

ORIGINAL ARTICLE

High prevalence of *TP53* mutations is associated with poor survival and an EMT signature in gliosarcoma patients

Sung-Yup Cho^{1,18}, Changho Park^{2,18}, Deukchae Na¹, Jee Yun Han³, Jieun Lee³, Ok-Kyoung Park², Chengsheng Zhang⁴, Chang Ohk Sung^{4,5}, Hyo Eun Moon^{6,7}, Yona Kim^{6,7}, Jeong Hoon Kim⁸, Jong Jae Kim⁵, Shin Kwang Khang⁵, Do-Hyun Nam^{9,10}, Jung Won Choi¹⁰, Yeon-Lim Suh¹¹, Dong Gyu Kim^{6,7}, Sung Hye Park^{12,13}, Hyewon Youn^{13,14}, Kyuson Yun¹⁵, Jong-Il Kim^{2,13,16}, Charles Lee^{3,4,19}, Sun Ha Paek^{6,7,19,20} and Hansoo Park^{17,19,20}

Gliosarcoma (GS) is a rare variant (2%) of glioblastoma (GBM) that poses clinical genomic challenges because of its poor prognosis and limited genomic information. To gain a comprehensive view of the genomic alterations in GS and to understand the molecular etiology of GS, we applied whole-exome sequencing analyses for 28 GS cases (6 blood-matched fresh-frozen tissues for the discovery set, 22 formalin-fixed paraffin-embedded tissues for the validation set) and copy-number variation microarrays for 5 blood-matched fresh-frozen tissues. *TP53* mutations were more prevalent in the GS cases (20/28, 70%) compared to the GBM cases (29/90, 32%), and the GS patients with *TP53* mutations showed a significantly shorter survival (multivariate Cox analysis, hazard ratio = 23.9, 95% confidence interval, 2.87–199.63, $P = 0.003$). A pathway analysis showed recurrent alterations in MAPK signaling (*EGFR*, *RASGRF2* and *TP53*), phosphatidylinositol/calcium signaling (*CACNA1s*, *PLCs* and *ITPRs*) and focal adhesion/tight junction (*PTEN* and *PAK3*) pathways. Genomic profiling of the matched recurrent GS cases detected the occurrence of *TP53* mutations in two recurrent GS cases, which suggests that *TP53* mutations play a role in treatment resistance. Functionally, we found that *TP53* mutations are associated with the epithelial–mesenchymal transition (EMT) process of sarcomatous components of GS. We provide the first comprehensive genome-wide genetic alternation profiling of GS, which suggests novel prognostic subgroups in GS patients based on their *TP53* mutation status and provides new insight in the pathogenesis and targeted treatment of GS.

Experimental & Molecular Medicine (2017) 49, e317; doi:10.1038/emm.2017.9; published online 14 April 2017

INTRODUCTION

Gliosarcoma (GS) is a rare type of brain tumor (annual incidence of 1 per 1 000 000), consisting of both malignant glial and

mesenchymal components.^{1–4} Although GS is currently treated in the same manner as glioblastoma (GBM), several lines of evidence suggest that GS is a distinct disease entity characterized

¹Ewha Institute of Convergence Medicine, Ewha Womans University Mokdong Hospital, Seoul, Korea; ²Department of Biomedical Sciences, Seoul National University College of Medicine, Seoul, Korea; ³Department of Life Science, Ewha Womans University, Seoul, Korea; ⁴The Jackson Laboratory for Genomic Medicine, Farmington, IL, USA; ⁵Department of Pathology, Asan Medical Center, University of Ulsan College of Medicine, Seoul, Korea; ⁶Department of Neurosurgery, Seoul National University College of Medicine, Seoul, Korea; ⁷Department of Neurosurgery, Seoul National University Hospital, Seoul, Korea; ⁸Department of Neurological Surgery, Asan Medical Center, University of Ulsan College of Medicine, Seoul, Korea; ⁹Department of Health Sciences and Technology, Samsung Advanced Institute for Health Science and Technology (SAIHST), Sungkyunkwan University, Seoul, Korea; ¹⁰Department of Neurosurgery, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, Korea; ¹¹Department of Pathology, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, Korea; ¹²Department of Pathology, Seoul National University Hospital, Seoul, Korea; ¹³Cancer Research Institute, Seoul National University College of Medicine, Seoul, Korea; ¹⁴Department of Nuclear Medicine, Seoul National University College of Medicine, Seoul, Korea; ¹⁵The Jackson Laboratory, Bar Harbor, ME, USA; ¹⁶Medical Research Center, Genomic Medicine Institute (GMI), Seoul National University, Seoul, Korea and ¹⁷Department of Biomedical Science and Engineering, Gwangju Institute of Science and Technology (GIST), Gwangju, Korea
¹⁸These authors contributed equally to this work and are considered co-first authors.

¹⁹These authors share senior authorship of this article.

²⁰These authors contributed equally to this work.

Correspondence: Professor SH Paek, Department of Neurosurgery, Seoul National University College of Medicine, 103 Daehak-ro, Jongno-gu, Seoul 03080, Korea. E-mail: paeksh@snu.ac.kr

or Professor H Park, Department of Biomedical Science and Engineering, Gwangju Institute of Science and Technology (GIST), 123 Cheomdangwagi-ro, Buk-gu, Gwangju 61005, Korea.

E-mail: hspark27@gist.ac.kr

Received 16 August 2016; revised 4 November 2016; accepted 29 November 2016

by a specific histology, a worse prognosis and more frequent metastases to extracranial lesions compared to GBM,^{1,3} which suggests that a different treatment approach for GS is needed.

The pathogenesis of GS is still largely unexplained. Early studies have suggested that the sarcomatous components originate from the mesenchymal components within the brain, such as endothelial cells.^{1,2} However, subsequent studies have failed to detect endothelial markers in the sarcomatous cells of GS.^{5–7} An alternative hypothesis is a monoclonal origin for both the glial and sarcomatous cells. The sarcomatous cells are considered to arise through the mesenchymal differentiation of the malignant glial cells,¹ which is also supported by similar genetic alterations in both the glial and mesenchymal components.^{4,8,9}

Previous studies have reported genetic alterations of the target genes for a limited number of GS samples. *PTEN* mutations (15–45%),^{4,9–11} *TP53* mutations (24–73%)^{4,9,11} and *TERT* promoter mutations (83%)¹⁰ were common, and *IDH1* mutations were rare or absent (0–7%).^{10,11} At the chromosome level, the copy-number alterations (CNAs) that were commonly detected included amplifications of chromosome 7 (75%) and X (20%) as well as deletions of chromosome 10q (88%) and 9p (35%).^{4,10} Amplifications of *EGFR* and homozygous deletions of *CDKN2A* were reported in 0–8%^{4,9} and 37–60%^{9,10} of the GS cases, respectively. However, a genome-wide characterization of the genetic alterations of GS is limited.

To provide a comprehensive view of the genomic alterations in GS, we generated an integrated genomic data set from whole-exome sequencing (WES) and a CNA analysis. Recurrent *TP53* mutations were detected in 71% of the GS patients, and these patients showed a significantly shorter survival. In addition, the *TP53* mutations were associated with the epithelial–mesenchymal transition process, suggesting that *TP53* mutations have a role in GS development, treatment resistance and poor prognosis.

MATERIALS AND METHODS

Patient samples and genomic DNA extraction

Four normal blood/GS paired samples and two normal blood/GS/GBM trios were obtained for the discovery set. Twenty-three formalin-fixed paraffin-embedded GS samples, including two samples from one patient, were obtained for the validation set. The patients were diagnosed with GS through a standardized review by two independent expert pathologists at the Seoul National University Hospital and the Samsung Medical Center. Total DNA was extracted from the sections using a QIAamp DNA Mini kit (Qiagen, Hilden, Germany). All samples were obtained with informed consent at the Seoul National University Hospital and the Samsung Medical Center, and the study was approved by the institutional review board in accordance with the Declaration of Helsinki.

Exome capture, library construction and sequencing

For all samples, 1 µg of DNA per sample was sheared using a Covaris SS Ultrasonicator (Covaris, Woburn, MA, USA). Exome capture was performed using an Agilent SureSelect Human All Exon Kit V5 (Agilent Technologies, Palo Alto, CA, USA). Each sample was sequenced on an

Illumina HiSeq 2000 instrument using a read length of 2 × 101 bp. Image analysis and base calling were performed using the Illumina pipeline (Illumina, San Diego, CA, USA) with the default settings.

Exome sequencing processing and variant calling

All FASTQ files were aligned to the human reference genome GRCh37/hg19 by Burrows–Wheeler Aligner mem.¹² Marking of the duplicate reads was performed by Picard tools. Indel realignment and base recalibration were performed using the Genome Analysis Tool kit.¹³ After processing the BAM files, MuTect was used to detect the somatic single nucleotide variants (SNVs), except for the *TTN* and *MUC16* genes.¹⁴ ANNOVAR was used to add the annotations.¹⁵ To select functional variants, variants within the coding regions were retained and filtered by a minor allele frequency if < 0.01 or not reported, based on the ExAC 65,000 exomes allele frequency data, the 1000 Genome Project for all population frequencies and the NHLBI Exome sequencing project 6500. Nonflagged SNPs, based on dbSNP138, were filtered out to sort out passenger calls, and possible false-positive calls in the segmental duplicate region were also removed. ADTEX and PaternCNV were used to detect somatic copy-number variations (CNVs) for the six blood/GS paired samples.^{16,17}

CNV microarray

The CNV microarray test was performed using the Affymetrix CytoScan HD array system according to the protocol provided by the manufacturer. The Affymetrix CytoScan HD array contains ~2.7 million probes throughout the human genome and includes 1 953 246 copy-number probes and 743 304 SNP probes. The current build used was the human genome reference build NCBI 37.3 (hg19). The build covers >36 000 RefSeq genes with 1 marker per 880 bases, complete International Standards for Cytogenomic Arrays (ISCA) constitutional coverage (1 marker per 384 bases), cancer gene coverage (1 marker per 553 bases), X chromosome genes (1 marker per 486 bases) and 12 000 OMIM genes (1 marker per 659 bases). Genomic imbalances were called using ChAS software (Affymetrix, Santa Clara, CA, USA) when a minimum of 25 consecutive probes were observed for a loss and when 50 consecutive probes were observed for a gain. To focus on the large chromosomal aberrations, a 2 Mb size cutoff is used for the CNV calls in this study.

Pathway analysis

Significantly altered pathways in GS were analyzed with a 201 gene list from the WES data of six normal/GS paired samples (discovery set) using the Kyoto Encyclopedia of Genes and Genomes analysis tool in the DAVID bioinformatics resource (<http://david.ncifcrf.gov>; $P < 0.1$).¹⁸

Cell culture

U373-MG GBM cells were obtained from the Korean Cell Line Bank and were maintained in Dulbecco's modified Eagle medium (Life Technologies, Gaithersburg, MD, USA) containing 10% fetal bovine serum (Life Technologies). The characteristics of the cells were tested and authenticated by the Korean Cell Line Bank, and cells in early passages were used for the experiment to minimize genetic changes. For the primary cell culture from the GS and GBM tissues, the samples were cut into pieces of ~1 mm on each edge and were incubated with trypsin-EDTA (33.3%; Life Technologies) and DNase I (20 U ml⁻¹)-containing phosphate-buffered saline for 1.5 h at 37 °C. After an additional incubation with DNase I (20 U ml⁻¹)-containing Dulbecco's modified Eagle medium (10% fetal bovine serum

included) for 15 min, the samples were triturated sequentially 20, 15 and 10 times through fire-polished glass pipettes with decreasing core diameters. After trituration, the samples were filtered through a 40- μm mesh, and the cells were eventually plated in T-75 culture flasks with Dulbecco's modified Eagle medium containing 10% fetal bovine serum. Penicillin (100 U ml⁻¹; Life Technologies) and streptomycin sulfate (100 μg ml⁻¹; Life Technologies) were supplemented to all cell culture media. All cells were maintained in a humidified incubator with 5% CO₂ at 37 °C.

Quantitative real-time PCR

Total RNA was purified using an RNeasy Plus Mini kit according to the manufacturer's instructions (Qiagen). One microgram of total RNA was transcribed into complementary DNA using a Maxime RT PreMix (Intron Biotechnology, Seoul, Korea) for 1 h at 45 °C. Quantitative real-time PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as an internal control for normalization. The sequences of the primers are summarized in Supplementary Table S1.

Gene set enrichment analysis

To identify the gene sets enriched in GS compared to GBM, we searched Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo/) for the gene expression profiling and selected the data set of GSE8692, contributed by Kenneth Kosik.¹⁹ From this data set, we chose a total of two GS samples and six GBM samples for further analysis. Each Affymetrix data set was background-adjusted and normalized by the Robust Multichip Averaging algorithm in the Affy package using R ver. 3.1.1.²⁰ Gene set enrichment analysis (GSEA) was performed using the javaGSEA desktop application (GSEA v2.1.0).^{21,22} For this analysis, the chemical and genetic perturbations (version 5.2) gene sets were used, and the gene sets with <50 genes and >100 genes were excluded. As the number of samples in this study was small, the *P*-values were calculated by permuting the data 1000 times for finding enriched gene sets. The GSEA software produces an enrichment score, a normalized enrichment score, nominal *P*-value and a false discovery rate (*Q*-value). The gene sets that were up- or downregulated with a *Q*-value <0.05 were considered significant. For the complementary DNA microarray data from the primary cell culture, the GSEA was performed with the same parameters except for using the pre-ranked options derived from the rank of fold changes of the GS samples compared to the GBM samples.

Complementary DNA microarray

Total RNA was extracted from the primary cells (<10 passages) using a TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The transcriptomic profiles of these glioma cells were characterized by an oligonucleotide microarray analysis using MA-human Agilent 44K (Agilent Technologies). The one-channel microarray data were analyzed by a global median normalization method using GeneSpring GX 7.3 (EBIOGEN, Seoul, Korea).

Site-directed mutagenesis and transfection of DNA

A QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) was used to generate point mutations in *TP53*, and the resulting mutations were verified by Sanger sequencing. The primers used in constructing the point mutations are listed in Supplementary Table S1. Transfection experiments were performed using Lipofectamine 2000 Transfection reagent according to the manufacturer's

protocol (Invitrogen). After 48 h, the cells were collected for real-time PCR and migration/invasion assays. The overexpression of the wild-type and mutants *TP53* was validated by western blot analysis.

Western blot analysis

Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (Thermo Scientific, Hudson, NH, USA) containing a protease inhibitor cocktail (Roche, Indianapolis, IN, USA) and a phosphatase inhibitor cocktail (Roche) and were centrifuged at 20 000 g for 10 min at 4 °C. After determining of the protein concentration in the cell extract by the bicinchoninic acid (BCA) method (Thermo Scientific), 20 μg of protein was resolved by SDS-polyacrylamide gel electrophoresis and was transferred to a polyvinyl difluoride membrane. Membranes were blocked for 1 h with 5% skim milk in Tris-buffered saline and were incubated with anti-Flag (Sigma-Aldrich Corporation, St Louis, MO, USA) and anti-actin (Sigma-Aldrich Corporation) antibodies. The membranes were washed and were incubated with a horseradish peroxidase-conjugated secondary antibody, followed by enhanced chemiluminescence development according to the manufacturer's instructions (Pierce, Rockford, IL, USA).

In vitro migration and invasion assay

Transfilter migration and invasion assays were performed using 8.0- μm pore inserts in a 24-well Transwell (BD Biosciences, San Jose, CA, USA). Cells were transfected with wild-type and mutant *TP53*-expressing vectors using Lipofectamine 2000 Transfection reagent. After 24 h of incubation, the cells were isolated and added to the upper chamber of a transwell (5×10^3 cells per well) with a non-coated filter and were incubated for 24 h in the migration assay. The invasion assays were performed using 12.5% Matrigel (Corning, New York, NY, USA)-coated filters at 5×10^3 cells per well, and the cells were incubated for 48 h. The migrated or invaded cells were stained with a Diff-Quik staining kit (Sysmex Corporation, Kobe, Japan). Each experiment was performed in triplicate.

Statistical analysis

Statistical calculations were performed using SPSS, version 22.0 (IBM Corporation, Somers, NY, USA) or Prism 4.0 (GraphPad, San Diego, CA, USA). Kaplan–Meier curves were constructed to evaluate overall survival. For univariate survival analysis, log-rank tests were used to explore the differences in survival according to the *TP53* mutation status. Multivariate Cox analyses were performed to evaluate the predictive factors of overall survival. The variables tested included sex (categorical), age (continuous), tumor size (continuous), tumor location (categorical), treatment (categorical) and *TP53* mutation status (categorical). The differences between multiple variables were assessed by a one-way analysis of variance with a Tukey's multiple comparison test. The difference was considered significant if the *P*-value was <0.05.

RESULTS

Genome-wide analyses of mutations and CNAs in GS

We explored genomic alterations in GS through WES of six paired tumor and normal samples (the discovery set). In addition, we analyzed additional 22 formalin-fixed and paraffin-embedded GS samples from a multicenter study (the validation set). Detailed clinical information and the magnetic resonance imaging images for these patients are provided in Table 1, Supplementary Table S2 and Supplementary Figure S1. On average, 99.9% of the reads mapped for

all samples, with an average read coverage of $124.64\times$ and $117.95\times$ for the six matched normal samples and the six GS samples, respectively. Similarly, the average read coverage for the 22 formalin-fixed paraffin-embedded samples was $126.76\times$.

In the discovery set, exome sequencing identified a total of 295 somatic mutations (199 missense mutations, 9 nonsense mutations, 77 synonymous mutations, 5 splice sites mutations and 5 unclassified mutations). The 213 non-silent mutations were matched to 201 genes (Supplementary Table S3). Somatic mutations in *TP53* were the most frequent alterations (5/6), and mutations of *PTEN*, *SETD2*, *PLCL1*, *BCAM* and *PCDH19* were also recurrently detected (Figure 1a; Supplementary Table S3).

In the 22 patient validation set, 5904 somatic or germline mutations (5619 nonsynonymous mutations, 185 nonsense mutations, 5 stoploss mutations and 95 splice sites mutations; Supplementary Table S4) were identified. To preclude

the possible germline mutations, we further focused on the 201 genes detected to have non-silent mutations in the discovery set. The *TP53* mutation was also found as the predominant mutation type in the validation set (15/22, 68.2%), and all *TP53* mutations, except for one, were already reported in the COSMIC database (Supplementary Table S5). Therefore, alterations in *TP53* were detected in 71% of all GS cases (20/28; Figure 1a; Supplementary Table S6), which were significantly higher than in cases of Korean GBM patients (29/90, 32%, Supplementary Table S7). The mutation abundance was not significantly different between the *TP53* wild-type and the *TP53*-mutated groups ($P=0.3884$). In addition, we identified 43 recurrently mutated genes in three or more cases from the total 28 GS samples. *PTEN* mutations were the second most frequent alteration observed (8/28, 28.6%; Figure 1a; Supplementary Table S6).

Clinically, *TP53*-mutated GS appeared to have a poorer prognosis than non-mutated *TP53* GS in both a univariate analysis (log-rank test, $P=0.019$; Figure 1b) and the multivariate analyses (multivariate Cox analysis, hazard ratio = 23.9, 95% confidence interval, 2.87–199.63, $P=0.003$; Table 2), which suggest that *TP53*-mutated GS is a unique subgroup of GS. Other clinical parameters, such as location, size, gender and GS type (primary or secondary), showed no association between the *TP53* wild-type and the *TP53*-mutated subgroups (Supplementary Table S8). By contrast, no significant survival difference was observed between the *TP53* wild-type and the *TP53*-mutated subgroups in the Korean GBM patient data ($n=90$, log-rank test, $P=0.368$; Figure 1b, detailed clinical information is in Supplementary Table S7). Patients with mutations in *PTEN* from our GS group showed little difference in overall survival compared to those without mutations (log-rank test, $P=0.819$, Supplementary Figure S2).

A gain of chromosome (chr) 7 and a loss of chr 10 are well-known events that occur during the evolution of cells into GBM.²³ For the somatic CNA analysis, we applied the genome-wide CNV microarray platform for the five paired tumor and normal samples (Supplementary Figure S3; Supplementary Table S9). Chr 7 was amplified in all the GS samples, and chr 10p was deleted in two out of the five cases analyzed from the discovery set (Figure 1c). These alterations were consistent with the copy-number analysis results using the WES data (Supplementary Figure S4). The copy-number analysis using WES also showed well-known CNAs, such as amplification events of *EGFR*, *CDK6* and *MET*, as well as heterozygous deletions of *PTEN*, *RBI* and *NFI* in the GS samples from the discovery set (Supplementary Table S10). These findings imply that GS has some shared alterations with GBM in terms of CNA.

A biallelic loss of *TP53* probably has profound effects on cancer cell development. Out of the five patients (patient #1–5) whose WES and CNV microarray data were available, we found one patient (patient #2) showing biallelic alterations of *TP53* with both mutation (D149G) from WES data and copy-number loss from the CNV microarray data (Supplementary Figure S5; Supplementary Table S11).

Table 1 Demographic and clinical characteristics of the 28 gliosarcoma patients

Variables	No. (N = 28)	%
<i>Gender</i>		
Male	19	67.9
Female	9	32.1
<i>Age, years (median = 51.5)</i>		
≤ 50	11	39.3
> 50	17	60.7
<i>TP53 mutation</i>		
<i>TP53</i> mutation	20	71.4
<i>TP53</i> wild-type	8	28.6
<i>Diagnosis</i>		
Primary gliosarcoma	21	75.0
Secondary gliosarcoma	7	25.0
<i>Tumor size, cm³</i>		
≤ 30	15	53.6
> 30, ≤ 60	7	25.0
> 60	6	21.4
<i>Location</i>		
Frontal	10	35.7
Temporal	9	32.2
Parietal	5	17.9
Occipital	2	7.1
Others	2	7.1
<i>Treatment</i>		
Surgery	4	14.3
Surgery+RT	2	7.1
Surgery+RT+CTx	13	46.4
Surgery+RT+CTx+GKS	9	32.2

Abbreviations: CTx, chemotherapy; GKS, gamma knife surgery; RT, radiotherapy.

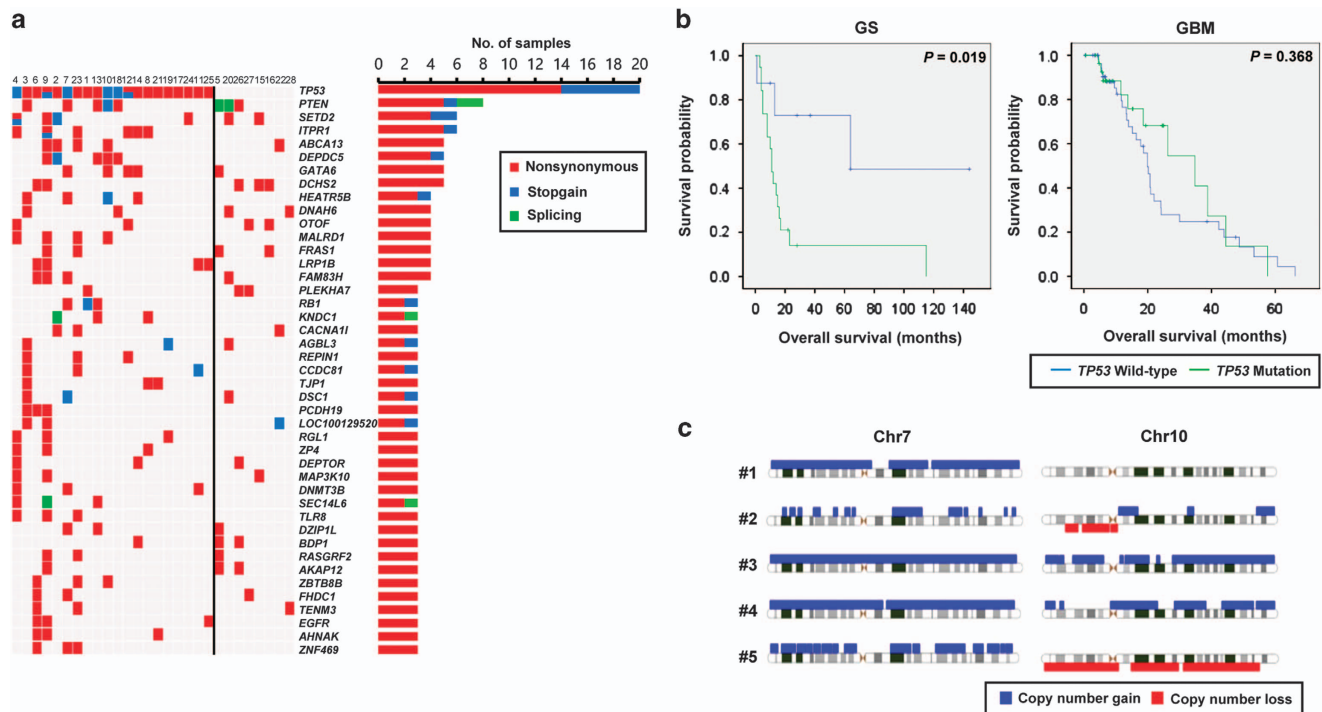


Figure 1 Genomic alterations of gliosarcoma (GS) patients. **(a)** Mutational profiles for 28 GS patients by whole-exome sequencing. Matrix of the altered genes colored by types of mutations. Each column stands for an individual cancer patient (#1–6 for discovery set and #7–28 for validation set), and each row denotes a gene. The right bar graph represents the number of samples with mutations for each gene in the 28 GS patients. **(b)** Left panel: Kaplan–Meier plot for the overall survival of the GS patients with ($n=20$; green line) and without *TP53* mutations ($n=8$; blue line). Right panel: Kaplan–Meier plot for the overall survival in the Korean glioblastoma (GBM) patients with ($n=30$; green line) and without *TP53* mutations ($n=61$; blue line). **(c)** Copy-number alterations for chromosome 7 (Chr7) and chromosome 10 (chr 10) in five GS patients. The amplifications (blue) and deletions (red), determined by a copy-number variation (CNV) microarray, are displayed for chromosomes 7 and 10.

Table 2 Multivariate Cox regression analysis of the association between features of the patients and overall survival

Variables	Multivariate Cox analysis		
	HR	95% CI	P-value
Sex (female vs male)	0.348	0.095–1.282	0.113
Age (per year)	0.930	0.846–1.023	0.137
Tumor size	0.986	0.967–1.005	0.149
<i>Location</i>			
Temporal vs frontal	0.669	0.173–2.588	0.560
Parietal vs frontal	0.085	0.007–1.001	0.050
Occipital vs frontal	1.030	0.061–17.460	0.984
Others vs frontal	1.500	0.097–23.118	0.771
<i>Treatment</i>			
Surgery+RT vs Surgery	0.176	0.009–3.474	0.254
Surgery+RT+CTx vs Surgery	0.009	0.000–0.223	0.004 ^a
Surgery+RT+CTx+GKS vs Surgery	0.002	0.000–0.071	0.001 ^a
<i>TP53</i> (mutated vs non-mutated)	23.933	2.869–199.634	0.003 ^a

Abbreviations: CI, confidence interval; CTx, chemotherapy; GKS, gamma knife surgery; HR, hazard ratio; RT, radiotherapy.
^aIndicate significance at $P < 0.05$.

Significantly altered pathways in GS

We performed pathway analysis on the 201 altered genes identified in the discovery set using the Kyoto Encyclopedia of Genes and Genomes analysis tool²⁴ in the DAVID bioinformatics resource (<http://david.ncicrf.gov>).²⁵ Several important pathways, such as MAPK signaling, phosphatidylinositol/calcium signaling and focal adhesion/tight junction pathways, were significantly altered in the GS cases (Figure 2a and 2b; Supplementary Tables S12 and S13). Mutations in genes of the MAPK signaling pathway were detected in 79% (22/28) of the GS cases, which included *EGFR*, *FGFR1*, *RASGRF2* and *TP53*. We also found mutations in genes of the phosphatidylinositol/calcium signaling pathway, which included *CACNA1F/1I*, *PLCB3/L1* and *ITPR1/3*, in 64% (18/28) of the GS cases. Finally, we found mutations in the genes of the focal adhesion/tight junction pathway, which included *COL5A1*, *ITGB7*, *PAK3* and *PTEN*, in 57% (16/28) of the GS cases (Figure 2a and 2b; Supplementary Table S13).

Analysis of genomic alteration profiles in the matched initial and recurrent GS samples

Among our GS patient cohort, we found three patients with matched initial and recurrent tumors after treatment (surgery and concurrent chemoradiotherapy with temozolomide), and then we compared the mutational profiles between

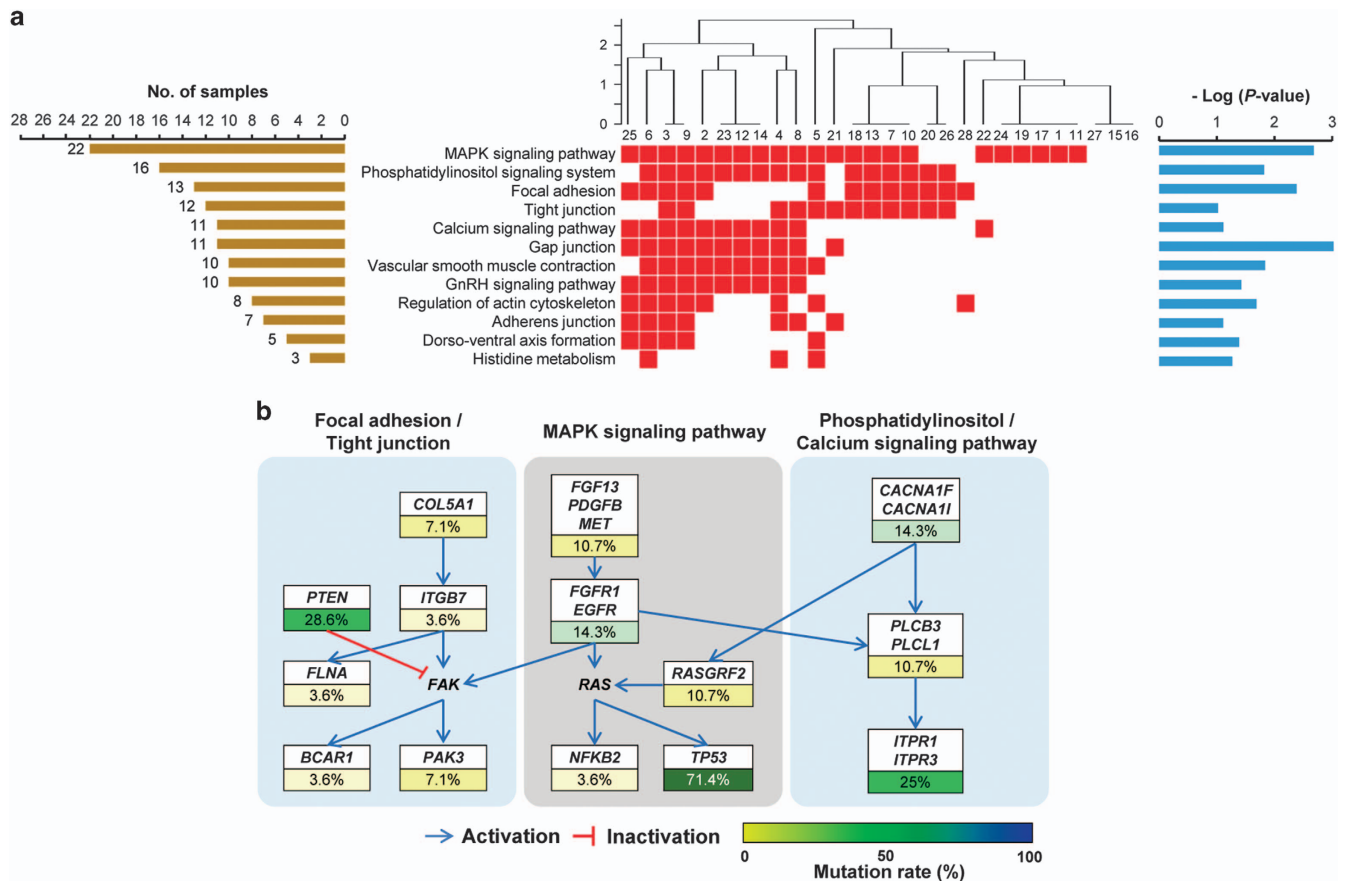


Figure 2 Pathway analysis in gliosarcoma (GS). (a) List of the somatically mutated pathways analyzed by the Kyoto Encyclopedia of Genes and Genomes database ($P < 0.1$). The left side shows the number of affected patients out of the 28 patients in each pathway. The middle matrix indicates the alteration events in each individual (column), and the patients were clustered according to altered pathways by a hierarchical cluster analysis. The right side shows the P -values from the pathway analysis using DAVID bioinformatics resource. (b) Mutated genes in the MAPK signaling, phosphatidylinositol/calcium signaling and focal adhesion/tight junction pathways. Mutation frequencies are expressed as a percentage of cases ($n = 28$).

the initial and recurrent samples. Patient #5 was first diagnosed as having GS through a pathological examination; after treatment, the tumor recurred as a same-site GBM at the second operation and a same-site GS at the third operation (Figure 3a and 3b). We detected 56 and 21 recurrence-specific mutations in first recurrent sample (GBM) and second recurrent sample (recurrent GS), respectively (Figure 3b; Supplementary Table S14). Interestingly, the WES analysis detected a *TP53* mutation only in the recurrent GS sample (Figure 3b; Supplementary Table S14). A comparison of the CNAs between the initial GS and the recurrent GBM in patient #5 showed increased CNAs in the recurrent sample, especially in chromosomes 4, 5 and 21 (Supplementary Figure S6), suggesting that CNAs are also associated with treatment resistance.

Patient #6 was first diagnosed as having GBM; however, after treatment, the tumor recurred as a same-site GS (Figure 3b). We found only one recurrence-specific mutation (*ZNF469*) in patient #6, and the *TP53* mutation was detected in both the initial and recurrent GS samples (Figure 3b; Supplementary Table S15). To preclude the possibility of false positives in the

WES, we performed Sanger sequencing for mutations found in one of the matched samples, and four initial tumor-specific mutations (*HEPACAM2*, *CCDC83*, *PTPRT* and *FTSJ1*) and one recurrence-specific mutation (*ZNF469*) were validated (Supplementary Table S15).

An additional recurrent sample from patient #22 was determined as GS in the pathological investigation of both the initial and same-site recurrent lesions. Of the 201 genes found in the discovery set, the WES analysis detected three mutations (*ITPR3*, *GIMAP8* and *TP53*) only in the recurrent sample, likely allowing these clones to overcome therapy (Figure 3b; Supplementary Table S16). Interestingly, *TP53* mutations were found in all three recurrent GS samples, and the WES analysis detected *TP53* mutations in the recurrent GS samples from patient #5 and #22 (Figure 3b). Moreover, the amino-acid changes of these two patients were in the same residue (D281G; Figure 3b). Therefore, *TP53* mutations are likely associated with the outgrowth of aggressive GS subclones that are resistant to previous treatments, such as concurrent chemoradiotherapy with temozolomide.

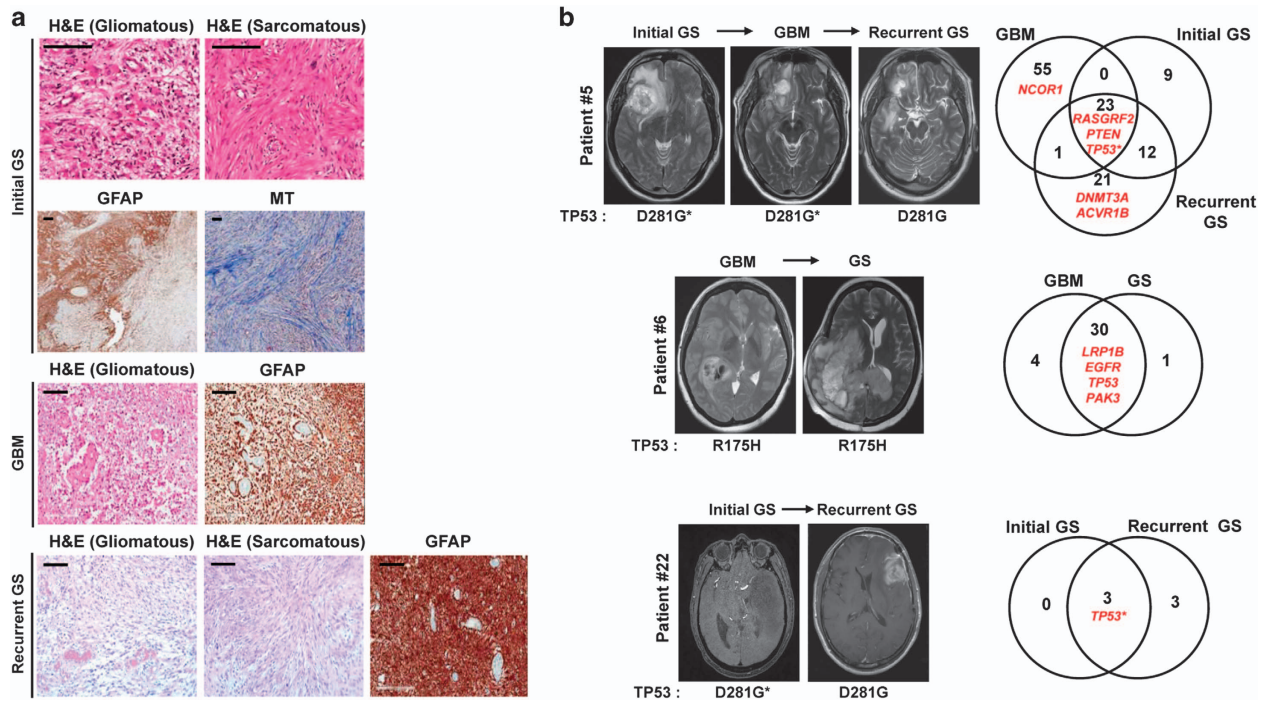


Figure 3 Changes in histology, magnetic resonance imaging (MRI) images and the mutational profile in recurrent gliosarcoma (GS) patients. **(a)** Representative microscopic images of the GS and glioblastoma (GBM) tissues of patient #5, who had same-site recurrent samples showing the conversion of pathological diagnosis (initial GS to the same-site recurrent GBM after the first treatment and GBM to the same-site recurrent GS after the second treatment). The gliomatous and sarcomatous areas were visualized by H&E staining ($\times 200$ in initial GS, and $\times 100$ in GBM and recurrent GS). Gliomatous area was positive for glial fibrillary acidic protein (GFAP) staining ($\times 40$ in initial GS, and $\times 100$ in GBM and recurrent GS), and sarcomatous area showed Masson trichrome (MT)-positive collagen deposit ($200\times$). Scale bar, $100\mu\text{m}$. **(b)** MRI images (left panel) and Venn diagrams representing the number of mutated genes (right panel) in three patients (patients #5, #6 and #22) who had matched initial and recurrent tumors after treatment. The genes in the Venn diagram are the mutated genes selected from the modified JAX Cancer Treatment Profile.⁴⁴ Asterisks indicate mutations detected by droplet digital PCR. WT, wild type.

To investigate whether the *TP53* mutations in the recurrent tumors existed as a clone in the initial tumors, we adopted droplet digital PCR, which is a highly sensitive method to detect mutations to a 0.1% frequency within a sample.²⁶ We detected a D281G *TP53* mutant allele at a frequency of 0.24% and 0.12%, in the initial GS and the same-site recurrent GBM samples of patient #5, respectively (Supplementary Figure S7a). Similarly, we found a D281G *TP53* mutation at a frequency of 1.7% in the initial GS of patient #22 (Supplementary Figure S7b). After treatment of the initial GS of patient #22, we detected the *TP53* mutations at a frequency of 82.2% in the recurrent sample (Supplementary Figure S7b). These results suggest that *TP53* mutations exist as a minor clone at a low allele frequency in the initial tumors, and that *TP53* mutations may play a role in treatment resistance.

Association of *TP53* mutations with the EMT process

Given that EMT is the conversion of cells with epithelial characteristics into cells with mesenchymal properties,²⁷ we investigated the role of the EMT process in GS development. Concordantly, the EMT signature gene expressions, including N-cadherin, vimentin and α -smooth muscle actin,²⁸ were

elevated in the GS tissues of patients #5 and #6 compared to the GBM tissues (Figure 4a). In addition, the GSEA using the microarray data from the Gene Expression Omnibus database (GSE8692) showed that the two cancer EMT-associated gene sets (ANASTASSIOU_CANCER_MESENCHYMAL_TRANSITION_SIGNATURE²⁹ and JECHLINGER_EPITHELIAL_TO_MESENCHYMAL_TRANSITION_UP³⁰) were significantly enriched in GS compared to GBM (Supplementary Figure S8; Supplementary Table S17). In addition, the GSEA analysis showed that inflammation-related signatures, including HECKER_IFNB1_TARGETS, SANA_RESPONSE_TO_IFNG_UP, SANA_TNF_SIGNALING_UP, MORI_MATURE_B_LYMPHOCYTE_UP, LU_IL4_SIGNALING and COATES_MACROPHAGE_M1_VS_M2_UP, were enriched in the GS samples compared to the GBM samples. An enrichment of the cancer EMT-associated gene sets in the GS samples was also reproduced in the GSEA analysis using a complementary DNA microarray data from the primary culture cells of the GS and GBM tissue from patient #6 (Supplementary Table S18), suggesting that EMT processes are involved in the mesenchymal differentiation in GS.

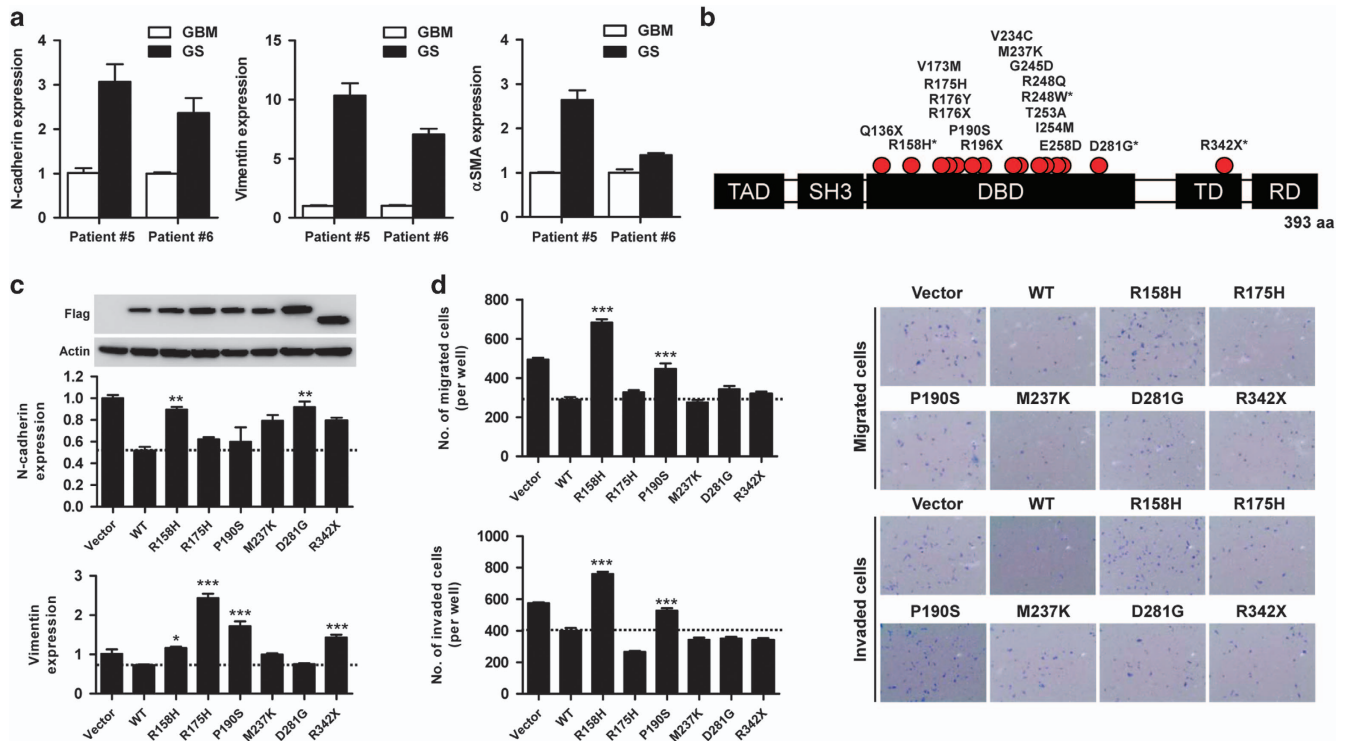


Figure 4 Association of the epithelial–mesenchymal transition signature with *TP53* mutations. (a) Relative mRNA expression levels of EMT-associated genes in gliosarcoma (GS) and glioblastoma (GBM) tissues from patients #5 and #6. The amount of mRNA for each gene was determined by real-time PCR. (b) The locations of somatic mutations of *TP53* in the 28 GS samples (red circles). The protein of *TP53* consists of a transactivation domain (TAD), an SH3 domain, a DNA-binding domain (DBD), a tetramerization domain (TD) and a regulation domain (RD). Asterisks indicate recurrently mutated residues in the *TP53* gene. (c) Regulation of EMT-associated genes by wild-type and mutant *TP53* in the mutant *TP53* (R273H)-expressing cells (U373-MG). The effects of wild-type and mutant *TP53* on mRNA expression of EMT-associated genes were estimated by real-time PCR. (d) Effect of the wild-type and mutant *TP53* on cell migration and invasion in the mutant *TP53* (R273H)-expressing cells (U373-MG). *In vitro* cell migration and invasion were estimated by a transfilter migration and invasion assay. Left graphs show the number of migrating (upper) and invading (lower) cells from triplicate experiments. The right panel is the representative images of the migrating (upper) and invading (lower) cells. Asterisks indicate significant differences (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$) compared to wild-type *TP53*.

We found 18 mutated residues of the *TP53* gene in 20 GS patients, and four mutations (R158H, R248W, D281G and R342X) were recurrently detected in two or more cases of GS (Figure 4b; Supplementary Table S5). All *TP53* mutations, except one stopgain mutation (R342X), were detected in the DNA-binding domain of *TP53* (Figure 4b). To investigate the role of *TP53* mutations in the EMT signature of GS, we examined the effects of the *TP53* mutants on EMT-associated gene expression. The overexpression of wild-type *TP53* in the mutant *TP53*-expressing cells (U373-MG) attenuated the expression of N-cadherin and vimentin (Figure 4c). Among the randomly selected six *TP53* mutants (five mutants from DNA-binding domain and the one mutant from tetramerization domain), five mutants (R158H, R175H, P190S, D281G and R342X) showed a decreased inhibitory effect on the mRNA expression of either or both N-cadherin and vimentin compared to wild-type *TP53* (Figure 4c). In addition, these mutants have functional defects in inhibiting cell viability compared to the wild-type *TP53* in clonogenic assays (Supplementary Figure S9).

EMT increases the migration and invasion of cancer cells, resulting in increased metastasis.³¹ The overexpression of wild-type *TP53* decreased *in vitro* migration and invasion of the mutant *TP53*-expressing cells (U373-MG; Figure 4d). Out of the randomly selected six *TP53* mutants, the R158H and P190S mutants had reduced inhibitory effects on cell migration and invasion compared to the wild-type *TP53* (Figure 4d), which are characteristics of EMT. Therefore, the high prevalence of *TP53* mutations in GS is associated with EMT features.

DISCUSSION

In this study, we found dominant mutations of the *TP53* gene (20/28, 71%) in GS, and somatic mutations of the *TP53* gene are associated with a poor patient survival, suggesting that the GS patients can be divided into two main molecular subgroups according to their *TP53* mutation status. By comparing the paired initial/recurrent samples from the same patient, we found two cases in which the *TP53* mutations were detected only in the WES analysis of the recurrent samples, which are

likely associated with treatment resistance. In addition, we showed that the *TP53* mutations rendered the cells more vulnerable to mesenchymal differentiation via cancer EMT-like processes (Supplementary Figure S10).

GS is considered a rare variant of GBM,¹ and extensive studies of the genomic alterations in GBM have been performed.^{32,33} Mutations in *IDH1* were found to be a molecular marker that distinguished primary and secondary GBM.³⁴ The most frequent genetic alteration of GBM (~60%) is a deletion at 10q,^{35,36} and *TP53* mutations are common in secondary GBM (~25%) but are rare (<15%) in primary GBM.³⁷ Using the expression profiles of GBM, proneural, neural, classical, mesenchymal and proliferative patterns were reported.^{33,38} Most *IDH1*-mutated GBM (92%) show a proneural expression signature, and high frequencies of *TP53* (54%) and *NF1* (37%) mutations are characteristic of the proneural and mesenchymal subtypes, respectively.³³ We found one patient with an *IDH1* mutation and another patient with an *NF1* mutation out of the 28 GS patients (Supplementary Table S6) and detected a hemizygous deletion of *NF1* in three of the GS samples within the discovery set (Supplementary Table S10). Therefore, GS showed distinctive genomic alteration profiles in the known cancer-driver genes compared to the subtypes of GBM.

EMT is a process by which epithelial cells undergo changes into mesenchymal cells, which are usually associated with cancer aggressiveness.^{27,39} EMT is often induced by epigenetic reprogramming mediated by EMT-inducing transcription factors, such as Twist, Snail, Slug and Zeb1.³⁹ A previous study reported significant nuclear localization of Twist and Slug in the mesenchymal tumor area of GS samples, suggesting that GS is associated with EMT.⁴⁰ Notably, the MAPK signaling, phosphatidylinositol/calcium signaling and focal adhesion/tight junction pathways, which are significantly altered in GS cases (Figure 2a), are known to cooperate in the initiation and progression of EMT.^{41,42} In addition, losses or mutations in the *TP53* gene were previously shown to correlate with the activation of EMT reprogramming as well as increased stem cell properties by modulating microRNAs.⁴³ Therefore, *TP53* mutations induce the epigenetic reprogramming of EMT genes, which could be the molecular mechanism of the mesenchymal differentiation, increased aggressiveness and poor prognosis of GS.

In addition to the roles of *TP53* mutations in the development of GS via mesenchymal differentiation, our data suggested that *TP53* mutations were also associated with the treatment resistance of GS. We found two patients (#5 and #22) in whom *TP53* mutations were only found in the recurrent GS samples by the WES analysis. However, the more sensitive droplet digital PCR method detected *TP53* mutations in the preceding lesions, although the allele frequencies were very low. In these patients, most of the cells in preceding GS regions did not have *TP53* mutations, suggesting that the development of the initial GS was not highly associated with *TP53* mutations. However, minor clones with *TP53* mutations survived after treatment and became one of major

populations in the recurrent GS sample, suggesting that *TP53* mutations were associated with treatment resistance.

In summary, our study provides the first understanding of the genomic alterations specific to GS and elucidates the relationship among predominant mutations of *TP53*, EMT process and poor prognosis in GS. These results shed light on the understanding of the pathogenesis and clinical characteristics of GS for future therapeutic applications.

Data deposition

The sequence reported in this paper has been deposited in the European Nucleotide Archive (accession no. PRJEB12063).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We thank Y Dorsett for comments on this manuscript. This work was supported in part by grants to CL, an Ewha Womans University Research Grant; the Korean Healthcare Technology R&D project through the Korean Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare, Republic of Korea (grant no. HI13C2148); and the International Research & Development Program of the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT & Future Planning (grant no. 2015K1A4A3047851). Grants to SHP, including the Korean Healthcare Technology R&D project through the Korean Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare, Republic of Korea (grant no. HI11C21100200); the Korea Institute of Planning & Evaluation for Technology in Food, Agriculture, Forestry, and Fisheries, Republic of Korea (grant no. 311011-05-3-SB020); the Technology Innovation Program (grant no. 10050154, Business Model Development for Personalized Medicine Based on Integrated Genome and Clinical Information) funded by the Ministry of Trade, Industry & Energy (MI, Korea); and the Bio & Medical Technology Development Program of the NRF funded by the Korean government, MSIP (grant no. 2015M3C7A1028926). A grant to D-HN included the Korean Healthcare Technology R&D project through the Korean Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare, Republic of Korea (grant no. HI14C3418).

- Han SJ, Yang I, Tihan T, Prados MD, Parsa AT. Primary gliosarcoma: key clinical and pathologic distinctions from glioblastoma with implications as a unique oncologic entity. *J Neurooncol* 2010; **96**: 313–320.
- Feigin IH, Gross SW. Sarcoma arising in glioblastoma of the brain. *Am J Pathol* 1955; **31**: 633–653.
- Kozak KR, Mahadevan A, Moody JS. Adult gliosarcoma: epidemiology, natural history, and factors associated with outcome. *Neuro Oncol* 2009; **11**: 183–191.
- Actor B, Cobbers JM, Buschges R, Wolter M, Knobbe CB, Lichter P *et al*. Comprehensive analysis of genomic alterations in gliosarcoma and its two tissue components. *Genes Chromosomes Cancer* 2002; **34**: 416–427.
- Grant JW, Steart PV, Aguzzi A, Jones DB, Gallagher PJ. Gliosarcoma: an immunohistochemical study. *Acta Neuropathol* 1989; **79**: 305–309.
- Perry JR, Ang LC, Bilbao JM, Muller PJ. Clinicopathologic features of primary and postirradiation cerebral gliosarcoma. *Cancer* 1995; **75**: 2910–2918.

- 7 Sreenan JJ, Prayson RA. Gliosarcoma. A study of 13 tumors, including p53 and CD34 immunohistochemistry. *Arch Pathol Lab Med* 1997; **121**: 129–133.
- 8 Biernat W, Aguzzi A, Sure U, Grant JW, Kleihues P, Hegi ME. Identical mutations of the p53 tumor suppressor gene in the gliomatous and the sarcomatous components of gliosarcomas suggest a common origin from glial cells. *J Neuropathol Exp Neurol* 1995; **54**: 651–656.
- 9 Reis RM, Konu-Lebleblicioglu D, Lopes JM, Kleihues P, Ohgaki H. Genetic profile of gliosarcomas. *Am J Pathol* 2000; **156**: 425–432.
- 10 Oh JE, Ohta T, Nonoguchi N, Satomi K, Capper D, Pierscianek D *et al*. Genetic alterations in gliosarcoma and giant cell glioblastoma. *Brain Pathol* 2015; **125**: 401–410.
- 11 Cachia D, Kamiya-Matsuoka C, Mandel JJ, Olar A, Cykowski MD, Armstrong TS *et al*. Primary and secondary gliosarcomas: clinical, molecular and survival characteristics. *J Neurooncol* 2015; **125**: 401–410.
- 12 Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics* 2009; **25**: 1754–1760.
- 13 Van der Auwera GA, Carneiro MO, Hartl C, Poplin R, Del Angel G, Levy-Moonshine A *et al*. From FastQ data to high confidence variant calls: the Genome Analysis Toolkit best practices pipeline. *Curr Protoc Bioinformatics* 2013; **11**: 11.10.1–11.10.33.
- 14 Cibulskis K, Lawrence MS, Carter SL, Sivachenko A, Jaffe D, Sougnez C *et al*. Sensitive detection of somatic point mutations in impure and heterogeneous cancer samples. *Nat Biotechnol* 2013; **31**: 213–219.
- 15 Wang K, Li M, Hakonarson H. ANNOVAR: functional annotation of genetic variants from high-throughput sequencing data. *Nucleic Acids Res* 2010; **38**: e164.
- 16 Amarasinghe KC, Li J, Hunter SM, Ryland GL, Cowin PA, Campbell IG *et al*. Inferring copy number and genotype in tumour exome data. *BMC Genomics* 2014; **15**: 732.
- 17 Wang C, Evans JM, Bhagwate AV, Prodduturi N, Sarangi V, Middha M *et al*. PatternCNV: a versatile tool for detecting copy number changes from exome sequencing data. *Bioinformatics* 2014; **30**: 2678–2680.
- 18 Huang da W, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc* 2009; **4**: 44–57.
- 19 Liu T, Papagiannakopoulos T, Puskar K, Qi S, Santiago F, Clay W *et al*. Detection of a microRNA signal in an in vivo expression set of mRNAs. *PLoS ONE* 2007; **2**: e804.
- 20 Gautier L, Cope L, Bolstad BM, Irizarry RA. affy - analysis of Affymetrix GeneChip data at the probe level. *Bioinformatics* 2004; **20**: 307–315.
- 21 Mootha VK, Lindgren CM, Eriksson KF, Subramanian A, Sihag S, Lehar J *et al*. PGC-1 α -responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. *Nat Genet* 2003; **34**: 267–273.
- 22 Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA *et al*. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci USA* 2005; **102**: 15545–15550.
- 23 Ozawa T, Riestter M, Cheng YK, Huse JT, Squatrito M, Helmy K *et al*. Most human non-GCIMP glioblastoma subtypes evolve from a common proneural-like precursor glioma. *Cancer Cell* 2014; **26**: 288–300.
- 24 Kanehisa M, Goto S, Sato Y, Kawashima M, Furumichi M, Tanabe M. Data, information, knowledge and principle: back to metabolism in KEGG. *Nucleic Acids Res* 2014; **42**: D199–D205.
- 25 Jiao X, Sherman BT, Huang da W, Stephens R, Baseler MW, Lane HC *et al*. DAVID-WS: a stateful web service to facilitate gene/protein list analysis. *Bioinformatics* 2012; **28**: 1805–1806.
- 26 Watanabe M, Kawaguchi T, Isa S, Ando M, Tamiya A, Kubo A *et al*. Ultra-sensitive detection of the pretreatment EGFR T790M mutation in non-small cell lung cancer patients with an EGFR-activating mutation using droplet digital PCR. *Clin Cancer Res* 2015; **21**: 3552–3560.
- 27 McDonald OG, Wu H, Timp W, Doi A, Feinberg AP. Genome-scale epigenetic reprogramming during epithelial-to-mesenchymal transition. *Nat Struct Mol Biol* 2011; **18**: 867–874.
- 28 De Craene B, Bex G. Regulatory networks defining EMT during cancer initiation and progression. *Nat Rev Cancer* 2013; **13**: 97–110.
- 29 Anastassiou D, Rumjantseva V, Cheng W, Huang J, Canoll PD, Yamashiro DJ *et al*. Human cancer cells express Slug-based epithelial-mesenchymal transition gene expression signature obtained in vivo. *BMC Cancer* 2011; **11**: 529.
- 30 Jechlinger M, Grunert S, Tamir IH, Janda E, Ludemann S, Waerner T *et al*. Expression profiling of epithelial plasticity in tumor progression. *Oncogene* 2003; **22**: 7155–7169.
- 31 Thiery JP, Acloque H, Huang RYJ, Nieto MA. Epithelial-mesenchymal transitions in development and disease. *Cell* 2009; **139**: 871–890.
- 32 Cancer Genome Atlas Research Network. Comprehensive genomic characterization defines human glioblastoma genes and core pathways. *Nature* 2008; **455**: 1061–1068.
- 33 Verhaak RG, Hoadley KA, Purdom E, Wang V, Qi Y, Wilkerson MD *et al*. Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1. *Cancer Cell* 2010; **17**: 98–110.
- 34 Yan H, Parsons DW, Jin G, McLendon R, Rasheed BA, Yuan W *et al*. IDH1 and IDH2 mutations in gliomas. *N Engl J Med* 2009; **360**: 765–773.
- 35 Ohgaki H, Dessen P, Jourde B, Horstmann S, Nishikawa T, Di Patre PL *et al*. Genetic pathways to glioblastoma: a population-based study. *Cancer Res* 2004; **64**: 6892–6899.
- 36 Fujisawa H, Reis RM, Nakamura M, Colella S, Yonekawa Y, Kleihues P *et al*. Loss of heterozygosity on chromosome 10 is more extensive in primary (de novo) than in secondary glioblastomas. *Lab Invest* 2000; **80**: 65–72.
- 37 Tohma Y, Gratas C, Biernat W, Peraud A, Fukuda M, Yonekawa Y *et al*. PTEN (MMAC1) mutations are frequent in primary glioblastomas (de novo) but not in secondary glioblastomas. *J Neuropathol Exp Neurol* 1998; **57**: 684–689.
- 38 Phillips HS, Kharbanda S, Chen R, Forrest WF, Soriano RH, Wu TD *et al*. Molecular subclasses of high-grade glioma predict prognosis, delineate a pattern of disease progression, and resemble stages in neurogenesis. *Cancer Cell* 2006; **9**: 157–173.
- 39 Tam WL, Weinberg RA. The epigenetics of epithelial-mesenchymal plasticity in cancer. *Nat Med* 2013; **19**: 1438–1449.
- 40 Nagaishi M, Paulus W, Brokinkel B, Vital A, Tanaka Y, Nakazato Y *et al*. Transcriptional factors for epithelial-mesenchymal transition are associated with mesenchymal differentiation in gliosarcoma. *Brain Pathol* 2012; **22**: 670–676.
- 41 Lamouille S, Xu J, Derynck R. Molecular mechanisms of epithelial-mesenchymal transition. *Nat Rev Mol Cell Biol* 2014; **15**: 178–196.
- 42 Davis FM, Azimi I, Faville RA, Peters AA, Jalink K, Putney JW Jr *et al*. Induction of epithelial-mesenchymal transition (EMT) in breast cancer cells is calcium signal dependent. *Oncogene* 2014; **33**: 2307–2316.
- 43 Chang CJ, Chao CH, Xia W, Yang JY, Xiong Y, Li CW *et al*. p53 regulates epithelial-mesenchymal transition and stem cell properties through modulating miRNAs. *Nat Cell Biol* 2011; **13**: 317–323.
- 44 Ananda G, Mockus S, Lundquist M, Spoltow V, Simons A, Mitchell T *et al*. Development and validation of the JAX Cancer Treatment Profile (TM) for detection of clinically actionable mutations in solid tumors. *Exp Mol Pathol* 2015; **98**: 106–112.



This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivs 4.0 International License. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit <http://creativecommons.org/licenses/by-nc-nd/4.0/>

Supplementary Information accompanies the paper on Experimental & Molecular Medicine website (<http://www.nature.com/emm>)