Characterization of iPSCs derived from low grade gliomas revealed early regional chromosomal amplifications during gliomagenesis

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Abstract

Introduction.—IDH1 mutation has been identified as a early genetic event driving low grade gliomas (LGGs) and it has been proven to exerts a powerful epigenetic effect. Cells containing IDH1 mutation are refractory to epigenetical reprogramming to iPSC induced by expression of

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Yamanaka transcription factors, a feature that we employed to study early genetic amplifications or deletions in gliomagenesis.

Methods.—We made iPSC clones from freshly surgically resected *IDH1* mutant LGGs by forced expression of Yamanaka transcription factors. We sequenced the IDH locus and analyzed the genetic composition of multiple iPSC clones by array-based comparative genomic hybridization (aCGH).

Results.—We hypothesize that the primary cell pool isolated from LGG tumor contains a heterogeneous population consisting tumor cells at various stages of tumor progression including cells with early genetic lesions if any prior to acquisition of IDH1 mutation. Because cells containing IDH1 mutation are refractory to reprogramming, we predict that iPSC clones should originate only from LGG cells without IDH1 mutation, i.e. cells prior to acquisition of IDH1 mutation. As expected, we found that none of the iPSC clones contains IDH1 mutation. Further analysis by aCGH of the iPCS clones reveals that they contain regional chromosomal amplifications which are also present in the primary LGG cells.

Conclusions.—These results indicate that there exists a subpopulation of cells harboring gene amplification but without IDH1 mutation in the LGG primary cell pool. Further analysis of TCGA LGG database demonstrates that these regional chromosomal amplifications are also present in some cases of low grade gliomas indicating they are reoccurring lesions in glioma albeit at a low frequency. Taken together, these data suggest that regional chromosomal alterations may exist prior to the acquisition of *IDH* mutations in at least some cases of LGGs.

Introduction

Glial tumors are among the most fatal human tumors [1]. Monoallelic R132 (Arginine 132) mutations of *IDH1* or R172 (Arginine 172) mutations of *IDH2*, which encode isocitrate dehydrogenases (IDH) that convert isocitrate into α-ketoglutarate (αKG), have been detected in nearly all secondary glioblastoma multiforme (GBM) and 80% of low grade gliomas (LGGs) [2–4]. The mutant IDH, which forms a heterodimer with the wild-type enzyme, acquires neomorphic activity to catalyze αKG into D-2-Hydroxyglutarate (D2HG) [5,6]. Because of the structural similarity, D2HG effectively inhibits αKG-dependent enzymes including the TET-family DNA methyl hydroxylases, Jumonji-family histone demethylases, and the hydroxylases that regulate HIF1α stability [7]. The *IDH* mutations are therefore considered as drivers for gliomagenesis because of the acquired capacities to alter the epigenome and respond to hypoxia.

Clonal evolution is a major force to drive tumor progression, in which cancer cells accumulate genetic lesions stepwise, generate intratumoral heterogeneity, and selectively expand the fittest clones [8]. Besides the *IDH* mutations, progression of LGGs involves gradual accumulation of additional genetic alterations including mutations in *TP53, ATRX, TERT* and chromosome 1p/19q co-deletion [4,9–13]. Analyses of multiple LGG biopsies

collected from the same patients indicated sequential acquisition of genetic alterations, in which *IDH* mutations occur invariably prior to the other aforementioned mutations/chromosomal deletions [14]. However, whether there are LGG-associated genetic lesions that occur even prior to the *IDH* mutations remains unknown. One approach to gain insight is to make induced pluripotent stem cell (iPSC) clones from pooled primary tumor cells and then examine their genetic composition including amplifications or deletions by aCGH. The iPSC clones collectively should reflect the primary tumor cell pool if each cell has similar potential to be induced to iPSCs. Interestingly, we observed that cells containing IDH1 mutation are refractory to reprogramming by forced expression of Yamanaka transcription factors Oct4, Sox2, Klf4, and c-Myc. Therefore, iPSC clones originated from primary cell pool of IDH1 mutant glioma should exclude all the cells harboring IDH mutations and thus amplify cells containing early genetic lesions.

In this study, we generated iPSC (LGG-iPSC) clones from primary cells from freshly surgically resected IDH1 mutant LGGs. Because cells with IDH mutation is blocked from forming iPCS, we hypothesized that analyzing the genome of LGG-iPSCs would reveal early genetic changes in gliomagenesis. We established multiple iPSC clones from *IDH1* mutation-bearing primary LGGs. As expected, we found that none of the iPSC clones carried the *IDH1* mutations suggesting that tumor cells cannot be reprogrammed as long as they contain *IDH1* mutation. Importantly, we found that the LGG-iPSCs contained regional chromosomal amplifications and these amplifications are also present in the primary LGG cells. These data suggest that the LGG-iPSCs are originated from tumor cells not yet acquiring the *IDH1* mutations. Furthermore, the observed chromosomal alterations were detected in a fraction of LGGs in the Cancer Genome Atlas (TCGA) database. Taken together, by reprogramming freshly resected LGG cells, we demonstrated that *IDH1* mutation-bearing LGG cells are resistant to become iPSCs and that certain regional chromosomal alterations exist prior to the acquisition of *IDH* mutations at least in a fraction of LGGs. The functional role of the amplification await further study.

Materials and Methods

Preparation and culture of primary low-grade glioma cells

De-identified LGG samples were acquired in accordance with the policies of Institutional Review Board (IRB) of University of Alabama at Birmingham. Freshly resected tumors were minced with #11 scalpel blades and minced tumors were disaggregated via gentleMacs Dissociator per standard tumor protocol supplied by Tumor Dissociation Kit (Miltenyi Biotec, Auburn, CA). Collected cells were washed twice with serumless DMEM/F12 (300×G, 7 minutes, 4°C), counted in the presence of 0.04% trypan blue to enumerate viable cells. Dissociated LGG cells were cultured in cell culture plates coated with poly-L-ornithine (Sigma-Aldrich) and laminin (EMD-Millipore) in LGG medium (Neurobasal-A medium containing 1% N-2, 2% vitamin A-free B-27, 2 mM L-Glutamine [Thermo Fisher], 20 ng/ml basic FGF [Stemgent], and 20 ng/ml human EGF [Cell Signaling]) as described [15,16]. Medium was exchanged as needed, and cells grew adherently to the bottom of flasks. All cell cultures were maintained at 37°C, 20.8% O_2, and 5% CO_2.

Immunohistochemistry and DNA FISH

Immunohistochemistry was performed as described [17]. In brief, paraffin sections were deparaffinized in xylene and rehydrated in decreasing concentration of ethanol solutions. Antigen is retrieved by incubation in Target Retrieval Solution (Agilent) at 95 °C for 20 min and endogenous peroxidase and alkaline phosphatase activity were quenched using BLOXALL blocking solution (Vector Laboratories) at RT for 10 min. Slides were blocked by with horse serum, incubated with the IDH1 R132H antibody (DIA-H09, Dianova) at 4°C overnight, and secondary antibody at RT for 30 min. FISH was performed as per manufacturer instructions. Chromosome arms 1p and 19q status was determined by Vysis LSI 1p36/1q25 and LSI 19q13/19p13 Dual Color probe (Abbott Molecular).

Lentiviral preparation and iPSC derivation

STEMCCA lentivirus, which express all reprogramming factors from a polycistronic transcript [18], was prepared as described [19]. Primary LGG cells were transduced with the STEMCCA lentivirus at an MOI of 3 as described [20]. Three days after transduction, the LGG cells were reseeded onto cell culture plates-coated with hESC-qualified Geltrex basement membrane matrix (Thermo Fisher) at a density of $1-3\times10^5$ cells/cm^2 in LGG medium. The cells were then cultured in E7 medium (E8 medium without TGF-β) from the following day for two weeks and in E8 medium for one to two weeks as described [20–22]. Independent clones of iPSCs were manually picked into Geltrex-coated 12-well tissue culture plates and maintained in E8 medium as described [23,24].

DNA isolation and Sanger sequencing

Genomic DNA of primary LGG cells and LGG-iPSCs were prepared using the Quick-gDNA miniPrep kit (Zymo Research). DNA fragments containing the R132 mutation of *IDH1* and R172 mutation of *IDH2* were PCR amplified. Primers used include IDH1f (5' - AAT GAG CTC TAT ATG CCA TCA CTG – 3'), IDH1r (5' - TTC ATA CCT TGC TTA ATG GGT GT – 3'), IDH2f (5' - TTG TTG CTT GGG GTT CAA AT – 3'), and IDH2r (5' - AAG GAA AGC CAC GAG ACA GA – 3'). PCR products were purified using the DNA Clean & Concentrator-5 kit (Zymo Research) and sequenced using the forward primer. Chromatograms were viewed using the Sequence Scanner v1.0 software (Applied Biosystems).

RNA isolation and quantitative RT-PCR analysis

Total RNA was isolated with the DirectZol RNA Kit (Zymo Research) and cDNA were synthesized by the Verso cDNA Synthesis Kit (Thermo Fisher Scientific). Quantitative PCR was performed using 2x Absolute Blue Q-PCR Master Mix (Thermo Fisher Scientific) on a ViiA7 real-time PCR system (Thermo Fisher Scientific). The primers used to quantify REST include: REST-F 5' – CAT ACA GGA GAA CGC CCA TAT AA – 3'; REST-R 5' – ATG GCT TCT CAC CTG AAT GAG – 3'; bActin-F 5' – TCA GAA GGA TTC CTA TGT GGG CGA – 3'; bActin-R 5' -TTT CTC CAT GTC GTC CCA GTT GGT – 3'.

Stem cell marker analysis

For alkaline phosphatase staining, iPSCs were fixed in 4% paraformaldehyde (PFA) and stained using the Leukocyte Alkaline Phosphatase Kit (Sigma-Aldrich) per manufacturer's instruction. For immunostaining, iPSCs were fixed in 4% PFA, blocked in Protein Block (Agilent), stained with primary antibodies at 4 °C overnight and secondary antibodies at room temperature for 1 hour as described [15,25]. Primary antibodies used were OCT4 (sc-5279, Santa Cruz Biotech), SOX2 (561469, BD Biosciences), and NANOG (AF1977, R&D Systems). Images were acquired by a Nikon Ti-S microscope and processed by the Adobe Photoshop CS6 software. For flow cytometry analysis, iPSCs were trypsinized and stained on ice with Tra-1–60 antibody (sc-21705, Santa Cruz Biotech) for 1 hour and FITC-conjugated anti-mouse-IgM secondary antibody (555988, BD Biosciences) for 30 min. Cells were analyzed on a BD Fortessa flow cytometer and data were analyzed by the Flowjo V10 software as described [20,26].

Teratoma formation assay

All experiments involving lab animals were conducted in accordance with guidelines from the University of Alabama at Birmingham (UAB) and National Institute of Health. Teratoma formation assay was performed as described [20,27]. In brief, $2–5\times10^6$ iPSCs were suspended in 100 μl of DMEM/F12 medium containing 30% of human ESC-qualified Geltrex membrane matrix (Thermo Fisher) and injected subcutaneously into 4–6 week-old NOD *scid* gamma (NSG) mice (005557, Jackson Laboratory). Teratomas were harvested, fixed in 10% Formalin (Sigma-Aldrich), and processed by the Comparative Pathology Laboratory at UAB.

Array-based Comparative Genomic Hybridization (aCGH) analysis

Frozen pellets or genomic DNA of primary LGG cells or iPSCs were submitted to Cell Line Genetics (Madison, WI) for aCGH analysis using the SurePrint G3 60K Human Genome CGH microarray (Agilent). Data were analyzed by the Agilent CytoGenomics software. Only amplifications and deletions not observed in the healthy population, as determined by referencing against the Database of Genomic Variants (DGV) [28], were considered as chromosomal aberrance.

TCGA data mining

TCGA low-grade glioma database was queried using the cBioportal tools [29,30]. Genetic regions of interest were analyzed for copy number variation in low-grade gliomas with and without *IDH1* mutations. Tumors with regional chromosomal gain or loss were presented as a percentage of the total tumor samples.

Results

Characterization of primary LGGs

To investigate the early genetic alteration of LGGs, we acquired two samples of freshly surgically resected LGGs (BT01 and BT03). Both LGGs were from the frontal lobe in male patients (Fig. 1A–D). Histochemical analysis indicated that BT01 is an astrocytoma that is

not reactive to the antibody specifically recognizing the IDH1 R132H mutation (Fig. 1E and 1F), while BT03 is an oligodendroglioma strongly expressing the IDH1 R132H mutation (Fig. 1G and 1H). Fluorescence in situ hybridization (FISH) revealed reduced copy number of chromosomes 1p and 19q in BT03 cells (Fig. 1I–K) but not in BT01 cells (data not shown), which demonstrated 1p/19q co-deletion in BT03 and is consistent with the molecular feature that 1p/19q co-deletion occurs in oligodendrogliomas [4,10,11,13]. Sequencing of the IDH1 gene demonstrated that BT01 carries an IDH1 R132C mutation while BT03 carries an IDH1 R132H mutation (Fig. 1L). Sequencing of IDH2 gene confirmed that both BT01 and BT03 have the wild type IDH2, which is consistent with the notion that mutations of IDH1 and IDH2 are mutually exclusive in LGGs [2–4].

Generation of iPSC clones from primary LGG cells

To generate iPSCs from primary LGG cells, we transduced primary BT01 and BT03 cells with lentivirus expressing the reprogramming factors OCT4, SOX2, KLF4, and c-MYC [23,31]. Multiple clones of LGG-iPSCs, which have typical iPSC colony morphology and growth property, were isolated from both BT01 and BT03 cells (Fig. 2A–D). We confirmed that the LGG-iPSC clones are fully reprogrammed because they expressed the pluripotency-associated markers alkaline phosphatase, OCT4, NANOG, SOX2, and Tra-1–60 (Fig. 2E–I) and can differentiate into tissues from all the three embryonic germ layers (Fig. 2J–L). Sequencing analysis of the genomic DNA of at least 15 LGG-iPSC clones from each tumor revealed that none of the clones contained the *IDH1* or *IDH2* mutations (Fig. 2M and Fig. S1). These data indicate that the *IDH1* mutations inhibit somatic cell reprogramming.

To further test whether IDH1 mutation inhibits somatic cell reprogramming, we attempted to generate iPSCs from the brain tumor cell line BT142 (ATCC #ACS-1018), which was originally established from a grade III oligoastrocytoma carrying the monoallelic *IDH1* R132H mutation but subsequently became homozygous of the *IDH1* mutation because of loss of heterozygosity [32]. Although we can propagate BT142 cells in culture, we were unable to make iPSCs (data not shown), suggesting that the *IDH1* mutation and/or other genetic aberrations in BT142 cells are incompatible with iPSCs. Because the *IDH1* mutations acquire the neomorphic activity to produce D2HG, which inhibits all αKG-dependent epigenetic modifying enzymes, we then tested whether human PSCs could tolerate D2HG. Our data discovered a deterioration of colonies of human H9 ESCs or BJ-iPSCs after a 24-hour treatment of D2HG (Fig. S2), which further supports the notion that iPSCs cannot tolerate D2HG and the *IDH* mutations.

To rule out the possibility that we may selectively eliminate LGG cells carrying the *IDH1* mutations during tumor dissociation or cell culture, we compared *IDH1* mutation status in freshly dissociated primary LGG cells and the same cells that have been cultured. As expected, we were unable to expand the primary LGG cells because *in vitro* culture these cells remains as a major challenge to the entire field. However, we found that the proportion of *IDH1* mutation-carrying cells, as judged by the relative peak heights on the DNA sequencing chromatograms, remained relatively stable over a 4-month period for the BT01 cells or a 14-day period for the BT03 cells (Fig. S3). Therefore, our observation of none of

the LGG-iPSCs carrying the *IDH1* mutations is unlikely explained by selective loss of mutation-bearing cells during sample processing and the subsequent culture.

Because REST is known as a transcriptional regulator that plays critical roles in stem cells, neural differentiation, and glioblastoma aggressiveness [33–37], we examined how REST is expressed in LGG-iPSCs. Quantitative RT-PCR data confirmed that REST is expressed at similar levels in LGG-iPSCs as in human H9 ESCs and in the skin fibroblast-derived BJ-iPSCs (Fig. S4). These data suggest that REST expression in iPSCs is not affected by their somatic tissue origins. These data also support the notion that once reprogrammed iPSCs are indistinguishable from ESCs [38].

LGG-iPSCs contain regional chromosomal alterations

Because primary tumors likely consist of normal brain cells and a mixture of tumor cells at various stages of tumor progression, the LGG-iPSCs could originate from normal cells or tumor cells prior to acquiring the *IDH1* mutations. To distinguish these possibilities and to investigate whether the LGG-iPSCs carry other genomic abnormalities, we performed array-based comparative genomic hybridization (aCGH) analysis on primary LGG cells and the derived LGG-iPSCs. The BT01 primary cells contained large amplifications on chromosomes 8q and 17q (Fig. 3A). An 8q gain is a common chromosomal abnormality reported in *IDH1* mutated gliomas [39]. However, these large chromosomal amplifications were not detected in BT01 LGG-iPSC clones. One of the BT01-iPSC clones, BT01-iPS16, contains no chromosomal abnormality (data not shown), suggesting that the BT01-iPS16 cells originated from a normal cell in the tumor mass. The other clones contained a 2.1 Mb-amplification on chromosome Xq23 and a representative clone BT01-iPS6 is shown (Fig. 3B). Importantly, we found that the observed Xq23 amplification is present in the primary BT01 cells (Fig. 3C) but absent from over 22,300 genomes of healthy individuals, as determined by referencing the Database of Genomic Variants (DGV) [28]. To examine whether the Xq23 amplification is recurrent in LGGs, we analyzed TCGA LGG database [29,30]. We found that 2.5% of the LGGs (7 of 283) showed the Xq23 amplification, as determined by co-amplification of genes within this region such as *LUZP4, LRCH2*, and *HTR2C* (Fig. 3D and Table 1). Therefore, frequency of the Xq23 amplification in LGGs is significantly higher than that in healthy genomes ($p = 4.5 \times 10^{-14}$, Fisher's exact test). Furthermore, among the subset LGGs carrying the Xq23 amplification, most (6 of 7) also have the *IDH1* mutation (Fig. 3D). Taken together, these data demonstrated that the BT01-iPSCs originated either from normal cell or tumor cells carrying the Xq23 amplification but not the *IDH1* mutation. Because tumor cells gradually accumulate multiple genetic lesions during tumorigenesis, these data suggest that the Xq23 amplification occurred before the *IDH1* mutation in BT01.

Similarly, aCGH analysis of the primary BT03 cells revealed amplification of chromosome 11 and deletion of chromosome 1p, 4, 19q, and Y (Fig. 4), which was consistent with the FISH data showing 1p/19q co-deletion of this tumor (Fig. 1I–K). However, analysis of randomly selected clones of iPSCs revealed that none of these major chromosomal aberrations were present in iPSCs. The aCGH data demonstrated that BT03-iPSC clones amplified a 1.4 Mb region on chromosome 7q31, which is also present in the primary BT03

cells (Fig. 5A) but absent from the over 22,300 genomes of healthy individuals in DGV [28]. A search of TCGA LGG database revealed that 3.2% of LGGs (9 of 283) showed the 7q31 amplification, as demonstrated by co-amplification of genes within this region such as *PTPRZ1* and *FEZF1* (Fig. 5B and Table 1). Similarly, frequency of the 7q31 amplification in LGGs is significantly higher than that in healthy genomes (p = 6.7×10^{-18}, Fisher's exact test). Among the nine LGGs carrying the 7q31 amplification, all carried mutations in *IDH1* (8 of 9) or *IDH2* (1 of 9) (Fig. 5B). Furthermore, genes on chromosome 7 such as *PDGFA* and *EGFR* that are known drivers of primary GBM were not amplified in this subset of LGG samples (Fig. 5B). Because the 7q31 amplification presents in both iPSCs and the primary tumor cells, these data indicated that the BT03-iPSC cells originated from tumor cells. Because these cells do not carry the *IDH1* mutations, the 7q31 amplification in BT03 is mostly likely an early genetic amplification exist before the *IDH1* mutations. Taken together, we conclude that the regional amplifications on chromosome Xq23 (BT01) and 7q31 (BT03) are early genetic lesions and is likely acquired prior to the *IDH1* mutations (Fig. 6). These lesions could represent predisposing conditions for *IDH1* mutations and/or be cooperating with the *IDH1* mutations in gliomagenesis.

Discussion

Clonal evolution is a major force to drive tumor progression and an improved understanding to the process is critical to novel target identification and cancer treatment. Cancer cell-derived iPSCs, which involves epigenetic reprogramming of cancer cells at various stages of cancer progression, have recently been used to deconvolute the phylogenetic history of leukemia and to test stage-specific responses to cancer therapies [40]. Here, we applied a similar method to LGG research by reprograming primary LGG cells. Although *IDH1* mutations can be clearly detected in primary BT01 and BT03 cells (Fig. 1A), none of the derived iPSC clones carried the mutations. Because reprogramming of iPSCs involves global erasure of epigenetic marks of the differentiated cells and establishment of the marks of pluripotent cells [41], it is likely that the *IDH* mutations, which produce high levels of D2HG and inhibit epigenetic remodeling enzymes such as the TET-family DNA demethylases and Jumonji-family histone demethylases, completely block the transition of brain tumor cells into iPSCs. Furthermore, it is likely that pluripotent cells rely on the activities of these αKG-dependent enzymes to maintain its status because treatment with D2HG caused rapid death of PSCs (Figure S2). Consistent with this notion, human patients carrying the neomorphic *IDH1* R132 germline mutations have never been reported, suggesting that the *IDH1* mutations are detrimental to early embryonic cells.

The monoallelic mutations of *IDH1* or *IDH2* have been identified in most LGGs and secondary GBMs [2–4]. Comparing to the *IDH2* mutations, the *IDH1* mutations (e.g., R132H and R132C) are more prevalent and have been identified in over 90% of all patients carrying *IDH* mutations [2–4]. Furthermore, mutations in *IDH1* or *IDH2* are mutually exclusive in patients [2–4], suggesting that they play similar roles in driving gliomagenesis. Our data agree with the previous findings because BT01 and BT03 carry the *IDH1* mutations but they have wild type *IDH2* (Fig. 1L and 1M).

Besides the *IDH* mutations, additional genetic alterations have been identified to be closely associated with subtypes of LGGs. The recurrent genetic changes include mutations in *TP53* and *ATRX* in over 80% of *IDH* mutation-bearing astrocytomas, and *TERT* promoter mutation and chromosome 1p/19q co-deletion in over 80% of oligodendrogliomas [4,11]. The *IDH1* mutations are considered as an early event acquired prior to these other major genetic changes [14]. Our data agree with this conclusion because the LGG-iPSCs contained neither the *IDH1* mutations nor the major chromosomal gains/losses detected in primary LGG cells (Fig. 3A and 4). It has been reported that the *IDH1* mutations could be deleted in later stage of gliomagenesis [42,43], which raises the possibility that the LGG-iPSCs may originate from these cells. However, we argue this is unlikely because we did not detect loss or amplification of large regions of multiple chromosomes presented in the primary LGG cells (Fig. 3 and 5). Instead, we detected small regional chromosomal amplifications in BT01 and BT03-derived iPSCs (Fig. 3B–C and 5). Because these regional amplifications were found in the primary tumor cells, we conclude the LGG-iPSCs must originate from tumor cells at early stage of gliomagenesis before acquisition of the *IDH1* mutations.

Several lines of evidences demonstrated that the observed chromosomal aberration is unlikely resulted from lentiviral transfection or other experimental artifact. First, we found no chromosomal aberration in BT01-iPS16 (data not shown), which was established by identical experimental conditions as all other iPS cell lines. Secondly, we found the primary LGG cells also contain amplifications in the same chromosomal regions (Fig. 3B–C and 5A), demonstrating that the observed chromosomal aberration is not generated by lentiviral transduction or other experimental manipulation. Third, amplifications in the same chromosomal regions were also identified in a fraction of TCGA LGG samples (Fig. 3D and Fig. 5B), suggesting that these amplifications are recurrent events and functionally important to LGGs. Consistent with this notion, several recent studies implicated potential roles of the genes within the amplified regions, such as *FEZF1* [44], *PTPRZ1* [45,46], *AASS* and *TAS2R16* [47], in gliomagenesis (Table 1). Therefore, more research would be necessary to fully appreciate the functional consequence of these regional chromosomal amplifications in gliomagenesis.

Previous genome-wide association studies (GWAS) have identified rare inherited variants on chromosome 8q24 and 11q23 that may increase the risk of *IDH*-mutant gliomas [48,49]. In a similar way, the regional chromosomal amplifications of Xq23 and 7q31 may represent early genetic aberrations, which serve as predisposing conditions for gliomagenesis. It is also possible that these regional chromosomal changes represent early genetic events in gliomagenesis that can cooperate with *IDH1* mutations. Indeed, there are examples of chromosomal alteration proceeding acquisition of driver mutations. In primary glioblastoma (GBM), it has been reported that the earliest genetic alterations are polysomy of chromosome 7 and monosomy of chromosome 10, which occur in 86% and 90% of GBM, respectively [50]. Although this is clearly different from the regional chromosomal amplifications reported in this study, which were only observed in a small fraction of LGGs (Fig. 3D and 5B), it is possible that many different genetic lesions may exist to facilitate the acquisition of *IDH1* mutations and/or cooperate with the *IDH* mutations to initiate LGGs. The method established in current study enables identification of genetic lesions that occur

prior to acquisition of the *IDH* mutations in gliomagenesis, which would provide novel insights into conditions leading to the initiation of LGGs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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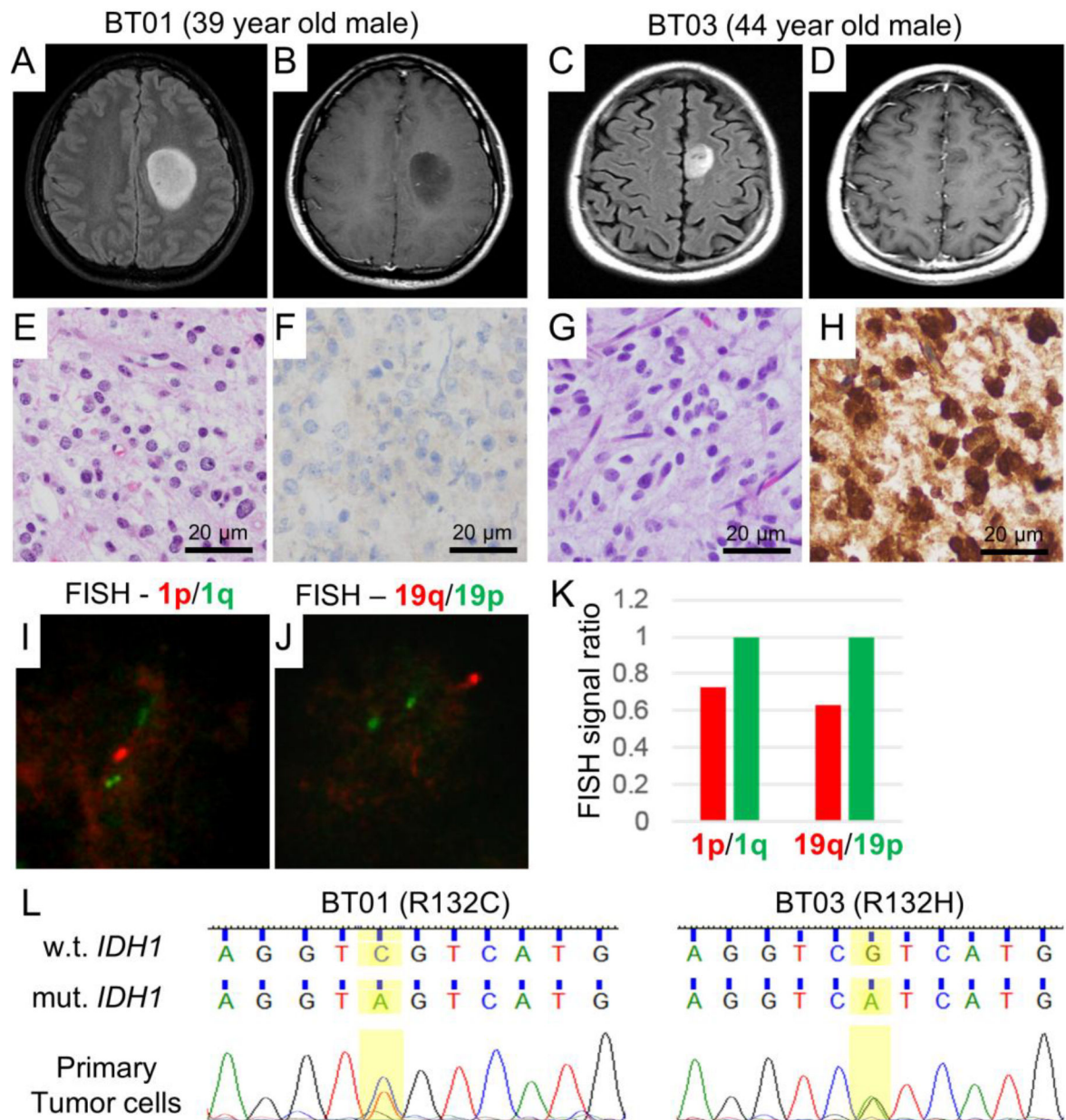
FIG. 1.

Characterization of primary LGGs. (**A-D**) MRI images FLAIR (A and C) and T1 with contrast (B and D) of BT01 and BT03. (**E-H**) Analysis of primary tumor tissues. (E, G) H&E stain and (F, H) immunohistochemistry of the IDH1 R132H mutant of paraffin-embedded BT01 and BT03 tissue, respectively. (**I-K**) Fluorescence *in situ* hybridization (FISH) of chromosomes 1p and 19q of primary BT03 cells. (I, J) Representative FISH images of chromosome 1 and 19. Red, 1p and 19q, respectively; green, 1q and 19p (control), respectively. (K) Quantification of FISH analysis of 75 cells for 1p and 80 cells for 19q. (**L**) Sequencing of genomic DNA identified monoallelic *IDH1* R132C and R132H mutations in BT01 and BT03, respectively. Shown are sequences of wild-type (w.t.) and mutant (mut.) *IDH1* sequences and the chromatograms of BT01 (left) and BT03 (right) primary cells. (**M**)

Sequencing of genomic DNA confirmed that BT01 and BT03 have wild type *IDH2*. Shown are wild-type *IDH2* DNA sequences encoding the R172 residue (highlighted) and the chromatograms of BT01 (left) and BT03 (right) primary cells.
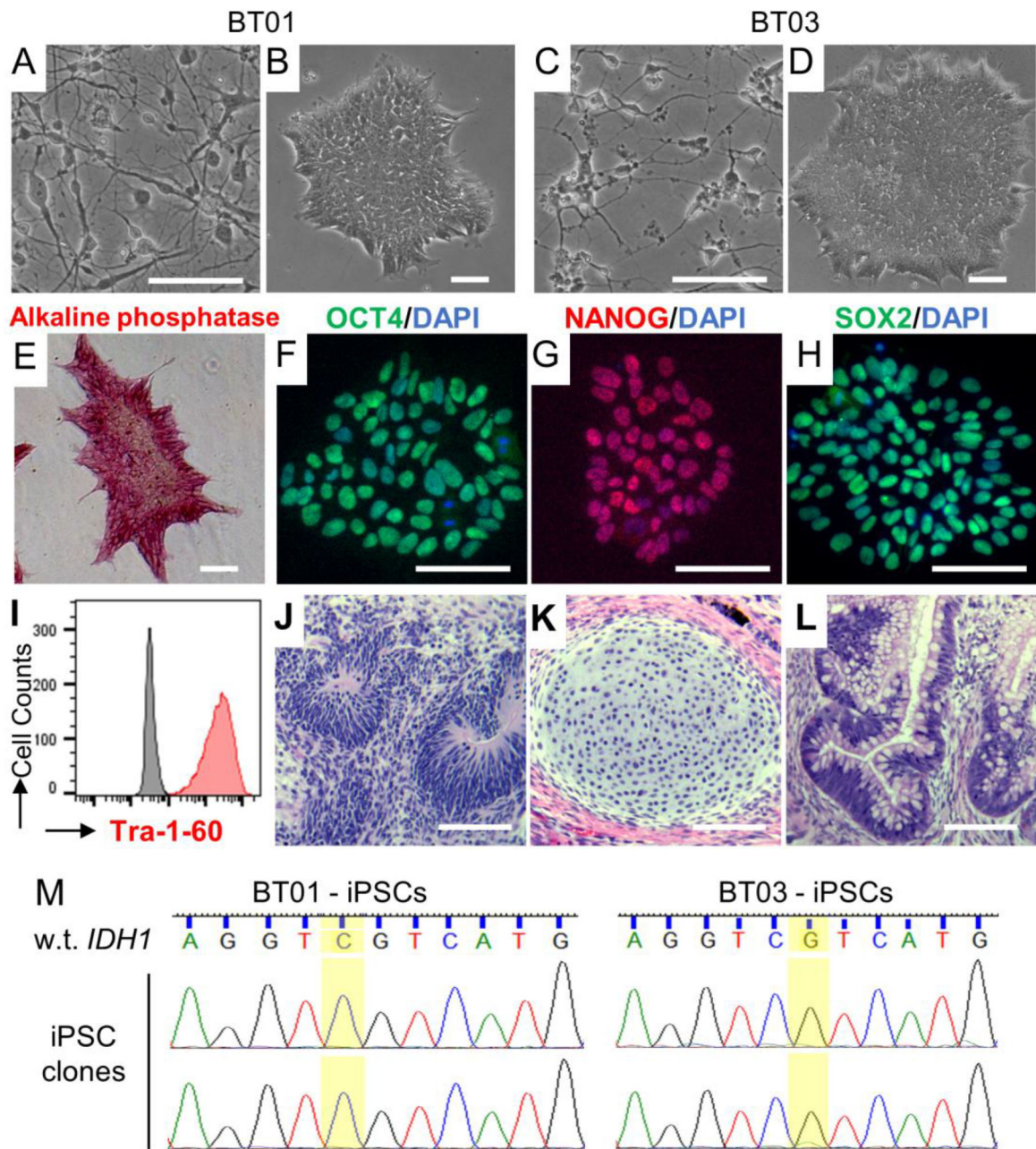
FIG. 2.

Generation of iPSCs from primary LGG cells. (**A, C**) Bright field images of primary BT01 and BT03 cells. (**B, D**) Images of representative iPSC colonies derived from BT01 and BT03 cells. (**E**) LGG-iPSCs express alkaline phosphatase. (**F-H**) Immunostaining demonstrated that LGG-iPSCs express pluripotency-associated transcription factors (F) OCT4, (G) NANOG, and (H) SOX2. (**I**) Flow cytometry analysis confirmed LGG-iPSCs expressing the pluripotency-specific surface marker Tra-1–60. (**J-L**) Teratoma formation assay demonstrated LGG-iPSCs can differentiated into (J) ectoderm (neuroepithelium), (K) mesoderm (cartilage), and (L) endoderm (glandular structure). Scale bars, 100 μm. (**M**) Representative sequencing results of the BT01- and BT03-iPSC clones. More than 15

independent clones of each LGG samples were sequenced. None carried the *IDH1* mutations.
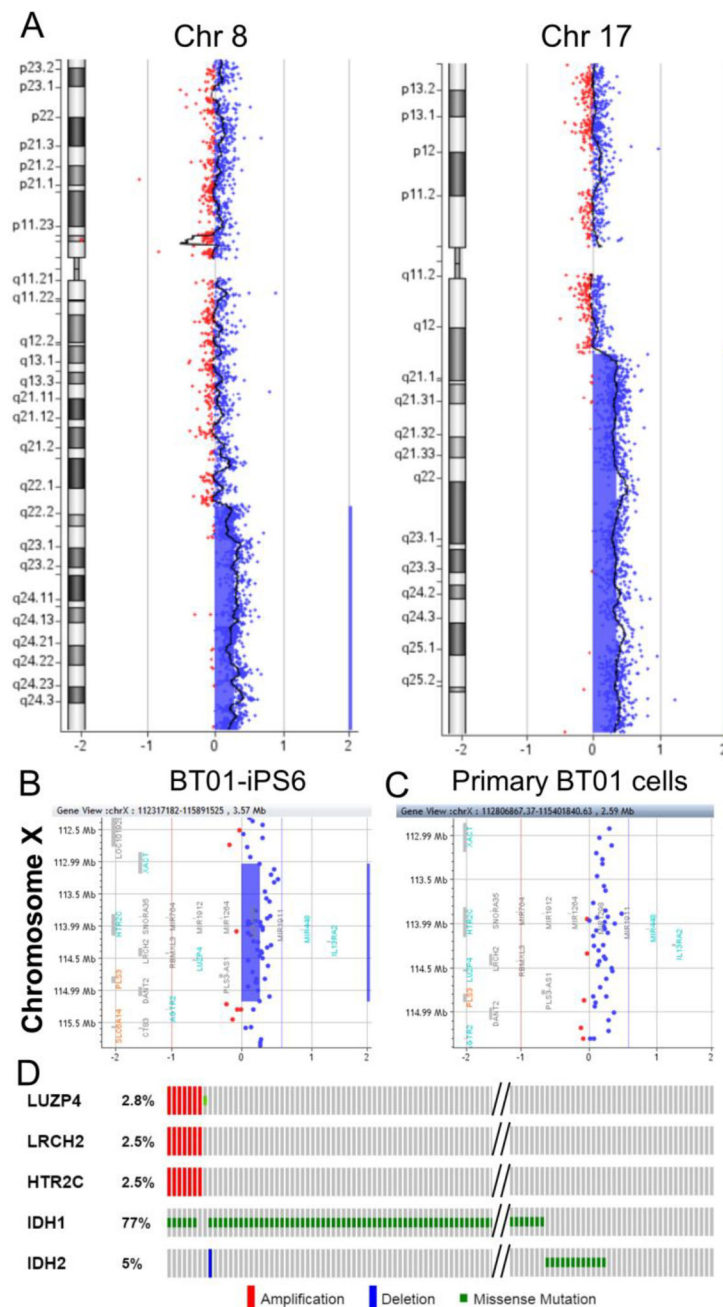
FIG. 3.

aCGH analysis of primary BT01 cells and the derived iPSCs. (**A**) aCGH analysis of primary BT01 cells demonstrated amplification of chromosome 8q and 17q. (**B-C**) aCGH analysis revealed that (B) BT01-iPS6 cells contained a 2.1 Mb-amplification on chromosome Xq23, which was also detected in (C) primary BT01 cells. Red, deletion; blue, amplification. (**D**) Analysis of TCGA low-grade glioma database revealed that 2.5% of LGGs (7 of 283) were amplified at the 2.1 Mb region and most of them (6 of 7) also have the *IDH*1 mutation. *LUZP4, LRCH2*, and *HTR2C* are genes within the amplified region on chromosome Xq23.
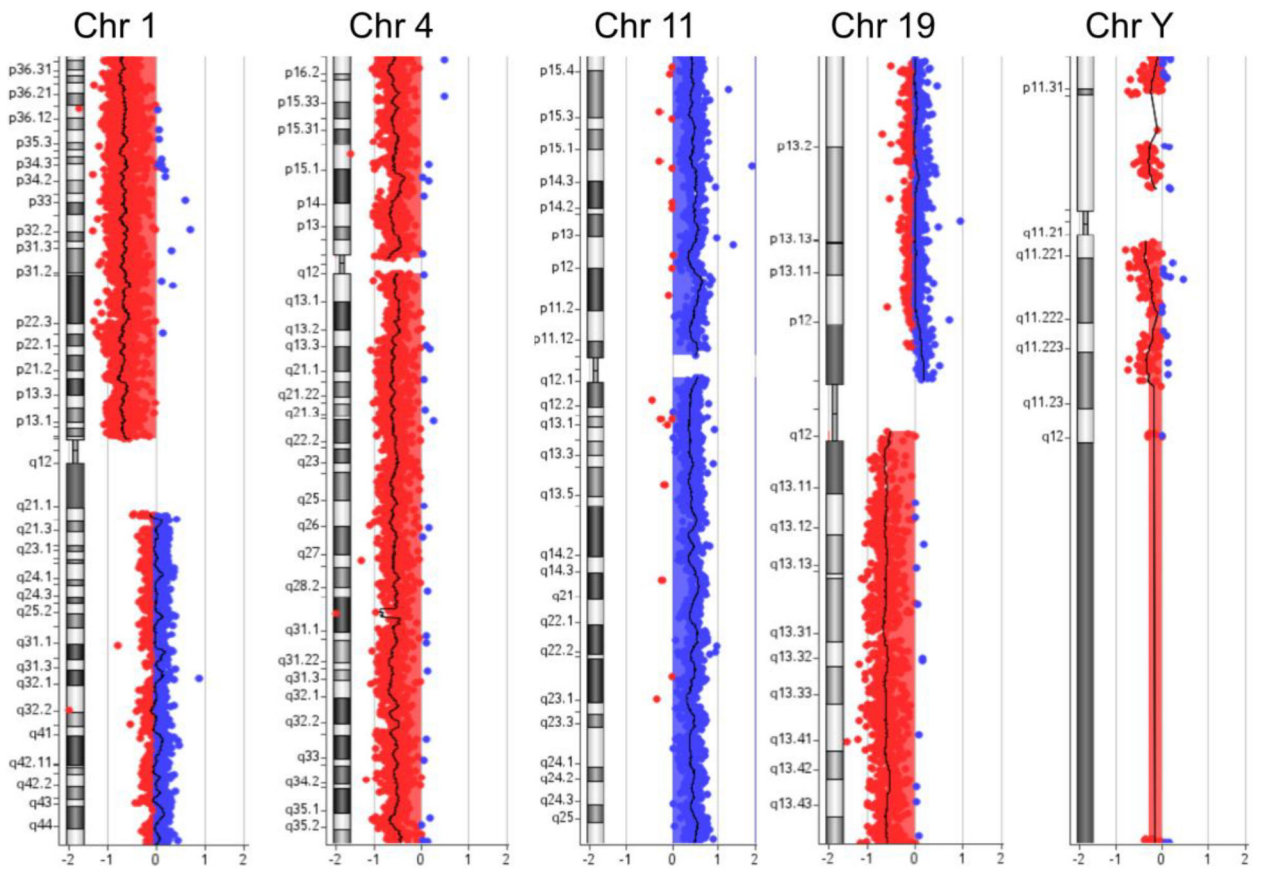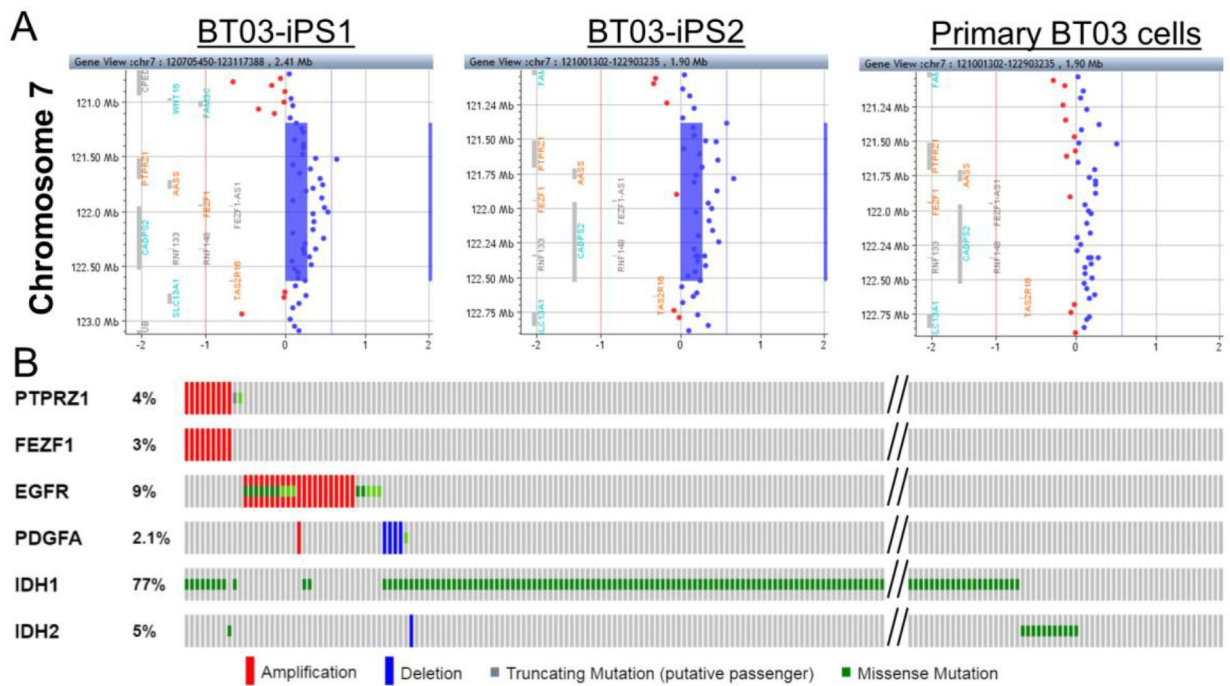
FIG. 4.
aCGH analysis of primary BT03 cells revealed amplification of chromosome 11 and deletion of chromosome 1p, 4, 19q, and Y. The results are consistent with a diagnosis of oligodendroglioma. Red, deletion; Blue, amplification.

FIG. 5.
aCGH analysis of BT03-iPSCs. (**A**) aCGH analysis revealed BT03-iPS1 (left) and BT03-iPS2 cells (center) shared a common 1.4 Mb-amplification on chromosome 7, which was also detected in the primary BT03 cells (right). (**B**) Analysis of the TCGA data revealed that 3% of LGGs (9 of 283) have amplification at the 1.4 Mb region on chromosome 7 and all of them also have the *IDH1* (8 of 9) or *IDH2* (1 of 9) mutation. *PTPRZ1* and *FEZF1* are genes within the amplified region. Note that genes on chromosome 7 that known to drive brain tumors, such as *PDGFA* and *EGFR*, were not amplified in this subset of tumor cells.
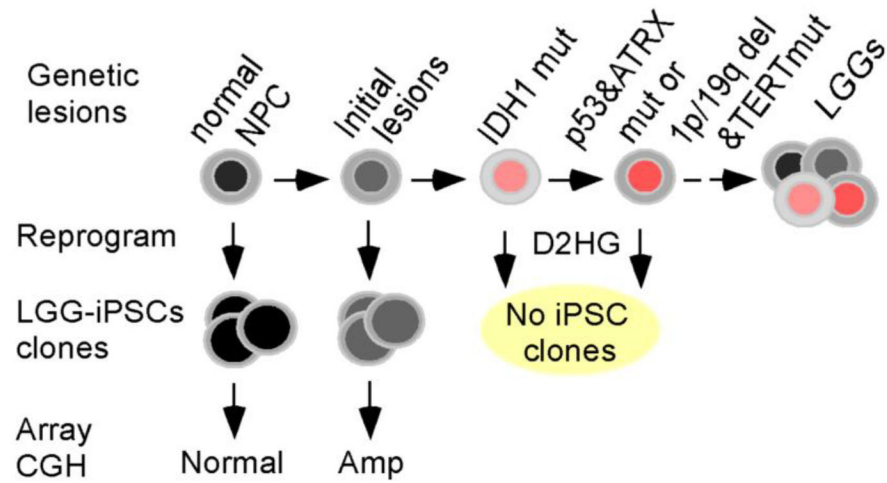
FIG. 6.
A model illustrating sequential acquisition of genetic lesions. Previous studies have established that *IDH1* mutations are the key drivers for gliomagenesis and precede other major genetic alterations such mutations in p53, ATRX, and TERT and co-deletion of chromosome 1p/19q. We discovered the presence of regional chromosomal alterations in at least a subset of LGGs before acquiring the *IDH1* mutations, which may represent predisposing conditions for *IDH1* mutations and/or be cooperating with the *IDH1* mutations in gliomagenesis.

Table 1.

All genes that are within the amplified chromosomal regions

LGG sample	Amplified chromosomal region	Genes
BT01	Xq23 Start (bp) - 113,032,051 Stop (bp) - 115,176,657	XACT, HTR2C, SNORA35, MIR764, MIR1912, MIR1264, MIR1298, MIR1911, MIR448, IL13RA2, LRCH2, RBMXL3, LUZP4, PLS3-AS1, PLS3, DANT2
BT03	7q31 Start (bp) - 121,187,838 Stop (bp) - 122,635,001	PTPRZ1, AASS, FEZF1, FEZF1-AS1, CADPS2,RNF133, RNF148, TAS2R16