based on the product of orthogonal diameter on the image with the largest Gd-enhancing tumor area.¹⁸ If multiple lesions were present, the sum of the products of individual measurable lesions was calculated. Radiological studies were performed at regular 3-month intervals during the follow-up period or if there was clinical suspicion of disease progression.

Immunohistochemical Staining and Its Interpretation

All tissue specimens were examined for expression of UTX and MLL4 proteins. We obtained 3 or 4 sections sequentially from 1 FFPE GBM block per patient. For this analysis, the labeled streptavidin-biotin method was performed on sections from paraffin-embedded tissues that

had been used for disease diagnosis. The following monoclonal or polyclonal primary antibodies were used: UTX (1:200, Atlas Antibodies AB) and MLL4 (1:200, Abcam).

Appropriate positive and negative immunohistochemical controls were used throughout the study. Negative controls were samples in which the primary antibody had been omitted. Sections from normal brain cortex obtained from autopsy specimens were used as positive controls for UTX and MLL4 detection. Ten fields were selected from the regions with the highest concentrations of immunopositive nuclei and were examined at high-power magnification ($\times 400$). Each field corresponded to a total cell number ranging from 700 to 1000 cells relative to the cellularity of the tumor specimen, and areas of necrosis, normal glial cells, and endothelial cells were excluded. Considering 1000 cells by manual counting, we recorded the immunoreactivity of proteins and markers as the percentage of immunopositive cells.

Two different neuropathologists, who were both blinded to patient clinical and radiological information, reviewed all slides. If the difference between the percentages of immunopositive cells calculated independently by the 2 pathologists was less than 5%, the mean of the 2 percentages was used. If the difference was 5% or more, defined as discordance, 2 reviewers determined the mean percentage of immunoreactivity after repeatedly counting the cells and discussion; there was only 1 discordant case (1.3%) of immunoreactivity. Digital images were captured with a microscope (model BX41TF, Olympus) and digital camera (model DP70, Olympus).

The purpose of analyzing the immunoreactivity of UTX and MLL4 in GBM tissues was to determine whether any differences in expression of these 2 protein markers among patients were associated with the length of survival of these patients. The area under the receiver operating characteristic (ROC) curve was used to determine the optimal threshold of the mean percentage of immunopositive cells from 1000 cells. Sensitivity was calculated as the true positive rate (number of true positives divided by the sum of the numbers of true positives and false negatives), specificity was estimated as the true negative rate (the number of true negatives divided by the sum of the numbers of true negatives and false positives), and accuracy as the sum of the number of true positives and true negatives divided by the total number of positives and negatives. True positives were those in which the immunoreactivity percentage above the cutoff value had a positive influence on OS and true negatives those in which the immunoreactivity percentage below the cutoff value had a negative influence on OS.

We determined the threshold of immunoreactivity with the greatest sensitivity and specificity. Through a sensitivity-specificity analysis, a cutoff point for immunoreactivity at which sensitivity and specificity crossed and that was correlated with tumor recurrence was determined for each protein marker. According to the cutoff value established for these 2 proteins, a sequential correlation analysis for OS among GBM patients was performed.

Methylation-Specific Polymerase Chain Reaction

The methylation status of the *O*-6-methylguanine DNA

methyltransferase (*MGMT*) gene promoter in FFPE material was determined with methylation-specific PCR (MSP) as described by Palmisano et al.¹⁵

DNA Extraction and Bisulfite Conversion for Pyrosequencing

Because MSP is not a quantitative method for measuring gene methylation, we verified our PCR data with pyrosequencing (PSQ). This method uses the sequencing-by-synthesis principle and is a sensitive, highly reproducible, and cost-effective technique for analyzing DNA methylation. Genomic DNA was extracted from three 10- μ m-thick slices of FFPE material with the QIAamp DNA FFPE extraction kit and the QIAcube automated DNA extraction machine (Qiagen) and quantified by UV absorption (Nanodrop, Thermo Scientific), typically yielding more than 1 μ g of genomic DNA per specimen. Genomic DNA (200 ng) was used in the bisulfite conversion reactions with the EpiTect bisulfite kit (Qiagen), in which unmethylated cytosine is converted to uracil according to the manufacturer's instructions.

The PSQ protocol was performed as described by Kim et al.⁷ In brief, primer sets in which 1 primer was labeled with biotin were used to amplify the aforementioned bisulfite-converted DNA samples. The PyroMark Q96 CpG *MGMT* kit^{5,11} (Ensembl ID: OTTHUMT00000051009) (Qiagen) can detect methylation at positions 17–39 in Exon 1 of the *MGMT* gene, which contains 5 CpGs. A cytosine not followed by a guanine and which was unmethylated was used as an internal control in the analysis of the PSQ96MA 2.1 software (Biotage) to assess the efficiency of the bisulfite conversion. In theory, the internal control must yield 0% methylation because all cytosines that are not followed by a guanine are converted to uracil during bisulfite conversion, followed by conversion of uracil to thymine in PCR. Less than 5% methylation was considered an acceptable value for the internal control according to the manufacturer's protocol. If the internal control gave a value of greater than 5%, all procedures (from bisulfite conversion to PSQ) were repeated.

We performed PCR by using a bisulfite-converted genomic DNA equivalent of approximately 5000 cells and the PyroMark PCR kit (Qiagen). Throughout all steps, a standard PSQ sample preparation protocol was used.²⁶ PyroGold reagents were used for the PSQ reaction, and the signal was analyzed with the PSQ 96MA System (Biotage). Target CpGs were evaluated with PSQ96MA 2.1 instrument software (Biotage), which converts pyrograms of DNA to numerical values based on peak heights in analytical traces and calculates the proportion of methylation at each base as a cytosine-to-thymine ratio. Unmethylated (300 ng, Qiagen) and hypermethylated (Millipore) DNAs were used as standard controls and were bisulfite converted as described above.

Statistical Analysis

Differences between subgroups were analyzed with the Student t-test for normally distributed continuous values and with the Mann-Whitney U-test for nonnormally distributed continuous values. The chi-square test was used

to analyze categorical variables. To define the immunoreactivity cutoff value, the performance of each protein as a prognostic factor of survival with GBM was investigated with ROC curve analysis and sensitivity-specificity analysis.³ The PFS was calculated according to the Kaplan-Meier method, and comparisons among groups were performed with log-rank tests. Variables found to be statistically significantly associated with recurrence of GBM in the univariate analyses were examined in a multivariate analysis. Moreover, several additional variables that have been associated with OS in the literature and that were of interest to us were also included in the multivariate analysis. In this analysis, the Cox proportional-hazards regression model was used to assess the independent effects of specific factors on PFS and OS and to define the hazard ratios of significant covariates. Two-sided *p* values < 0.05 were considered statistically significant. SPSS version 18.0 (SPSS Inc.) was used for the statistical analysis.

Results

Patient and Tumor Characteristics

From a total of 81 cases of GBM recorded in the selected study period, data from 76 patients (47 men and 29 women) were eligible for inclusion in our analysis (Table 1). The mean age of these patients at the time of a new diagnosis of GBM was 52.4 years (range 29.4–81.6 years). The most frequent chief presenting symptoms were headache in 40 patients (52.6%), altered mentation in 16 (21.1%), focal neurological deficits such as motor weakness or dysphasia in 14 (18.4%), and seizures in 6 (7.9%). Thirty patients (39.5%) had a WHO performance status score of 0. Sixty-six patients (86.8%) had undergone radical section of tumors, and 10 (13.2%) had a diagnosis of GBM after a biopsy. For postoperative adjuvant treatment, 35 patients (46.1%) underwent nitrosourea-based combination chemotherapy with or without radiotherapy, and 41 (54.0%) underwent concurrent chemoradiotherapy with temozolomide.

All GBMs were located in the supratentorial area, and the main locations of involvement were as follows: 39 tumors (51.3%) were located in the frontal lobe, 18 (23.7%) in the temporal lobe, 8 (10.5%) in the parietal lobe, 5 (6.6%) in the occipital lobe, and 6 (7.9%) in the thalamus and basal ganglia. The mean maximal tumor diameter in the T1-weighted, Gd-enhancing image was 4.4 cm (range 1.5–8.3 cm), and the mean extent of peritumoral edema was 4.0 cm (range 2.1–6.4 cm). The *MGMT* promoter was methylated in 49 patients (64.5%) and unmethylated in 27 (35.5%).

After tumor progression, 34 patients (44.7%) underwent a second resection, and 9 (11.8%) were treated with repeated radiotherapy, 43 (56.6%) with salvage chemotherapy, and 9 (11.8%) with supportive care only (Table 1).

Results of Immunohistochemical Staining and Pyrosequencing

Interestingly, the pattern of immunohistochemical staining for UTX was identical to that of MLL4 in each section; the percentage of positive immunoreactive cells to UTX and MLL4 was completely identical in 2 different

TABLE 1. Characteristics of the patients with GBM

Variable	No. of Patients (%) (n = 76)
Age in yrs	
<50	25 (32.9)
≥50	51 (67.1)
M/F ratio	47 (61.8):29 (38.2)
WHO performance status score	
0	30 (39.5)
1	38 (50.0)
2	8 (10.5)
Extent of surgery	
Biopsy	10 (13.2)
Subtotal resection	30 (39.5)
Gross-total resection	36 (47.4)
RPA class	
III	17 (22.4)
IV	43 (56.6)
V	16 (21.0)
Status of <i>MGMT</i> promoter	
Methylated	49 (64.5)
Unmethylated	27 (35.5)
Postop adjuvant Tx	
Nitrosourea-based combination chemotherapy	35 (46.1)
Concurrent chemoradiotherapy w/temozolomide	41 (54.0)
Salvage treatment after progression*	
Second resection	34 (44.7)
Repeated radiotherapy	9 (11.8)
Salvage chemotherapy	43 (56.6)
Supportive treatment only	9 (11.8)

Tx = therapy.

* Some patients were treated with more than one modality.

sections obtained sequentially from 1 FFPE-GBM block. Increased expression of these 2 proteins was observed in 34 samples (44.7%) and decreased expression in 42 (55.3%) (Table 2). The percentage of cells with immunohistochemical staining relative to all cells in the stained sample was similar for UTX and MLL4, and the mean percentage for both was 27.6% (95% CI 8.6%–46.4%) (Fig. 1). An ROC curve analysis of the mean percentage of immunohistochemical staining predicting longer survival indicated an area under the curve of 0.653. The optimal threshold of the mean percentage of immunohistochemical staining for distinguishing between patients with longer survival and those with shorter survival was 34%, and this threshold yielded a sensitivity of 60.2% (95% CI 54.6%–65.8%), a specificity of 69.4% (95% CI 53.8%–84.8%), and an accuracy of 70.4% (95% CI 55.8%–86.0%). UTX and MLL4 were immunohistochemically overstained above the cutoff value in 34 samples (44.7%). Considering the UTX and MLL4 immunoreactivity results, we found that chi-square tests indicated no significant differences in clinical characteristics between the GBM patients with overexpressed

TABLE 2. Results of immunoreactivity analysis of MLL4-UTX in GBM stratified by clinical patient characteristics*

Variable	Immunoreactivity of MLL4-UTX Expression	
	Increased (n = 34)	Decreased (n = 42)
Age in yrs		
<50	12 (48.0)	13 (52.0)
≥50	22 (43.1)	29 (56.9)
Sex		
Male	22 (46.8)	25 (53.2)
Female	12 (41.4)	17 (58.6)
WHO performance status score		
0	14 (46.7)	16 (53.3)
1	17 (44.7)	21 (55.3)
2	3 (37.5)	5 (62.5)
Extent of surgery		
Biopsy only	4 (40.0)	6 (60.0)
Subtotal resection	15 (50.0)	15 (50.0)
Gross-total resection	15 (41.7)	21 (58.3)
RPA class		
III	8 (47.1)	9 (52.9)
IV	18 (41.9)	25 (58.1)
V	8 (50.0)	8 (50.0)
Status of <i>MGMT</i> promoter		
Methylated	23 (46.9)	26 (53.1)
Unmethylated	11 (40.7)	16 (59.3)
Postop adjuvant Tx		
Nitrosourea-based chemotherapy	15 (42.9)	20 (57.1)
CCRT w/ temozolomide	19 (46.3)	22 (53.7)

CCRT = concurrent chemoradiotherapy.

* Data in this table represent number of patients; row percentages are shown.

UTX and MLL4 and those with underexpressed UTX and MLL4 (Table 2).

The mean ($\pm$ SD) percentage of methylation of the *MGMT* gene was determined by PSQ analysis and was $14.0\% \pm 16.8\%$ for all GBM samples, $39.0\% \pm 14.7\%$ for the methylated samples, and $3.2\% \pm 1.8\%$ for the samples determined by the qualitative MSP method to be unmethylated ($p < 0.001$). A statistically significant linear correlation was detected between the methylation extent of the *MGMT* promoter and the immunoreactivity of UTX-MLL4 in GBM samples with methylated *MGMT* promoter ($r = 0.321$, $p = 0.026$), but no such correlation was observed in the samples with unmethylated *MGMT* promoter (Fig. 2).

Progression-Free Survival

All patients were followed up for at least 3 months, with a mean follow-up length of 18.1 months (range 4.1–43.5 months). During the follow-up period, 73 patients (96.1%) had GBM progression, and the mean length of PFS was 9.2 months (95% CI 6.8–11.6 months). In the multivariate analysis, gross-total resection (vs biopsy or

subtotal resection, $p = 0.018$), RPA Class III (vs Class IV or V, $p = 0.048$), and methylated *MGMT* promoter (vs unmethylated *MGMT* promoter, $p = 0.026$) were statistically significantly associated with longer PFS. Age, sex, WHO performance status score, method of postoperative adjuvant therapy, and immunoreactivity of UTX and MLL4 were not significantly associated with changes in PFS (Table 3).

Survival Outcome

During the follow-up period, 68 patients (89.5%) died, and the mean length of OS was 18.6 months (95% CI 14.3–22.9 months). In the multivariate analysis, age younger than 50 years (vs age ≥ 50 years, $p = 0.004$), WHO performance status score of 0 (vs Score 1 or 2, $p = 0.019$), gross-total resection (vs biopsy or subtotal resection, $p = 0.007$), RPA Class III (vs Class IV or V, $p = 0.036$), methylated *MGMT* promoter (vs unmethylated *MGMT* promoter, $p = 0.010$), and increased expression of UTX and MLL4 (vs decreased expression of UTX and MLL4, $p = 0.001$) were statistically significantly associated with longer OS (Table 4). Kaplan-Meier survival curve analysis and the log-rank test indicated similar results (Fig. 3A–G). Patient sex and the method of postoperative adjunctive treatment were not significantly associated with changes in OS.

A survival analysis according to the immunoreactivity of MLL4–UTX and methylation status of the *MGMT* promoter in GBM yielded the following findings (Table 5). The mean OS was 13.4 months for patients with a GBM having an unmethylated *MGMT* promoter and underexpressed UTX-MLL4, 15.8 months for those with a GBM and an unmethylated *MGMT* promoter and overexpressed UTX-MLL4, 17.8 months for those with GBM and a methylated *MGMT* promoter and underexpressed UTX-MLL4, and 26.6 months for those with GBM and a methylated *MGMT* promoter and overexpressed UTX-MLL4. Immunoreactivity of UTX-MLL4 had no statistically significant effect on OS among patients with GBM having an unmethylated *MGMT* promoter (hazard ratio 1.44, $p = 0.350$). However, patients with a GBM and a methylated *MGMT* promoter and overexpressed UTX-MLL4 had significantly longer OS than patients with GBM and an unmethylated *MGMT* promoter and underexpressed UTX-MLL4 (hazard ratio 4.51, $p < 0.001$). A Kaplan-Meier survival curve analysis and the log-rank test confirmed these observations (Fig. 3H). Similarly, a Student t-test indicated no significant difference in 2-year survival rate between the patients with GBM and an unmethylated *MGMT* promoter and overexpressed UTX-MLL4 and those with GBM and an unmethylated *MGMT* promoter and underexpressed UTX-MLL4 ($p = 0.186$) (Table 5); however, a statistically significant difference was detected between the patients with GBM and an unmethylated *MGMT* promoter and overexpressed UTX-MLL4 and all the patients with GBM and a methylated *MGMT* promoter ($p = 0.038$, data not shown).

Discussion

In clinical practice, it is not unusual for neurooncologists to encounter GBM patients with methylated *MGMT*

FIG. 1. The features of immunohistochemical staining in GBM samples with decreased expression of UTX (A) (by 4%), increased expression of UTX (B) (by 80%), decreased expression of MLL4 (C) (by 12%), and increased expression of MLL4 (D) (by 85%). Figure is available in color online only.

FIG. 2. The relationship of UTX-MLL4 immunoreactivity with the extent of *MGMT* promoter methylation. A significant linear correlation between UTX-MLL4 immunoreactivity and the extent of *MGMT* promoter methylation was observed in GBM samples with methylated *MGMT* promoter (green data points), as determined by MSP ($p = 0.026$). However, no such correlation was observed between the 2 factors in GBM samples with unmethylated *MGMT* promoter (blue data points) ($p = 0.927$). Figure is available in color online only.

TABLE 3. Multivariate analysis of potential factors predicting length of PFS among patients with GBM*

Variable	Mean Length of PFS (mos)	HR	p Value
Age in yrs			
≥50	8.7 (6.8–10.5)	1.00	
<50	10.2 (8.6–12.3)	1.22 (0.90–1.53)	0.237
Sex			
Male	8.6 (6.3–10.8)	1.00	
Female	10.2 (8.2–12.2)	1.16 (0.88–1.45)	0.481
WHO performance status score			
1 or 2	8.2 (6.1–10.3)	1.00	
0	10.1 (7.5–12.7)	1.73 (0.96–2.49)	0.057
Extent of surgery			
Biopsy or subtotal resection	8.1 (5.4–10.8)	1.00	
Gross-total resection	10.4 (7.4–13.7)	2.67 (1.48–3.86)	0.018†
RPA class			
IV or V	8.8 (6.6–12.0)	1.00	
III	10.6 (7.8–13.4)	2.03 (1.08–2.98)	0.048†
Status of <i>MGMT</i> promoter			
Unmethylated	7.0 (5.5–8.5)	1.00	
Methylated	10.4 (8.1–12.7)	2.24 (1.28–3.19)	0.026†
Postop adjuvant Tx			
CCRT w/temozolomide	8.9 (6.8–11.1)	1.00	
Nitrosourea chemotherapy	9.5 (8.0–10.9)	1.21 (0.87–1.54)	0.352
Immunoreactivity of MLL4-UTX			
Decreased expression	8.8 (6.7–10.9)	1.00	
Increased expression	9.7 (7.9–11.5)	1.29 (0.95–1.63)	0.174

HR = hazard ratio.

* Analysis was performed with the Cox regression model; ranges are 95% CIs.

† Statistically significant p value.

promoter who do not survive longer than those with unmethylated *MGMT* promoter during conventional treatment under similar systemic conditions. The results of the present study have shown that if GBM involves underexpressed UTX-MLL4, the patients with a GBM tumor having a methylated *MGMT* promoter do not have a significantly longer OS than those with an unmethylated *MGMT* promoter. In other words, our results suggest a significant difference in OS only among patients with GBM and a methylated *MGMT* promoter, and this difference depended on UTX-MLL4 expression. In the present study, MLL4 expression was identical to that of UTX, an observation that is concordant with a previous report by Lee et al.¹¹

Although there have been no reports on the specific roles of these 2 histone-modifying enzymes in GBM biology, a few studies have reported on the oncogenic role of other enzymes, including SUV39H1, SUV39H2, KDM4A, UTX (KDM6A), and MLL4 in human breast cancer;^{8,16} MLL4 and UTX in leukemia;^{19,32} UTX in renal-cell carcinoma;²¹ TERT and UTX (KDM6A) in bladder cancer;¹³ MLL2 in medulloblastoma⁵ and gastric cancer;³¹ and MLL3 in esophageal cancer.³⁰ Recently, UTX and MLL4 were reported to coordinately regulate transcriptional programs for cell proliferation and invasiveness in breast cancer cells,⁸ and downregulation of MLL3 was reported

to be required for growth and metastasis in esophageal squamous cell carcinoma.³⁰ However, these studies did not investigate the clinical roles of these enzymes.

The histone residues H3K27 and H3K4 are among the best-characterized histone-methylation markers. Methylation of H3K27 is associated with repression of gene expression, whereas H3K4 methylation is linked to gene activation or poised states.^{1,17,20,24,29} Methylation of lysine residues in histones is catalyzed by histone methyltransferases.^{12,22} For example, H3K4 methylation is catalyzed by several specific H3K4 methyltransferases, such as MLL and SET1A/B,²⁰ whereas H3K27 methylation is catalyzed by EZH1- or EZH2-containing complexes. Methylation of a lysine residue in histones may be reversed by histone lysine demethylases.^{2,9} For instance, H3K4 methylation is undone by several H3K4 demethylases (e.g., LSD1/2 and JARID1a-d), whereas methylated K27 in H3 is demethylated by UTX (also known as KDM6A) and JMJD3 (also called KDM6B²).

Using breast cancer cells in vitro and in mouse xenograft models, Kim et al.⁸ reported that both UTX and MLL4 regulate transcription of genes for cell proliferation and invasiveness. The authors showed that levels of the UTX protein are generally higher in breast cancer cell lines than in normal cell lines, and UTX knockdown substantially reduces the proliferation of the breast cancer cells. These re-

TABLE 4. Multivariate analysis of potential factors predicting length of OS among patients with GBM*

Variable	Mean Length of OS (mos)	HR	p Value
Age in yrs			
≥50	15.8 (13.4–18.2)	1.00	
<50	24.2 (19.9–28.4)	3.34 (2.03–4.66)	0.004†
Sex			
Male	17.4 (14.1–20.4)	1.00	
Female	20.3 (16.5–23.7)	1.55 (0.89–2.20)	0.372
WHO performance status score			
1 or 2	14.2 (10.9–17.5)	1.00	
0	22.4 (17.8–27.1)	2.99 (1.86–4.12)	0.019†
Extent of surgery			
Biopsy or subtotal resection	14.6 (10.1–19.1)	1.00	
Gross-total resection	23.1 (19.5–26.8)	3.09 (1.82–4.36)	0.007†
RPA class			
IV or V	16.9 (10.8–22.9)	1.00	
III	24.3 (19.6–28.8)	2.65 (1.28–3.91)	0.036†
Status of <i>MGMT</i> promoter			
Unmethylated	14.8 (12.4–17.1)	1.00	
Methylated	20.7 (17.5–23.9)	3.68 (1.80–5.57)	0.010†
Postop adjuvant Tx			
CCRT w/temozolomide	17.0 (14.1–19.9)	1.00	
Nitrosourea chemotherapy	20.5 (16.8–24.1)	1.68 (0.91–2.46)	0.217
Immunoreactivity of MLL4-UTX			
Decreased expression	15.5 (13.5–17.5)	1.00	
Increased expression	22.5 (18.3–26.6)	3.90 (2.53–5.26)	0.001†

* Analysis was performed with the Cox regression model; ranges are 95% CIs.

† Statistically significant p value.

sults suggest that UTX is required for in vivo invasiveness and tumor formation competence of breast cancer cells. According to these observations, high UTX or MLL4 levels are associated with poor clinical prognosis of patients with breast cancer, a finding that is contrary to that of our study. In the present study, increased expression of UTX and MLL4 was associated with longer survival in patients with GBM having a methylated *MGMT* promoter. The main reason that could explain these conflicting results may be the inclusion of different target genes; moreover, the study by Kim and colleagues⁸ focused on the proliferation and invasiveness of breast cancer cells. Our study did not show any roles of UTX and MLL4 in the proliferation or invasiveness in GBM, and we did not determine a biological mechanism that could affect the prognosis of GBM patients. Similarly, although the study by Kim et al. showed that UTX and MLL4 are associated with proliferation and invasiveness of breast cancer cells, the authors did not determine the specific gene that is epigenetically controlled by UTX and MLL4 and therefore responsible for these phenotypes. Similarly, we did not determine the specific target gene or biological characteristics playing a role in the prognosis for patients with GBM. However, we observed that the extent of *MGMT* promoter methylation and immunoreactivity of UTX and MLL4 were linearly correlated and influenced OS in patients with GBM and a methylated *MGMT* promoter. This result suggests that

UTX and MLL4 regulate *MGMT* promoter methylation in GBM.

Previous studies have reported that the gene encoding the H3K27 demethylase UTX often undergoes somatic loss-of-function mutations in multiple cancers, including medulloblastoma, renal carcinoma, bladder cancer, leukemia, and prostate tumors.²⁶ In addition, in normal fibroblast cells, UTX transcriptionally activates Rb pathway genes to suppress cell growth.²⁸ Therefore, UTX is thought to be a tumor suppressor in the aforementioned cancers, which could explain the better outcome in GBM patients with increased expression of UTX. However, these studies showed only an alteration in the *UTX* gene rather than the extent of changes in UTX protein expression. A review by van der Meulen and colleagues reported that no genetic defect of *UTX* has been observed in GBM, but the authors did not comment on the expression of the *UTX* gene and UTX enzyme in GBM.²⁷ Similarly, several reports have focused on genetic mutations or deletion of MLL4 in other cancers, including prostate cancer, medulloblastoma, and lymphoma.²⁶ Xia et al.³⁰ suggested that downregulation of MLL3, a member of the MLL family, in esophageal squamous cell carcinoma is required for the growth and metastasis of cancer cells. However, the authors did not identify the specific target gene that was epigenetically controlled by MLL3 nor the clinical role of MLL3 in patients with esophageal cancer. Although we note that we performed only immu-

FIG. 3. Kaplan-Meier survival curves of patients with GBM, grouped by several variables. Age (A), WHO performance scale score (B), extent of tumor resection (C), RPA class (D), methylation status of the MGMT promoter (E), adjuvant treatment after surgery (F), and immunoreactivity of UTX-MLL4 (G). Four patient subgroups categorized according to methylation status of the MGMT promoter and immunoreactivity of UTX-MLL4 (H). Bx = biopsy; GTR = gross-total resection; STR = subtotal resection. Figure is available in color online only.

TABLE 5. Length of survival according to the immunoreactivity of MLL4-UTX and methylation status of the *MGMT* promoter in GBM

Variables	No. of Patients (%)	Mean Length of OS in Mos (95% CI)	HR (95% CI)	p Value*	2-Yr Survival Rate (%)	p Value†
Unmethylated <i>MGMT</i> promoter & decreased expression of MLL4-UTX	16 (21.0)	13.4 (9.8–17.0)	1.00	—	12.4	—
Unmethylated <i>MGMT</i> promoter & increased expression of MLL4-UTX	11 (14.5)	15.8 (12.7–18.9)	1.44 (0.88–2.00)	0.350	20.8	0.186
Methylated <i>MGMT</i> promoter & decreased expression of MLL4-UTX	26 (34.2)	17.8 (14.7–20.9)	2.28 (1.14–3.41)	0.042	32.2	0.047
Methylated <i>MGMT</i> promoter & increased expression of MLL4-UTX	23 (30.3)	26.6 (21.6–31.5)	4.51 (3.04–5.98)	<0.001	58.9	<0.001

— = no p value because variable was used as reference.

* p values determined with Cox regression hazard model.

† p values determined with Student t-test.

nohistochemical staining for UTX-MLL4 in GBM and no analysis of the expression of the genes for these 2 proteins, we showed that expression of the UTX and MLL4 proteins in GBM cells was increased by about 27%.

Although our study suggests a meaningful role of UTX-MLL4 in outcomes among GBM patients, we note several important limitations. First, an analysis of protein levels in the present study suggested epigenetic control of UTX-MLL4 in *MGMT* protein expression and that this control could influence the outcomes among patients with GBM. However, the genetic and molecular biological evidence for any effects on GBM outcomes was not supplemented with in vitro and in vivo functional studies at the levels of DNA and RNA. In addition, it will be essential to identify the specific target gene(s) of UTX-MLL4 to determine their role in GBM.

Second, although the immunoreactivity in samples was assessed by 2 different neuropathologists, we are not certain that the results obtained were absolutely correct because the assessment of immunohistochemical staining results is qualitative and often subjective. Proper interpretation of most immunohistochemical stains depends to some extent on estimating antigen content and on establishing cutoff levels between positive and negative results. Reasonable run-to-run reproducibility is essential for proper implementation of these cutoff levels. In addition, threshold levels require adjustment to the sensitivity of the method used. For this reason, we used specificity-sensitivity testing to determine the optimal cutoff level. However, to validate the reproducibility of our immunohistochemical staining method, additional studies are necessary. For instance, although the percentages of cells showing immunoreactivity to UTX and MLL4 were identical in 2 sections obtained sequentially from 1 FFPE-GBM block, we could not determine whether the same cells with immunoreactivity to UTX also had immunoreactivity to MLL4. To determine the identity of immunoreactivity for both UTX and MLL4 at the cell level, an in vitro study will be essential.

Third, in terms of clinical end points, for patients treated in the period between 2002 and 2005, several variables relating to the introduction of temozolomide as a standard chemotherapeutic agent for GBM management were not captured. In particular, the extent of resection was not

volumetrically determined in these patients. Moreover, the therapeutic modalities were also variable: some patients were treated with the Stupp regimen and others by nitrosourea-based combination chemotherapy as the first adjuvant treatment after surgery. Some patients received only radiation treatment to the brain without adjuvant chemotherapy. Last, the retrospective nature of the analysis in the present study is its main limitation. We attempted to partially mitigate this bias by extracting patient data from complete medical and radiological records and by including patients who were treated with the same treatment protocol. Despite these efforts, however, the conclusions drawn from our study need further validation through prospective and randomized clinical trials.

Conclusions

In this study, we investigated the prognostic value of using immunoreactivity of UTX, an H3K27 demethylase, and of MLL4, an H3K4 methyltransferase, for predicting the clinical outcomes among patients with a new diagnosis of GBM. We found that increased immunoreactivity of UTX-MLL4 significantly influenced the outcomes among patients with GBM and a methylated *MGMT* promoter but not among those with an unmethylated *MGMT* promoter. Therefore, assessing the immunoreactivity of UTX-MLL4 could be a useful tool for predicting the response to conventional treatment with radiotherapy, chemotherapy, or both in patients with GBM and a methylated *MGMT* promoter. Nonetheless, additional studies involving sophisticated and systematically developed genetic techniques are required to examine the specific target gene(s) of UTX-MLL4 in GBM to characterize any epigenetic changes involving these 2 enzymes and the effects of these changes on tumor genesis and progression.

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Disclosures

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Conception and design: YZ Kim, SH Lee. Acquisition of data: YZ Kim, J Kim, Jang. Analysis and interpretation of data: YZ Kim, SH Lee, MS Kim, EH Lee. Drafting the article: YZ Kim, J Kim. Critically revising the article: YZ Kim, J Kim. Reviewed submitted version of manuscript: all authors. Approved the final version of the manuscript on behalf of all authors: YZ Kim. Statistical analysis: YZ Kim, J Kim, Jang. Administrative/technical/material support: YZ Kim, SH Lee, MS Kim, EH Lee. Study supervision: all authors.

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