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Research Paper

SHOX2 is a Potent Independent Biomarker to Predict Survival of WHO Grade II–III Diffuse Gliomas

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ABSTRACT

Background: Diffuse gliomas, grades II and III, hereafter called lower-grade gliomas (LGG), have variable, difficult to predict clinical courses, resulting in multiple studies to identify prognostic biomarkers. The purpose of this study was to assess expression or methylation of the homeobox family gene *SHOX2* as independent markers for LGG survival.
Methods: We downloaded publically available glioma datasets for gene expression and methylation. The Cancer Genome Atlas (TCGA) (LGG, n = 516) was used as a training set, and three other expression datasets (n = 308) and three other methylation datasets (n = 320), were used for validation. We performed Kaplan-Meier survival curves and univariate and multivariate Cox regression model analyses.
Findings: SHOX2 expression and gene body methylation varied among LGG patients and highly significantly predicted poor overall survival. While they were tightly correlated, *SHOX2* expression appeared more potent as a prognostic marker and was used for most further studies. The *SHOX2* prognostic roles were maintained after analyses by histology subtypes or tumor grade. We found that the combination of *SHOX2* expression and *IDH* genotype status identified a subset of LGG patients with *IDH* wild-type (*IDH*wt) and low *SHOX2* expression with considerably favorable survival. We further investigated the combination of *SHOX2* with other known clinically relevant markers of LGG (*TERT* expression, 1p/19q chromosome co-deletion, *MGMT* methylation, *ATRX* mutation and *NES* expression). When combined with *SHOX2* expression, we identified subsets of LGG patients with significantly favorable survival outcomes, especially in the subgroup with worse prognosis for each individual marker. Finally, multivariate analysis demonstrated that *SHOX2* was a potent independent survival marker.
Interpretation: We have identified that *SHOX2* expression or methylation are potent independent prognostic indicators for predicting LGG patient survival, and have potential to identify an important subset of LGG patients with *IDH*wt status with significantly better overall survival. The combination of *IDH* or other relevant markers with *SHOX2* identified LGG subsets with significantly different survival outcomes, and further understanding of these subsets may benefit therapeutic target identification and therapy selections for glioma patients.

1. Introduction

Brain tumor gliomas include low grade (grade I) pilocytic astrocytomas, and the diffuse gliomas that include the grades II and III astrocytomas and oligodendrogliomas (referred to as lower-grade gliomas, LGG) and the highly malignant grade IV glioblastomas [GBM, grade IV, the World Health Organization (WHO) Classification of Tumors of the Central Nervous System (CNS)] (Louis et al., 2016; Louis et al., 2007).

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LGG are diffusely infiltrative tumors and have highly variable, difficult to predict clinical courses, further compounded by inter-observer variability in histologic classification and grading (van den Bent, 2010; Louis et al., 2007). While some LGG have indolent outcomes, others rapidly progress to high grade GBM. GBM patients almost always die from their disease (Louis et al., 2007; Ostrom et al., 2015). The evolution of gliomas from grade II to grade III or IV are characterized by the stepwise acquisition of genetic alterations and a considerable worsening of prognosis, justifying studies to identify genetic alterations as potential biomarkers for prognosis and selection of targeted therapy and overall clinical management (Ellison, 2015). A relatively recent finding of major biological and clinical importance was the identification of mutations in the isocitrate dehydrogenase (*IDH*) enzyme genes *IDH1* and *IDH2*. Somatic mutations, in particular of the *IDH1* gene, are present in the majority of LGG, especially oligodendrogliomas, and have a positive effect on overall survival (Turkalp et al., 2014; Yan et al., 2009). They are rare in primary GBM and absent in pilocytic astrocytomas and are often associated with *MGMT* promoter hypermethylation, *TP53* mutations as well as co-deletions of chromosome 1p or 19q (1p/19q codel). *IDH* mutations are an early, possibly driver, event for LGG (Watanabe et al., 2009), and clinical trials of *IDH* inhibitors are underway (Dimitrov et al., 2015). Many studies have demonstrated that survival outcome of LGG patients is significantly different based on the status of *IDH* gene mutation, 1p/19q codeletion, telomerase reverse transcriptase (*TERT*) promoter mutation, *ATRX* gene mutation, CpG island methylator phenotypes (CIMP), O-6-methylguanine-DNA methytransferase (*MGMT*) promoter methylation, the neural stem cell gene nestin (*NES*) expression and mRNA expression signatures by multiple genes (Cancer Genome Atlas Research et al., 2015; Eckel-Passow et al., 2015; Ceccarelli et al., 2016; Chan et al., 2015; Noushmehr et al., 2010; Turcan et al., 2012; Hatanpaa et al., 2014; Siegal, 2015; Bao et al., 2014; Zhang et al., 2015). The classification by CIMP status after filtering *IDH* mutation status revealed biologically discrete subsets having different clinic survival outcomes in diffuse gliomas (Ceccarelli et al., 2016), supporting the principle that *IDH* mutation status plus other molecular biomarkers can enhance the prognostic value for certain molecularly distinct subsets of LGG patients. The importance of combining tumor molecular features with traditional diagnostic features such as histology and grading was recognized in the recently revised 2016 WHO classification systems of CNS tumors (Louis et al., 2016).

The *SHOX2* gene, located on chromosome 3q, is a member of the homeobox family of genes that encodes a transcriptional regulator and its

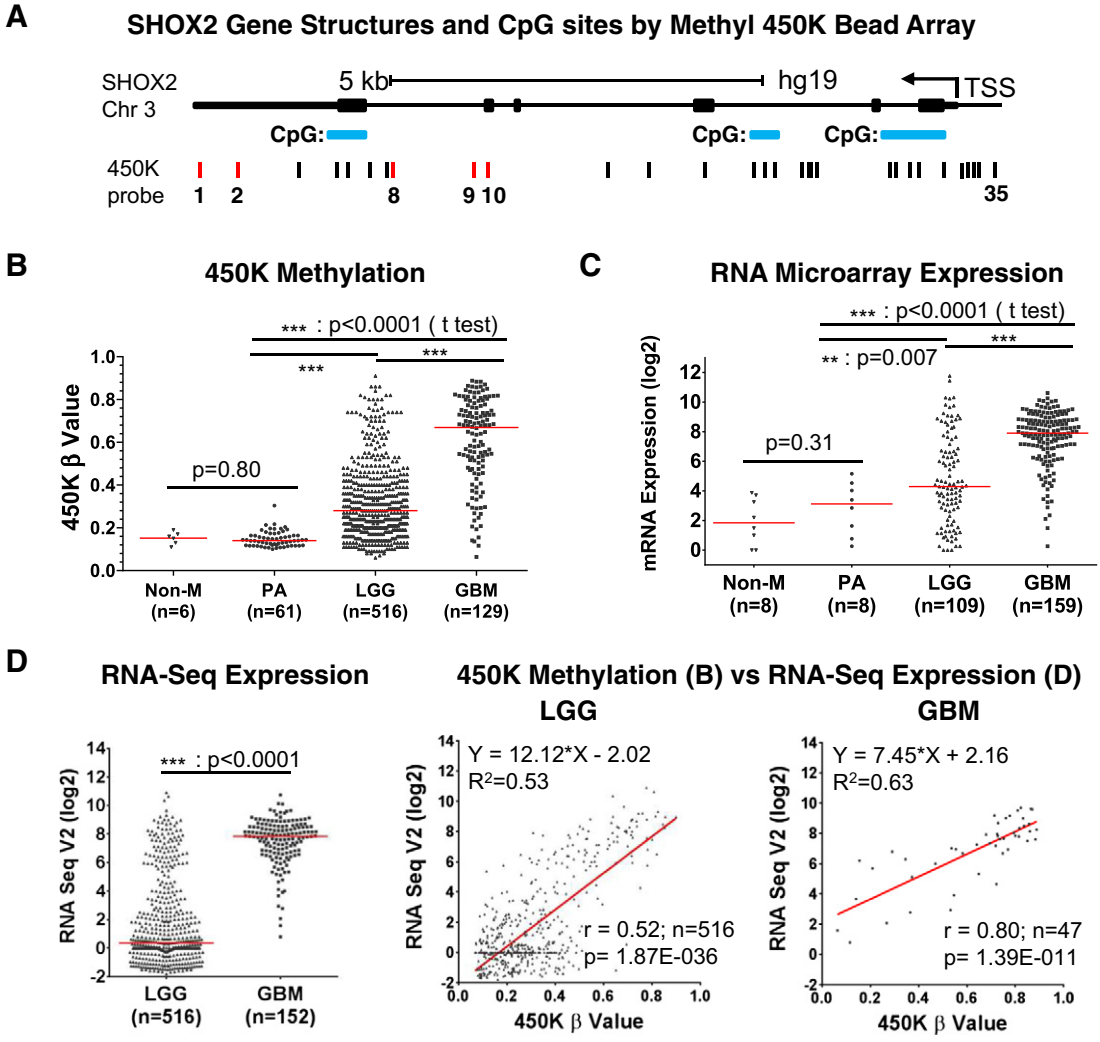


Fig. 1. Identification and correlation of differential *SHOX2* methylation and expression in glial tumors. (A) *SHOX2* gene structure and its CpG sites by the Infinium HumanMethylation450 BeadChip (Illumina) array (450 K) were based on the UCSC Genome Browser (http://genome.ucsc.edu/) and the NCBI Gene site (http://www.ncbi.nlm.nih.gov/gene/6474). The 35 designated 450 K probes are indicated by vertical lines scattered throughout *SHOX2* gene (Note: The 450K ProbeTarget ID cg00303223 was excluded due to its data unavailable in TCGA LGG and GBM 450K datasets). (B) Differential *SHOX2* methylation among non-malignant brains (Non-M), pilocytic astrocytomas (PA), lower grade gliomas (LGG) and glioblastomas (GBM) using TCGA LGG and GBM 450 K datasets. Five high differential methylation probes were found. The pooled mean beta value of four probes (Probes 1, 2, 8 and 9, Fig. 1A) was used. (C) Differential *SHOX2* mRNA expression using GSE16011 microarray data. (D) Correlation of *SHOX2* methylation and expression using TCGA LGG 450 K and RNA SeqV2 datasets (see also Appendix p2–9 for dataset source and data analyses). r: Spearman r, 2-tailed. Bars: median values.

expression is highly restricted to craniofacial, brain, heart, and limb development (Blaschke et al., 1998; Clement-Jones et al., 2000). *SHOX2* promoter DNA methylation has been identified as a diagnostic and prognostic biomaker for non-small cell lung cancer patients (Schmidt et al., 2010; Dietrich et al., 2012). Elevated *SHOX2* expression is associated with tumor recurrence of hepatocellular carcinoma (Yang et al., 2013) and with poor survival in breast cancers (Hong et al., 2014). In our experience, some genes such as *SCT* and *ITPKA*, are frequently methylated in many invasive cancers, but their methylation in certain low-grade tumors is variable (Zhang et al., 2016; Wang et al., 2016). The availability of a well-studied large set of LGG having molecular data including exome sequencing and genome wide methylation (Cancer Genome Atlas Research et al., 2015), permitted us to examine the prognostic role of *SHOX2* methylation and expression in gliomas and to correlate the data with other prognostic parameters. The primary aim of our study was to demostrate the prognostic role of *SHOX2* as a single indicator or in combination with *IDH* and other biomakers for improving survival predictions for LGG patients.

2. Materials and Methods

2.1. Datasets

We examined publically available genome-wide methylation and expression data of nonmalignant brains and glioma tissues, the associated pathological molecular markers, and clinical variables from the following main sources: The Cancer Genome Atlas (TCGA) data portal, National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO), ArrayExpress, and the data as described previously (Ceccarelli et al., 2016). The detailed list of data sources and accession dates and data process are provided in Appendix p1–4.

We used the TCGA LGG dataset as a training set to study the association between *SHOX2* methylation (or expression) and overall survival in LGG patients. These findings were independently validated in three external expression microarray datasets (GSE16011, GSE30336 and REMBRANDT) and three external methylation datasets (GSE61160, GSE30338 and GSE58218). Details of the clinical trials and cancer centers from which the data were extracted are provided in Appendix p3–4. We examined enhanced prognostic values of *SHOX2* in subsets of LGG classified by *IDH* or other known relevant markers using TCGA LGG datasets.

2.2. Statistical analysis

Two-tailed, Student *t*-test was performed to compare two groups of numerical values. To assess *SHOX2* as a prognostic biomarker, patient samples analyzed were dichotomized into two groups designed as high- and low- groups, based on a *SHOX2* cutoff value for their methylation or expression values. For the dataset containing numerical values, we used a data-driven approach to define an objective cutoff value by using model-based clustering method implemented by "mclust" package version 4.4 for R (Fraley and Raftery, 2002). By mclust clustering analysis, the *SHOX2* values (methylation or expression) were fitted into a mixture of two normal distributions, one with high *SHOX2* values and the other with low *SHOX2* values. The same approach was used to define the cutoff value for younger and older patient groups by age. Overall survival time was calculated from the date of diagnosis until death or the last follow-up contact. Survival curves were estimated using the product-limit method of Kaplan-Meier (Kaplan and Meier, 1958) with the log-rank test. Univariate and multivariate Cox tests were performed to assess the relative contribution of the risk group when assessed alone or after adjusting for clinical variables (or other indicator) (Andersen and Gill, 1982, Therneau and Grambsch, 2000).

3. Results

3.1. SHOX2 Methylation or mRNA Expression in Glial Tumors

A cartoon and description of the *SHOX2* gene are presented in Fig. 1A. From the TCGA datasets, varying degrees of methylation were noted at the 35 probes covering the entire gene (Fig. 1). We identified five probes, named as P1, P2, P8, P9 and P10 (Fig. 1A) which showed significantly high differential methylation in glial tumors (see details on Appendix p5–9). These probes were located between the last two exons

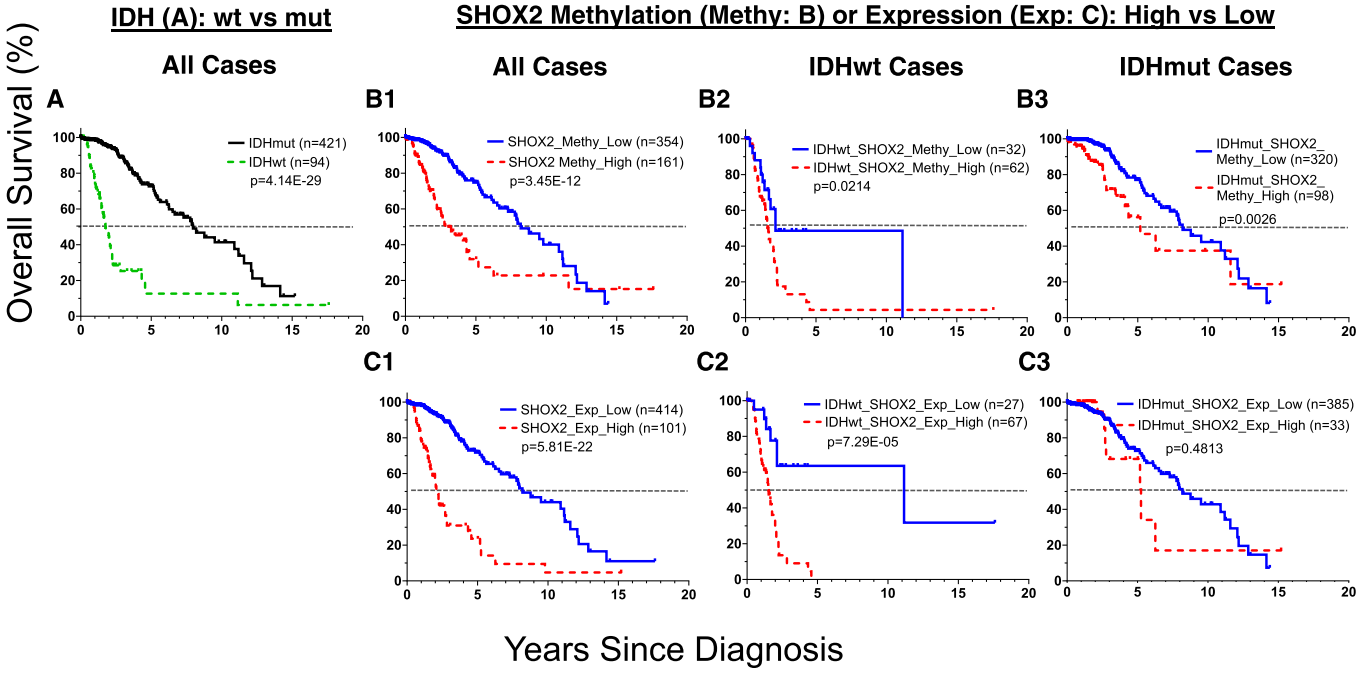
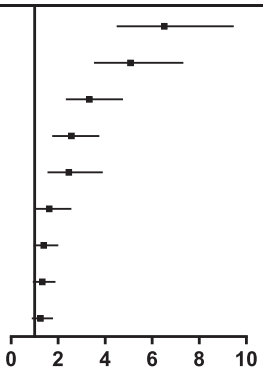


Fig. 2. Kaplan-Meier survival curve analyses of *SHOX2* alone and the combination of *SHOX2* with *IDH* mutation status in LGG patients using TCGA LGG datasets. The analyses of *SHOX2* 450K methylation (cutoff value of 0.357) and RNA-Seq expression data (cutoff value of 4.135) were presented in Figs. 2B1-3 and C1–3, respectively. *IDH*wt: *IDH* wild-type; *IDH*mut: *IDH* mutation (see also Appendix p2–9 for dataset source and data analyses).

A

Marker and Variable	Events/Patients	Median OS Years (95% CI)	p value (Log-rank test)	HR (95% CI)	p value (Cox model)
IDH: wt vs mut	50/94 vs 74/418	1.75 (1.53 to 2.23) vs 7.96 (6.52 to 11.59)	4.14E-29	6.52 (4.49 to 9.47)	<2.00E-16
SHOX2 mRNA: high vs low	51/101 vs 75/414	2.16 (1.78 to 2.83) vs 8.18 (7.29 to 11.58)	5.81E-22	5.08 (3.53 to 7.33)	<2.00E-16
SHOX2 methylation: high vs low	61/161 vs 65/354	2.83 (2.52 to 4.56) vs 8.19 (7.29 to 11.19)	3.45E-12	3.33 (2.33 to 4.76)	4.30E-11
MGMT: unmethy vs methy	39/90 vs 87/425	3.31 (2.08 to 8.77) vs 7.77 (6.26 to 10.90)	5.89E-07	2.56 (1.75 to 3.75)	1.40E-06
1p/19q: non-codel vs codel	104/346 vs 22/169	5.48 (4.32 to 7.88) vs 11.19 (7.96 to NA)	7.50E-05	2.46 (1.55 to 3.90)	0.0001
TERT promoter: mut vs wt	39/130 vs 35/162	6.52 (4.34 to NA) vs 6.26 (5.30 to NA)	0.0376	1.62 (1.02 to 2.56)	0.0394
ATRX: wt vs mut	79/325 vs 45/187	7.86 (5.17 to 14.20) vs 7.29 (5.48 to 10.90)	0.0810	1.39 (0.96 to 2.01)	0.8230
TERT mRNA: Exp .vs Not_Exp.	59/224 vs 67/288	6.52 (5.17 to NA) vs 7.29 (5.48 to 9.78)	0.1140	1.33 (0.93 to 1.89)	0.1150
NES mRNA: high vs low	68/255 vs 58/260	7.88 (5.30 to 11.20) vs 6.26 (5.25 to 11.60)	0.2060	1.25 (0.88 to 1.78)	0.2070



B

Subset	Events/Patients	Median OS Years (95% CI)	p value (Log-rank test)	HR (95% CI)	p value (Cox model)
NES mRNA High	34/65 vs 34/190	1.66 (1.47 to 2.23) vs 9.51 (7.96 to 12.86)	8.80E-25	13.30 (7.28 to 24.28)	<2.0E-16
MGMT_Unmethy	27/39 vs 12/51	1.47 (1.02 to 1.98) vs 8.77 (3.66 to NA)	3.75E-11	9.77 (4.48 to 21.30)	9.98E-09
TERT_Expressed	37/66 vs 22/158	1.66 (1.47 to 2.23) vs 11.19 (6.52 to NA)	4.44E-16	7.31 (4.22 to 12.66)	1.31E-12
1p/19q non-codel	50/91 vs 54/255	1.99 (1.62 to 2.63) vs 7.88 (6.12 to 11.59)	4.01E-24	6.72 (4.44 to 10.18)	<2.0E-16
ATRXwt	42/83 vs 37/242	1.98 (1.62 to 2.77) vs 11.14 (7.96 to NA)	1.11E-16	5.80 (3.66 to 9.19)	7.61E-14
IDHwt	43/67 vs 7/27	1.53 (1.19 to 1.99) vs 11.14 (2.13 to NA)	7.29E-05	4.99 (2.09 to 11.93)	0.0003
ATRXmut	8/17 vs 37/170	2.70 (2.63 to NA) vs 7.88 (6.12 to 11.6)	9.88E-05	4.28 (1.93 to 9.51)	0.0003
TERTwt	6/14 vs 29/148	5.25 (2.16 to NA) vs 7.29 (5.48 to NA)	0.0006	4.27 (1.729 to 10.55)	0.0017
TERTmut	21/39 vs 18/91	1.66 (1.47 to NA) vs 7.96 (5.38 to NA)	3.11E-06	4.22 (2.20 to 8.09)	1.46E-05
TERT_Not_Expressed	14/35 vs 35/253	2.77 (2.63 to NA) vs 7.88 (6.12 to 11.10)	2.35E-05	3.39 (1.86 to 6.21)	6.82E-05
MGMT_Methy	24/62 vs 63/363	2.83 (2.63 to 9.78) vs 8.19 (7.29 to 12.09)	1.18E-06	3.10 (1.92 to 5.01)	3.70E-06
NES mRNA Low	17/36 vs 41/224	2.83 (2.63 to NA) vs 7.29 (6.12 to NA)	0.0007	2.57 (1.46 to 4.53)	0.0011
IDHmut	7/33 vs 67/385	5.25 (2.77 to NA) vs 8.19 (7.29 to 11.58)	0.4815	1.33 (0.60 to 2.93)	0.4820
1p/19q codel	1/10 vs 21/159	NA (NA to NA) vs 11.2 (6.52 to NA)	0.2976	0.35 (0.04 to 2.78)	0.3160

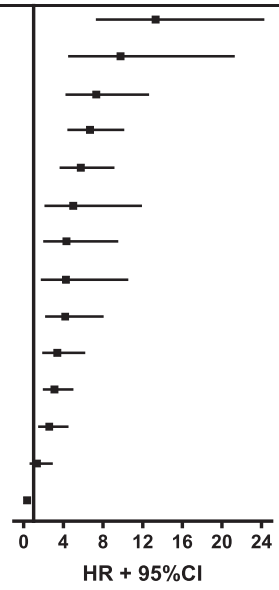


Fig. 3. The univariate Cox proportional-hazards model survival analyses of *SHOX2* and other individual markers (A), and *SHOX2* expression marker in the subsets for LGG patients when combined with other individual markers (B) using TCGA LGG datasets. (A) Comparison of *SHOX2* marker with other individual markers. (B) Comparison of enhanced prognostic values of *SHOX2* expression marker in the subsets of LGG sub-classified by other individual markers. Note: *NES* expression was dichotomized into high- (>13.35) and low- (≤13.35) subgroup by using median RNA-Seq value of 13.35 instead of the mclust clustering determined cutoff value (11.31) due to in part that the latter cutoff value resulted in a small number of samples (n = 30) for *NES* expression low subset with less statistical power in further analyses. HR: hazard ratio. NA: not available.

and not at CpG island sites. As shown in Fig. 1B, *SHOX2* methylation was absent or low in non-malignant brain tissues and pilocytic astrocytomas (PA), frequent and high in GBM, and with intermediate frequencies and values in LGG, respectively.

By microarray data, *SHOX2* expression patterns were similar to methylation: low in non-malignant brain and PA cases, high in GBM and intermediate but variable in LGG (Fig. 1C). The significantly higher expression of *SHOX2* expression in GBM compared to LGG was also

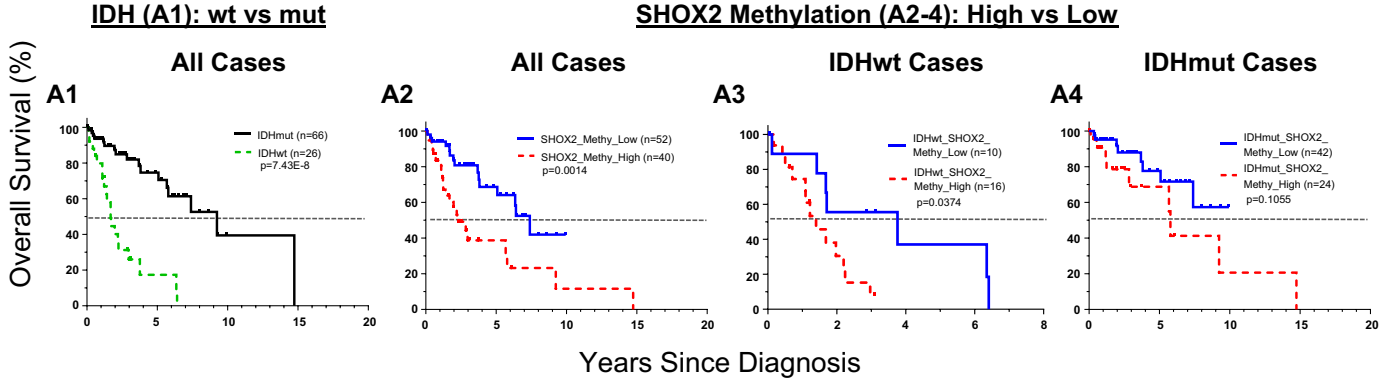


Fig. 4. Kaplan-Meier overall survival curve analyses of *SHOX2* methylation in additional external 450 K microarray methylation datasets besides the TCGA LGG dataset. The survival analyses were performed using *SHOX2* pooled mean methylation probe values (see Fig. 1) of LGG samples from the combined datasets (GSE61160 and GSE30338). p: Log-rank test. See Appendix p2–4, 13–14 for detailed list of datasets and data source and further analyses data.

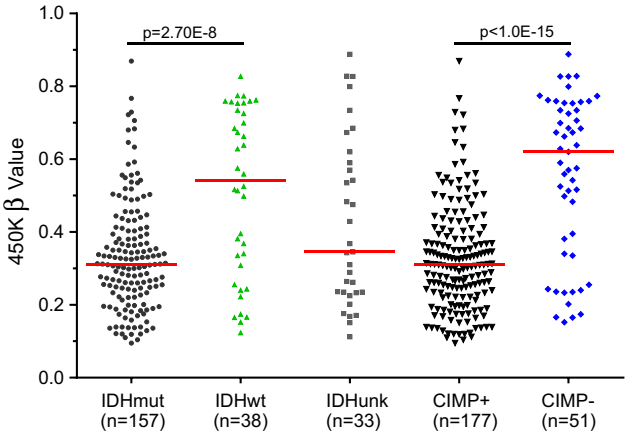


Fig. 5. Comparison of *SHOX2* methylation by *IDH* mutation or CIMP status in additional external 450K methylation dataset besides the TCGA LGG dataset. The *SHOX2* pooled mean beta value of four probes (Probes 1, 2, 8 and 9, Fig. 1A) was used, (GSE58218 dataset) were used and compared by *IDH* mutation or CIMP status. Bars: median values. wt: wild type; mut: mutation; unk: unknown. p: *t*-test. See Appendix p2–4 for detailed list of datasets and data source.

validated in additional microarray datasets (Appendix p10). The RNA-Seq data showed the same pattern of differential *SHOX2* expression (Fig. 1D). In addition, by correlating the RNA-Seq data with their corresponding sample methylation data available, we found that the *SHOX2* methylation and RNA-Seq expression values were highly positively correlated in both LGG and GBM tumors (Fig. 1D).

3.2. SHOX2 Methylation and mRNA Expression are Independent Potent Prognostic Markers for LGG

To explore whether *SHOX2* methylation and expression are related to LGG patient survivals, we first analyzed TCGA LGG training datasets containing 516 cases. The samples were dichotomized into either high or low subgroups when classified by *SHOX2* methylation or mRNA expression levels. We found that the methylation marker based on any of the five differentially methylated probes alone predicted overall survival of LGG patients, and use of the pooled mean beta values of four probes (Probes 1, 2, 8 and 9, Fig. 1A) showed significantly improved prognostic values as compared with the use of individual probes (Appendix p11–12) and other varied combinations of these probes (not shown). Thus the pooled mean beta values were used for further survival analyses of *SHOX2* methylation. Our analyses demonstrated that *SHOX2* methylation was associated with poor survival prognosis and was a potent independent prognosis marker for LGG [Figs. 2B1 and 3A, hazard ratio (HR) 3.33 (95% CI 2.33 to 4.76), p = 3.45E-12]. *SHOX2* high methylation predicted a poor overall survival of LGG patients comparable to *IDH* mutation status, and was significantly correlated with CIMP-negative marker, a poor survival prognosis marker for LGG (Wiestler et al., 2014; Noushmehr et al., 2010) as demonstrated in

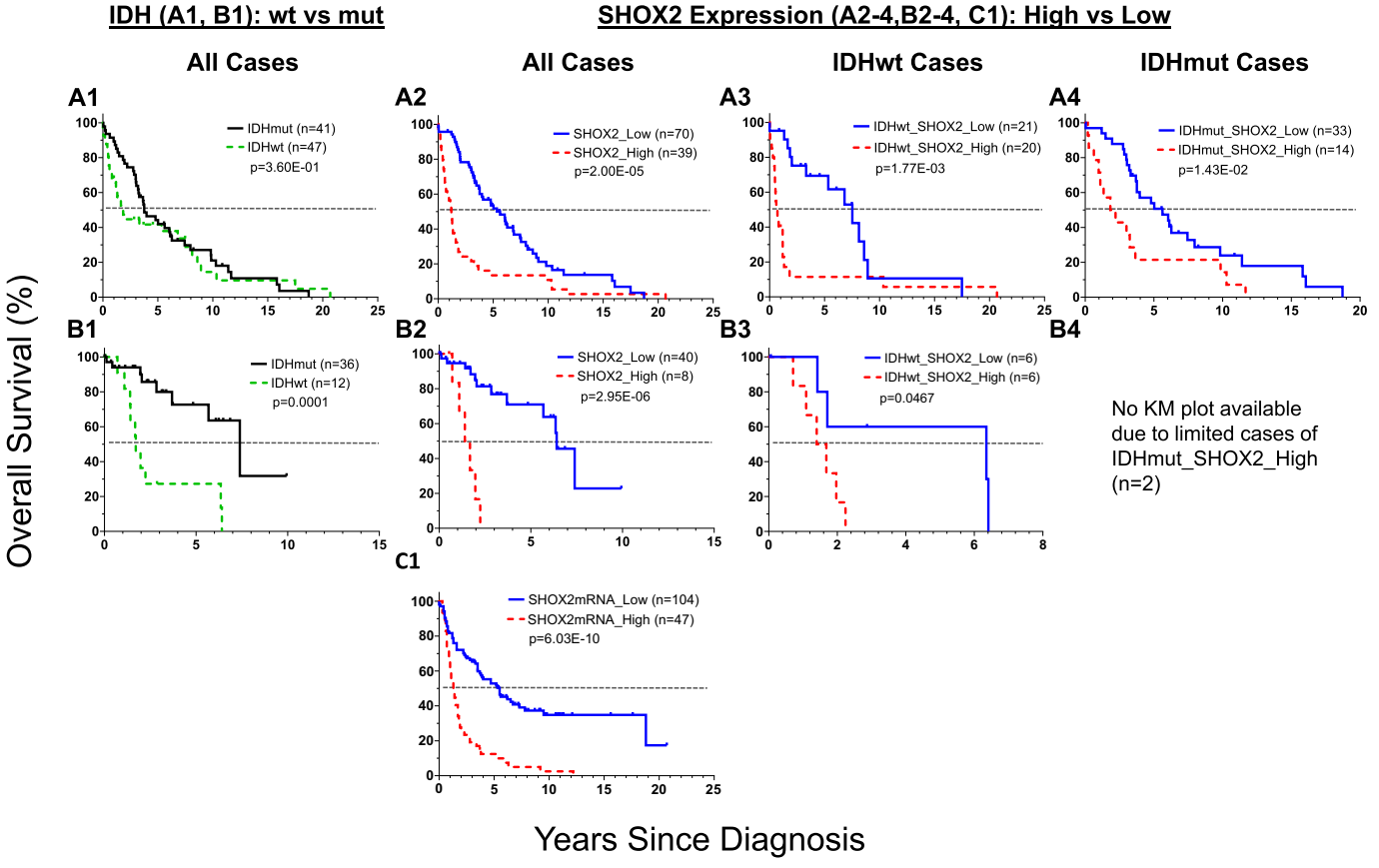


Fig. 6. Kaplan-Meier overall survival curve analyses of *SHOX2* expression in additional external mRNA microarray expression datasets besides the TCGA LGG dataset. The cutoff values of *SHOX2* mRNA expression were determined by mcluster analysis of datasets (see Materials/Methods) [*SHOX2* mRNA probe_210135_s_at: GSE16011(A): 5.922; GSE30336 (B): 5.678; REMBRANDT (C): 6.575, respectively]. Note: *IDH* mutation status was classified based on *IDH1* (R132) mutation status available in GSE16011 dataset. See Appendix p2–4, 15–16 for detailed list of datasets and data source and further analyses data.

three independent external 450 K methylation datasets (Figs. 4–5 and Appendix p13–14). *SHOX2* methylation (of the gene body associated with increased gene expression) was negatively correlated with CIMP status (methylation of promoter region of multiple genes associated with suppression of gene expression) (Fig. 5).

Similarly, *SHOX2* high mRNA expression (*SHOX2*_high) was an even more potent independent marker in predicting worse overall survival in the same LGG dataset using TCGA LGG RNA-Seq dataset [n = 516, HR 5.08 (95% CI 3.53 to 7.33), p < 2.0E-16] (Fig. 2C1, Fig. 3A). These findings were independently validated in three additional external expression microarray datasets, as demonstrated by using *SHOX2* as a single prognostic marker or in combination of *SHOX2* with *IDH* status data available (Fig. 6 and Appendix p15–16).

While *SHOX2* methylation and expression were highly correlated, *SHOX2* expression appeared more potent than methylation for predicting LGG survival and complete expression datasets were more widely available. For these reasons above, we used *SHOX2* expression marker for further studies.

We observed that *SHOX2* expression marker had a prognostic value comparable to *IDH* status marker, a widely-accepted potent prognostic marker for LGG [*IDH* wild type (*IDH*wt) vs mutant type (*IDH*mut) HR 6.52 (95% CI 4.49 to 9.47), p < 2.0E-16] (Figs. 2A, C1 and 3A). Interestingly, there was a significantly higher concordance rate (0.75) between *IDH*mut and *SHOX2* low expression than that (0.05) between *IDH*wt and SHOX2 low expression, as compared with a significant but less degree concordance difference between *IDH*wt and *SHOX2* high expression (0.13) vs *SHOX2* low expression (0.06) using TCGA LGG expression dataset (Appendix p17). As compared with other known markers, such as *TERT*, *MGMT*, 1p/19q codel, *TERT* and *NES*, *SHOX2* had the highest prognostic hazard ratio value as demonstrated in the univariate Cox proportional-hazards model analyses of each marker as a single indicator in TCGA LGG dataset (Fig. 3A).

We performed overall survival analyses of *SHOX2* expression by histology (astrocytoma, oligodendroglioma, excluding oligoastrocytoma which is not recognized as a separate entity in the 2016 CNS tumor classification system) (Louis et al., 2016) or tumor grade (grades II and III). We found that *SHOX2* expression was a potent survival indicator in both histology types and grades, especially in astrocytoma and grade III tumors (Fig. 7 and Appendix p18).

However, we did not find that *SHOX2* expression was a prognostic marker for overall survival in high grade GBM patients in TCGA GBM dataset (Appendix p19), possibly because the vast majority of GBM samples had high *SHOX2* expression values (Fig. 1D).

3.3. Combination of SHOX2 with IDH and Other Relevant Prognostic Markers

Next, we determined whether *SHOX2* can improve prognostic values of *IDH* in LGG patients. While the poor prognostic *SHOX2*_high and *IDH*wt subgroups were frequently present together (Appendix p20), we found that approximately one third (27 out of 94) of the *IDH*wt cases which had *SHOX2* low expression, were associated with an improved overall median survival of 9.6 years (p = 7.29E-05, log-rank test) (Figs. 2C2 and 3B). By contrast, combining *SHOX2* expression or methylation had no or minimal effect on the favorable prognostic *IDH*mut subgroup.

The findings of the enhanced prognostic combination of *SHOX2* and *IDH* markers led us to further study whether *SHOX2* can aid the prognostic values of other LGG prognostic markers (*NES* expression, *MGMT* methylation, *TERT* promoter mutation or expression, 1p/19q codel and *ATRX* mutation). Using the TCGA dataset, we analyzed each marker by itself, and compared with the prognostic values of *SHOX2* marker in the dichotomized subsets of LGG by these individual markers (Figs. 3B and 8). We found that 1p/19q codel or *MGMT* methylation as individual

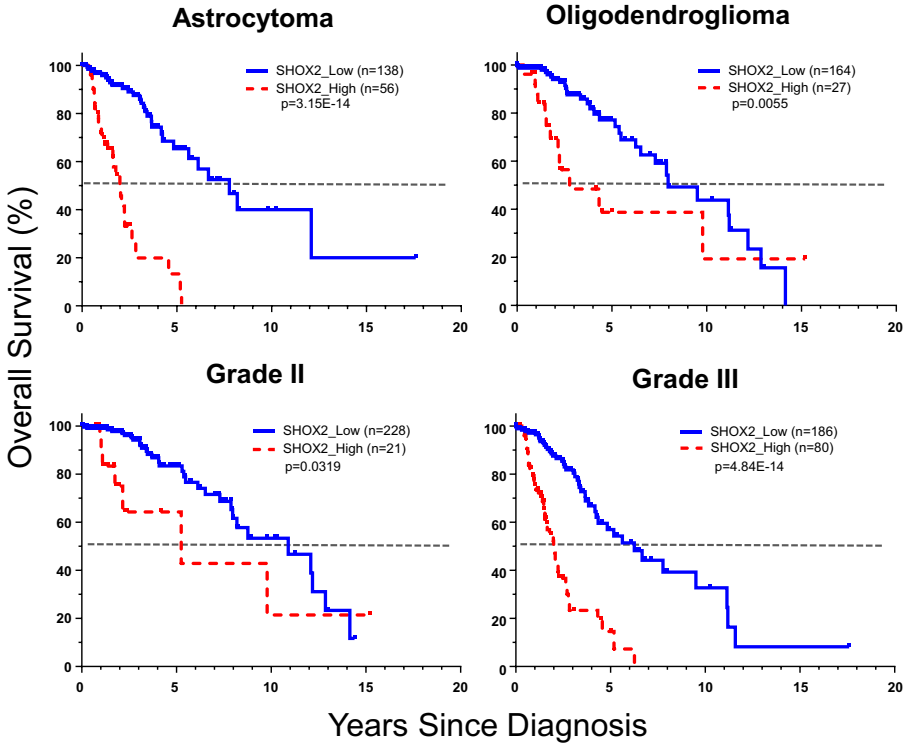


Fig. 7. Kaplan-Meier overall survival curve analyses of *SHOX2* expression in LGG by histology and tumor grades (TCGA LGG dataset). The cutoff value of *SHOX2* RNA-Seq expression data (4.135) was used to determine *SHOX2* expression high vs low. The grades II or III tumor samples included all histology types included in the TCGA LGG RNA-Seq datasets analyzed. See Appendix p2–4, 18 for detailed list of datasets and data source and further analyses data.

markers had a moderate prognostic effect, and *NES*, *TERT* and *ATRX* marker had no significant prognostic effect. In particular, we found that *SHOX2* expression identified favorable prognostic subsets more significantly in the unfavorable prognosis subgroup determined by each individual marker, which was similar to our findings in the subset of *IDH*wt LGG (Figs. 3 and 8).

We observed that the prognostic value of other markers was improved in combination with *IDH* mutation status, as compared to utilization of each marker alone, especially *TERT* expression or mutation, *MGMT* methylation, *ATRX* mutation and *NES* expression (Appendix p21). Comparing the effects of *SHOX2* and *IDH* makers while combining with other relevant markers, we found that *SHOX2* expression

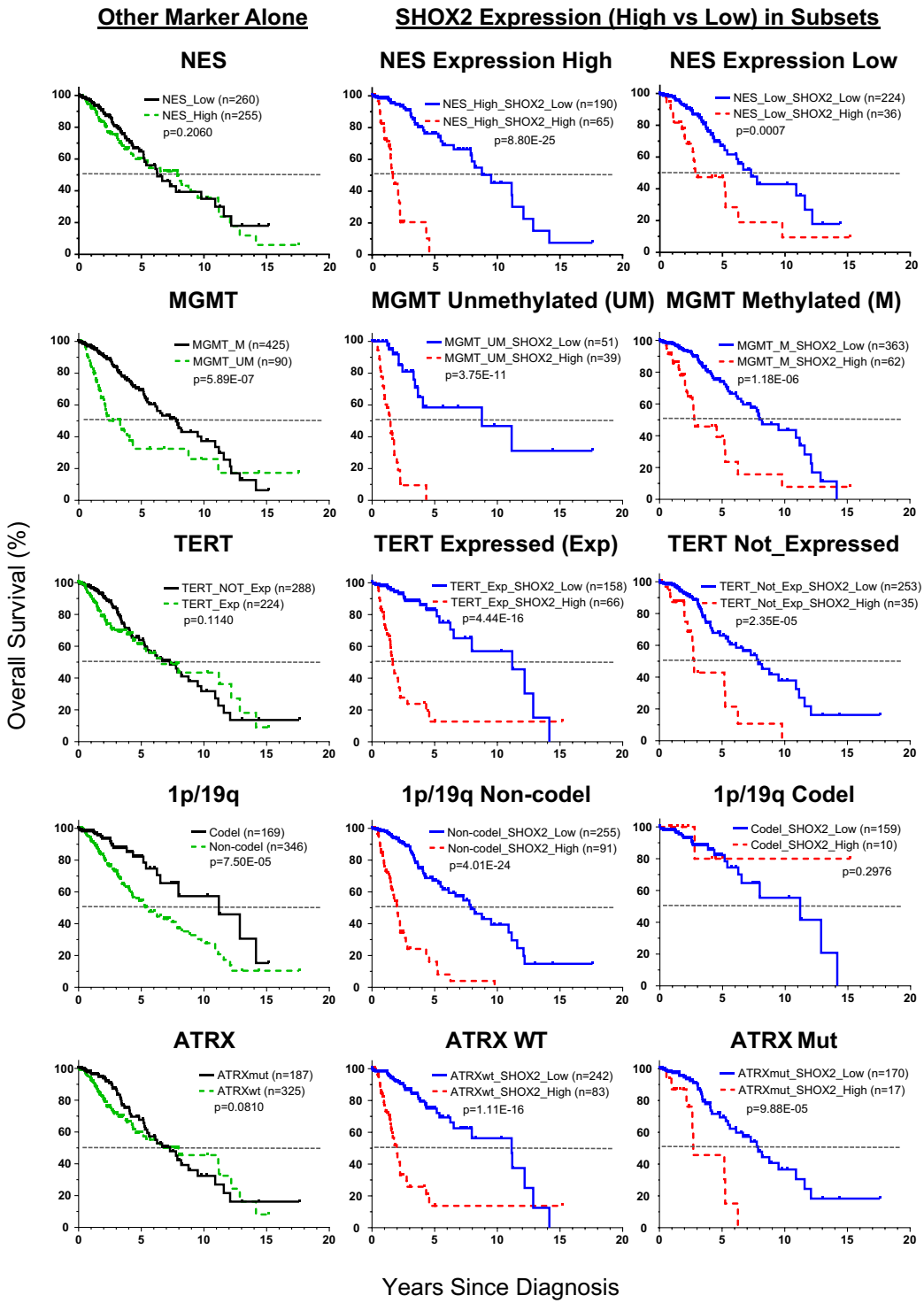


Fig. 8. Kaplan-Meier overall survival curve analyses of *SHOX2* expression in the subsets of LGG when combined with other individual markers (TCGA LGG dataset). This figure presented the result of survival analyses of other markers (*NES*, *MGMT*, *TERT*, 1p/19q and *ATRX*) individually, and *SHOX2* expression marker in the subsets of LGG sub-classified by these other individual markers. The cutoff value of *SHOX2* RNA-Seq expression data (4.135) was used to determine *SHOX2* expression high vs low. See Appendix p2–4 for detailed list of datasets and data source and Fig. 3B for *NES* expression cutoff value and additional data of each subset analyzed.

significantly enhanced the prognosis values of other relevant markers to a degree similar and comparable to *IDH* mutation status marker (Fig. 3B and Appendix p21).

3.4. Multivariate Cox Proportional-hazards Survival Analysis of SHOX2 and Other Markers

We performed multivariate Cox regression survival analysis to assess the relative contribution of the predicted risk groups classified by *SHOX2* expression, after adjusting for clinical variables including age, gender, stage and histology subtypes as well as other prognostic markers. We demonstrated that *SHOX2* expression and age were highly significant independent variables, while, gender, histology and tumor grade were of lesser significance (Table 1). Both *SHOX2* methylation and expression remained a significantly predictive marker and *SHOX2* expression had a stronger prognostic value comparable to *IDH* [methylation: HR 1.65 (95% CI 1.08 to 2.50, p = 1.94E-02); expression: HR 3.38 (95% CI 2.25 to 5.09, p = 5.12E-09; *IDH*: HR 3.82 (95% CI 2.47 to 5.90), p = 5.12E-09)] (Table 1 and Fig. 9).

To determine the relative contribution of the risk groups by *SHOX2* marker, after adjusting for the status of other known markers, we performed multivariate analyses under the same clinical variables setting as shown in Table 1 except including one of other prognostic markers (*IDH*, 1p/19q codel, *TERT*, *MGMT*, *NES* and *ATRX*) (Fig. 9). Fig. 8 shows a comparison of the hazard ratio values of *SHOX2* marker vs. other markers by multivariate analyses, and we demonstrated that under the combination of *SHOX2* with the other prognostic markers, *SHOX2* was also an independent prognostic factor. By contrast, *IDH*, 1p/19q codel and *MGMT* but not *TERT* and *NES* showed moderate prognostic significance after adjusting by *SHOX2* marker, based on their HR and p values (Fig. 9).

The overall survival of LGG patients was previously reported to be related with age and histology (Turcan et al., 2012; Cancer Genome Atlas Research et al., 2015) We found that *SHOX2* high methylation and expression were associated with astrocytoma histology type (Appendix p22). *SHOX2* methylation and expression levels were low and not age-dependent in normal and non-malignant brain tissues (Appendix p23–24), but appeared partially age-dependent in LGG tumors as reflected by a subset of LGG cases with high *SHOX2* methylation and expression values available in all age groups. Thus we performed multivariate analyses in younger (<48 years) or older (≥48 years) subgroups, respectively. The cutoff value (48 years) was objectively determined by model based clustering analysis of age data in TCGA LGG dataset (see Methods Statistical analysis). We found that *IDH* and *SHOX2* expression as individual markers both had higher HR values in the older patient subgroup than those in young patient subgroup, and *SHOX2* had a slightly higher HR value with a higher significant p value than *IDH* in the younger patient subgroup [*SHOX2*: HR 3.00 (95% CI 1.54 to 5.87), p = 0.0013; *IDH*: HR 2.65 (95% CI 1.19 to 5.90), p = 0.0171] (Appendix p25). While both *SHOX2* and *IDH* were included in multivariate analyses, *SHOX2* was more significant than *IDH* in the younger patient subgroup. The results of comparing *SHOX2* with other markers in different age subgroups are presented in (Appendix p25–27).

4. Discussion

In May of 2016, the latest version of 2016 WHO Classification was published, and the major changes in this version, as it relates to the present report, was combining molecular markers with traditional histology classification to introduce a more clinically relevant classification (Louis et al., 2016). In particular, a) most grades II and III diffuse gliomas have mutations in *IDH1* or *IDH2* genes; 2) most astrocytomas are 1p/19q intact and are often *ATRX* and *TP53* mutant; 3) most or all oligodendrogliomas are 1p/19q co-deleted; and 4) combined oligoastrocytomas are no longer recognized as an entity, but should be reclassified based on their molecular features. The grading system is maintained but its importance is de-emphasized. We present our analyses using traditional histology (omitting the category oligoastrocytoma) and grading, and the various molecular analyses including *IDH*, *ATRX* mutation status and 1p/19q co-deletions. The datasets we utilized were generated prior to the revised 2016 Classification, and from the sample data available, not all cases can be reclassified. We did not combine the traditional methods (histology and grading) with molecular methods for comparison with *SHOX2* expression or methylation, as that would introduce too many variables and would result in subsets that could not be reclassified according to the 2016 Classification. However, because our approach utilized both the traditional approaches and the important molecular features utilized in the 2016 Classification (*IDH* and *ATRX* mutation status, and 1p/19q co-deletion status) we believe it is relevant to the new 2016 WHO classification.

The role of multiple prognostic markers has been investigated in LGGs. We investigated the role of *SHOX2* expression and methylation in LGGs and their relationship to other prognostic and pathologic markers. We found approximately 20% of tumors had increased levels of expression and/or methylation. There was a high degree of positive correlation between the two parameters. At first this may appear paradoxical, as hypermethylation of the promoter region serves as a repressive epigenetic mark that down-regulates gene expression. However, the methylated regions of *SHOX2* were located in the gene body and previous studies as well as a recent one by us have noted that gene body methylation, which is more prevalent in the genome than promoter hypermethylation, may be associated with increased gene expression (Jones, 2012; Wang et al., 2016). Multiple recent studies have highlighted the importance and promise of using molecular markers for LGG prognosis and potentially as aids for therapeutic selection (Cancer Genome Atlas Research et al., 2015; Eckel-Passow et al., 2015; Turkalp et al., 2014; Dimitrov et al., 2015; Ceccarelli et al., 2016). To date, *IDH* mutation status has been the most widely accepted and powerful prognostic factor, either alone or in combination with other factors. In this study, we identified that *SHOX2* methylation or expression were potent independent prognostic indicator comparable to *IDH* for LGG patient survival. We used the TCGA dataset as our training set and six additional

Table 1
The multivariate Cox proportional-hazards models analyses of *SHOX2* methylation or expression for LGG patients (TCGA LGG dataset).

	SHOX2 methylation		SHOX2 expression	
Variable	Hazard ratio (95% CI)	p Value	Hazard ratio (95% CI)	p Value
Patient#/Event#: 512/126				
SHOX2 high vs low	1.65 (1.08 to 2.50)	0.0194	3.38 (2.25 to 5.09)	5.12E-09
Age	1.06 (1.04 to 1.07)	6.97E-11	1.05 (1.04 to 1.07)	7.88E-10
Gender (male vs female)	1.14 (0.79 to 1.64)	0.4778	1.30 (0.90 to 1.87)	0.1641
Histology (OA vs A)	0.65 (0.40 to 1.05)	0.0757	0.83 (0.51 to 1.34)	0.4451
Histology (OD vs A)	0.51 (0.33 to 0.78)	0.0019	0.53 (0.34 to 0.81)	0.0036
Tumor Grade (3 vs 2)	2.10 (1.37 to 3.23)	0.0007	2.24 (1.47 to 3.41)	0.0002

Hazard ratio for age variable: risk per 1 year. OA: oligoastrocytoma; A: astrocytoma; OD: oligodendroglioma.
See Appendix p3–4 for sample source and data.

Markers Compared	Variable	Events/Patients	HR (95% CI)	p value
SHOX2_alone	SHOX2 mRNA: high vs low	51/101 vs 75/414	3.38 (2.25 to 5.09)	5.12E-09
IDH_alone	IDH: wt vs mut	50/94 vs 74/418	3.82 (2.47 to 5.90)	1.44E-09
SHOX2_vs_IDH	SHOX2 mRNA: high vs low	51/101 vs 75/414	2.64 (1.61 to 4.34)	0.0001
SHOX2_vs_1p/19q	SHOX2 mRNA: high vs low	51/101 vs 75/414	2.87 (1.87 to 4.39)	1.27E-06
SHOX2_vs_TERT(promoter)	SHOX2 mRNA: high vs low	51/101 vs 75/414	2.99 (1.63 to 5.46)	0.0004
SHOX2_vs_MGMT	SHOX2 mRNA: high vs low	51/101 vs 75/414	3.08 (2.01 to 4.71)	2.09E-07
SHOX2_vs_TERT(expression)	SHOX2 mRNA: high vs low	51/101 vs 75/414	3.34 (2.20 to 5.06)	1.31E-08
SHOX2_vs_NES	SHOX2 mRNA: high vs low	51/101 vs 75/414	3.40 (2.25 to 5.14)	7.21E-09
SHOX2_vs_ATRX	SHOX2 mRNA: high vs low	51/101 vs 75/414	3.91 (2.50 to 6.10)	2.06E-09
IDH_vs_SHOX2	IDH: wt vs mut	50/94 vs 74/418	2.53 (1.52 to 4.20)	0.0003
1p/19q_vs_SHOX2	1p/19q: non -code vs codel	104/346 vs 22/169	2.30 (1.30 to 4.06)	0.0042
TERT(promoter)_vs_SHOX2	TERT promoter: mut vs wt	39/130 vs 35/162	1.09 (0.59 to 2.01)	0.7887
MGMT_vs_SHOX2	MGMT: unmethylated vs methylated	39/90 vs 87/425	1.82 (1.20 to 2.76)	0.0048
TERT(expression)_vs_SHOX2	TERT mRNA: Not_Expressed vs Expressed	67/288 vs 59/224	0.94 (0.61 to 1.44)	0.7632
NES_vs_SHOX2	NES mRNA: high vs low	68/255 vs 58/260	1.34 (0.93 to 1.93)	0.1189
ATRX_vs_SHOX2	ATRX: wt vs mut	79/325 vs45/187	0.96 (0.61 to 1.51)	0.8540

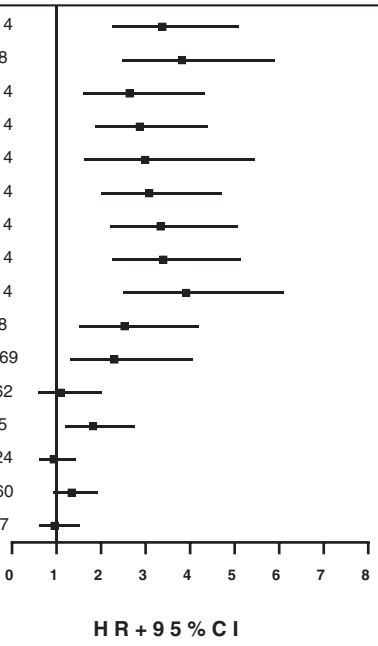


Fig. 9. Forest plot of the multivariate Cox proportional-hazards model survival analyses of *SHOX2* expression marker alone or in combination with other markers for LGG patients in TCGA LGG dataset. This figure presented a summary of the multivariate analyses of *SHOX2* expression marker alone, or *SHOX2* co-presence with one of other markers (*NES*, *MGMT*, *TERT*, 1p/19q and *ATRX*) after adjusting for the same clinical variables described in Table 1. The names in the first left column referred to the two markers (before or after "vs") compared in the same multivariate analyses, in which the analyzed result for the marker placed before "vs" was presented in the same row in the right columns. The cutoff value of *SHOX2* RNA-Seq expression data (4.135) was used to determine *SHOX2* expression high vs low. HR: hazard ratio.

independent LGG datasets for validation of expression or methylation. While *SHOX2* expression and gene body methylation were highly correlated, expression appeared to be the better prognostic marker and its data were more readily available. Thus we used expression for most additional studies. Importantly, we found that the combination of *SHOX2* and *IDH* status identified a subset of *IDH*wt LGG with a considerably improved survival time. Of interest, another study utilizing molecular profiling indicated the presence of a subtype with improved prognosis (Ceccarelli et al., 2016). Conceivably, *SHOX2* status can be determined by standard molecular assays such as PCR in a routine laboratory, which is clinically attractive.

We further investigated the combination of *SHOX2* with other known clinically relevant markers of LGG including *TERT*, 1p/19q, *MGMT*, *ATRX* and *NES*, and found that *SHOX2* was a potent indicator to identify subsets of LGG with significant better survivals especially in the worse prognosis subgroup determined by individual markers. Our study demonstrated that *SHOX2* not only is an independent potent prognostic marker, but also has potential to refine the molecular classification of LGG in combination with other well established markers. The addition of *SHOX2* expression identifies small but highly significant subgroups having good prognosis within the poor prognosis groups identified by *IDH* mutation status and several other commonly used prognostic markers. Thus, therapy options for these subgroups may be altered as a result of our observations, although prospective studies will be required to prove this. Finally, we demonstrated by multivariate survival analysis that *SHOX2* was a potent survival prognosis marker comparable to *IDH* after adjusting for age and other clinical variables and significantly different from all the other markers mentioned above.

As mentioned previously, *SHOX2* may play a prognostic role in breast and hepatocellular carcinomas (Yang et al., 2013; Hong et al., 2014). *SHOX2* was suggested to be a novel epithelial-to-mesenchymal transition inducer in breast cancer cells (Hong et al., 2014), and overexpression of *SHOX2* was able to induce canine mesenchymal stem cell differentiation into native pacemaker cells (Feng et al., 2016). We observed an upward trend of *SHOX2* aberrant hypermethylation and expression from clinically benign pilocytic astrocytomas, intermediate malignant LGG to high malignant GBM. This is in contrast to *IDH* mutations which are largely limited to LGG and only occasionally present in primary GBM (Yan et al., 2009). However, *SHOX2* expression was not found to have prognostic significance in the TCGA GBM dataset in this study, possibly because most GBM tumors have high *SHOX2* expression. Our current findings suggest a potential oncogenic role of *SHOX2* in glioma tumors, although the precise mechanism is largely unknown.

In conclusion, we have identified that *SHOX2* expression or methylation are potent independent prognostic indicators for predicting LGG patient survival, and have potential to identify an important subset of LGG patients with *IDH*wt status with significantly better overall survival. The combination of *IDH* or other relevant markers with *SHOX2* identified LGG subsets with significantly different survival outcomes, and further understanding of these subsets may benefit therapeutic target identification and therapy selections for glioma patients.

Author Contributions

YAZ and AFG contributed to the conception and study design, literature search, figures and tables' presentation, data collection and assembly and analysis and interpretation, writing and critical reading of manuscript. YZ, XL and GX contributed to the data collection and analysis and interpretations, figures and tables' presentation, and critical reading of manuscript. KS, XM, AS and LG contributed to the data collection and assembly and critical reading of manuscript.

Declaration of Interests

We declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ebiom.2016.10.040.

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