The Transcriptional Regulatory Network of Proneural Glioma Determines the Genetic Alterations Selected during Tumor Progression

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Abstract

Proneural glioblastoma is defined by an expression pattern resembling that of oligodendrocyte progenitor cells and carries a distinctive set of genetic alterations. Whether there is a functional relationship between the proneural phenotype and the associated genetic alterations is unknown. To evaluate this possible relationship, we performed a longitudinal molecular characterization of tumor progression in a mouse model of proneural glioma. In this setting, the tumors acquired remarkably consistent genetic deletions at late stages of progression, similar to those deleted in human proneural glioblastoma. Further investigations revealed that p53 is a master regulator of the transcriptional network underlying the proneural phenotype. This p53-centric transcriptional network and its associated phenotype were observed at both the early and late stages of progression, and preceded the proneural-specific deletions. Remarkably, deletion of p53 at the time of tumor initiation obviated the acquisition of later deletions, establishing a link between the proneural transcriptional network and the subtype-specific deletions selected during glioma progression. Cancer Res; 74(5); 1440–51. ©2014 AACR.

Introduction

Glioblastoma is remarkably heterogeneous in genotype and phenotype, which constitutes a major challenge for targeted therapies (1–3). Whereas much of the diversity in glioblastoma prognosis and clinical outcome has been attributed to its genetic heterogeneity, the determinants of such diversity remain obscure. Significant new evidence suggests that the acquisition of genetic alterations during tumor progression may be a deterministic rather than random process, influenced by multiple genetic and epigenetic factors (4–12). Thus, the phenotypic and genetic diversity of glioblastoma might result from differences in the cell of origin (13, 14), age (11, 15), and/or preexistent genetic background (4). Unfortunately, studying the factors that influence malignant progression is limited by poor reporting of relevant data in patient populations and by lack of data on cellular phenotype and genetic alterations in early stages of the disease.

Recent studies have revealed that glioblastoma can be divided into distinct subtypes based on gene expression profiling, and that some subtypes are associated with a specific set of genetic alterations (14, 16), suggesting a functional relationship between phenotype and genotype. However, the mechanistic and temporal dependencies between tumor phenotype and its genotype remain elusive (3). The proneural subtype, in particular, is defined by a distinctive gene expression profile (GEP; ref. 14), which resembles that of oligodendrocyte progenitor cells (OPC), a presumed cell of origin for this group of gliomas (13). Proneural gliomas bear characteristic genetic alterations, such as TP53 and IDH1 mutations, as well as PDGFRα amplification (14). The proneural phenotype is characteristic of the majority of low-grade gliomas, and secondary glioblastoma (14). Thus, although proneural gliomas present with specific genetic alteration patterns, suggesting direct dependency between genotype and phenotype, the identification and characterization of causal events continues to elude us.

To address this question, we used a murine model of proneural glioma induced by overexpression of the platelet-derived growth factor (PDGF) oncogene, and inactivation of the Pten tumor suppressor (13). PDGF signals stimulate proliferation of OPCs (17, 18) and its overexpression has also been implicated as a driver of proneural gliomas (13, 14, 19, 20). In
our model, tumor formation is induced by injecting PDGF-B–IRE5–Cre (PIC) retrovirus into the subcortical white matter of adult *Pten* \(_{lox/lox}\) transgenic mice (13). Thus, gliomagenesis starts as a small collection of retrovirus-infected glial progenitors and progresses to tumors that recapitulate the histologic features of glioblastoma with 100% penetrance. Cross-species comparison with human glioblastoma from The Cancer Genome Atlas (TCGA) revealed that this model matches the proneural GEP signature (13). In this study, we tracked the changes in gene expression, histologic appearance, and gene copy number alterations that occur during initiation and progression of murine proneural gliomas. This allowed for phenotypic and genotypic characterization of tumor progression at different time points, with experimental control of variables such as age, cell of origin, environmental exposures, and genetic alterations. Our analysis showed remarkable consistency in the pattern of spontaneously occurring copy number alterations, including a subset of genes commonly deleted in both human and murine proneural tumors, thus, establishing a temporal relationship between tumor phenotype and genotype. We also identified a proneural-specific transcriptional regulatory network in which p53 is a master regulator during both early- and late-stage glioma progression. Notably, upfront deletion of p53 facilitates glioma progression, without requiring accumulation of additional proneural-specific deletions. These results provide experimental evidence for a functional link between the pre-existing cellular phenotype and the acquisition of specific genetic alterations.

**Materials and Methods**

**Tumor models**

The *PTEN*, *PTEN/p53*, and *p53* tumors were generated by injecting PIC virus into the subcortical white matter of adult mice harboring floxed *Pten* or floxed *p53*, as previously described (13). For cell-transplantation experiments, \(2 \times 10^4\) primary tumor cells were resuspended in 2 \(\mu\)L of Opti-MEM (Invitrogen) and injected into brain with a flow rate of 0.2 \(\mu\)L/min (\(n = 20\)). Animals were followed, and upon signs of tumor-related morbidity were euthanized. Tumor DNA was collected at 21, 28, and 35 days post injection (dpi) for *PTEN*, *YFP*+ tumors, and upon death for *PTEN*, Luciferase+ and *PTEN/p53*, Luciferase+ tumors (\(n = 8/group\)). RNA was isolated from 21 dpi *PTEN* tumors, end-stage *PTEN*, and end-stage *PTEN/p53*, as well as mouse frontal lobe specimens were also collected for expression analysis (\(n = 6–8/group\)). All procedures were approved by the Institutional Animal Care and Use Committee of Columbia University (New York, NY).

**Cell lines**

*Ex vivo* gross resection of the tumor was performed and tumor cells were isolated using enzymatic digestion (21, 22), and cultured overnight in media containing Dulbecco’s Modified Eagle Medium, N2, T3, 0.5% FBS, and penicillin/streptomycin/amphotericin, PDGF-A (10 ng/mL) and basic fibroblast growth factor (10 ng/mL), and B104 conditioned media (1:2; ref. 23). Cell preparations were used for intracranial injection the following day. During these studies, we generated cell lines from *PTEN* tumors and propagated them in culture as described previously (13, 24).

**Comparative genomic hybridization**

Tumor and liver DNA was isolated [Qiagen DNA Mini Isolation Kit (51306) and Qiagen RNase A (19101)] and paired for comparative genomic hybridization (CGH) analysis on the Agilent 244A mouse array CGH platform. Balanced dye swap was performed in 50% of the samples. Samples were corrected by subtracting the background; zeros and negative intensities were then set to half the minimum value of positive corrected intensities for the array. We performed a within array global median normalization and subsequent segmentation with smoothing using CBS [PMID:15475419]. Minimal common regions were called by means of Plink [PMID:17701901] using a 10-kb window size and 30% copy number variation overlap. We found that a gene that is deleted in (6/8) 75% of samples was significant for deletion (\(P \leq 0.05\)), compared with a random occurrence null hypothesis model. We compared *PTEN* tumors against *PTEN/p53* with 50,000 sample permutations and an adjusted \(P\) value \(\leq 0.05\) for significance.

**Cre-mediated Pten deletion provides a validation of deletion differences between mouse tumor groups**

Given that comparisons made included tumors that were harvested through different techniques, and at different stages/sizes, we evaluated whether the relative tumor DNA content between these two cohorts was comparable. The log2 ratio signal for tumor/nontumor gene copy number for a probe that hybridizes the Cre/lox–mediated *Pten* deletion showed significantly lower log2 ratio for *PTEN* 35 dpi tumors than either *PTEN* or *PTEN/p53* end-stage tumors, suggesting a smaller fraction of tumor DNA on the former than the later groups, biasing against finding deletions in *PTEN* 35dpi tumors but not on *PTEN/p53* end-stage tumors (Supplementary Fig. S1). Thus, it is unlikely that time alone can explain the difference in copy number alterations seen between these two models.

**Cross-species genomic comparison**

For human gene copy number data acquisition, Affymetrix Genome-Wide Human SNP Array 6.0 data and Affymetrix U133A expression data for human glioblastoma specimens (\(n = 369\)) were queried, available through the TCGA (http://cancergenome.nih.gov/). Glioblastoma subtypes were defined on the basis of expression profile using a classifier based on the subtypes previously described (14), and by a list kindly provided by Dr. Verhaak (University of Texas MD Anderson Cancer Center, Houston, TX personal communication). Subgroup-specific copy number gene alterations were defined by the \(\chi^2\) test comparison between a subtype against the sum of all patients from the remaining three groups (\(P < 0.05\)) with a false discovery rate (FDR) <10%, and the presence of this deletion in at least 7% of that subgroup.

**Trp53 DNA-based sequencing**

*Trp53* was sequenced following PCR amplification of exons 5 to 9 using genomic DNA as template. A second reverse sense
confirmatory sequence for all positive samples was performed. Tp53 primers: exon 5 of 6 Fwd: 5'-CGGCCCTTTCTCTCTTCTCC-3', Rev: 5'-GGGCGCACCCCTCTCTCGG-3', exon 7 Fwd: 5'-GGGCGCACCCCTCTCTCGG-3', Rev: 5'-GGGCGCACCCCTCTCTCGG-3', and exon 8 of 9 Fwd: 5'-GGGCGCACCCCTCTCTCGG-3', Rev: 5'-GGGCGCACCCCTCTCTCGG-3'.

**Cross-species comparisons of phenotype and transcriptional regulatory network analysis**

We prepared RNA-Seq libraries from each mouse normal brain or tumor sample and obtained 15 to 30 million single-end, 100-base reads on an Illumina HiSeq 2000 sequencer (JP Sulzberger Columbia Genome Center). To assess the correlation with human glioblastoma subtype designation (14), we used the Spearman rank correlation coefficient using a previously described method for mouse RNA-Seq data (24). We use the median value of the correlation between an RNA-Seq data set and the TCGA microarray data for a given subtype as a similarity score for that subtype.

We applied ARACNe algorithm (25) to infer transcriptional regulatory network containing interactions between transcription factors and putative targets in human glioblastoma using expression data for 319 samples obtained from TCGA (http://cancergenome.nih.gov). Furthermore, we assessed the Spearman rank correlation coefficient using a previously described method for mouse RNA-Seq data (24). We performed transcriptional data analysis with mouse and human tumors, we compared the genes deleted in mouse and human proneural gliomas (http://cancergenome.nih.gov). To explore the similarities in the genetic alterations between mouse and human tumors, we compared the genes deleted in 75% to 100% of end-stage *PTEN tumors (Fig. 2B; Supplementary Fig. S2 and Supplementary Table S1). Copy number gains were also detected but were not as frequent as gene deletions (Fig. 1C; Supplementary Table S2). At 21 and 28 dpi, *PTEN tumors acquired only a few copy number alterations, with overall frequency significantly lower than in end-stage *PTEN gliomas (Fig. 1B and C). Notably, CGH was able to detect the Cre-mediated deletion of Pten as early as 21 dpi (Supplementary Fig. S1), arguing against the possibility that the differences in spontaneous deletions seen in early- versus late-stage tumors are merely due to changes in the fraction of retrovirus-infected tumor cells (harboring Cre-mediated Pten deletion). Rather, these results support the conclusion that the spontaneous deletions are acquired in a subset of tumor cells and then selected for during tumor progression.

**Cross-species comparisons identify gene deletions common to mouse and human proneural gliomas**

To explore the similarities in the genetic alterations between mouse and human tumors, we compared the genes deleted in 75% to 100% of end-stage *PTEN tumors (n = 541) to gene deletions reported in the TCGA human glioblastoma database (http://cancergenome.nih.gov). Furthermore, we assessed whether there is a statistically significant bias in the distribution of the deletions across the four glioblastoma subtypes (proneural, neural, mesenchymal, and classical; ref. 14). There were 108 genes that were deleted in 75% to 100% of end-stage *PTEN mouse tumors and deleted in greater than 10% of any human glioblastoma subtype (Supplementary Table S3). Of these gene deletions, 75 were significantly and selectively enriched in the proneural subtype of human glioblastoma (P ≤ 0.05; FDR ≤ 0.1; Fig. 2A; Supplementary Table S4).

The 75 proneural-specific deleted genes clustered into four loci on human chromosomes 11p, 14q, 17p, and 19q, all of which are within loci previously reported to be deleted in malignant gliomas (Fig. 2B; Supplementary Fig. S2 and Supplementary Table S3; refs. 26–28). When the threshold is relaxed to include genes deleted in more than 8% of any glioblastoma subtype, an additional 17 genes are identified as being selectively deleted in proneural glioblastoma, including 12 genes on chromosome 11q, three genes on chromosome 12q, and two genes on chromosome 19q (Fig. 2B; Supplementary Table S4). Using either of these thresholds, there were three genes on chromosome 10 that were significantly enriched in both the classical and neural subtypes and one gene on chromosome 6 that was significantly deleted only in
Figure 1. PDGF*PTEN<sup>−/−</sup> proneural gliomas acquire highly consistent gene deletions during tumor progression. A, hematoxylin and eosin (H&E) staining shows the characteristic glioblastoma histology, including vascular proliferation (V) and pseudopalisading necrosis (N) seen in end-stage *PTEN lesions, but not in *PTEN 21 dpi. Ki-67 labeling by immunofluorescence, and graphical representation of Ki-67 index shows significant difference between *PTEN 21 dpi and end-stage *PTEN tumors (P < 0.001). B, whole-genome view shows a significant number of genetic alterations seen in end-stage *PTEN but not in *PTEN 21 dpi. Green bars represent deletions and red bars represent amplifications. The height of the bar correlates with the frequency of the alteration per group. C, histogram comparing the frequency of copy number alterations in *PTEN tumors at different time points (n = 8/group). Frequencies of 75% or greater are significantly higher than that expected by a random permutation test (P < 0.05).
the classical subtype. Thus, in addition to the similarities seen at gene expression level (13), PTEN tumors also most closely resemble the proneural subtype of human glioblastoma in the pattern of genetic deletions that they acquire.

Interestingly, the putative tumor suppressor gene CIC was deleted in 6 of 8 PTEN 35 dpi and 5 of 8 PTEN end-stage mouse tumors. Situated in the 19q locus in the human genome, CIC is often deleted or mutated in human oligodendrogliomas (29), as well as in 13.6% of proneural glioblastoma from TCGA dataset.

Within the TCGA dataset, there were significant positive correlations among the proneural-specific deletions, including 17p with 11q (P ≤ 0.01), and 17p with 14q deletion (P ≤ 0.05; Supplementary Table S5). We also compared the distribution of the proneural-specific deletions with other genetic alterations characteristic of proneural glioma, including PDGFRα amplification, TP53 mutations, and IDH1 mutations, which coincide with a CpG island hypermethylation phenotype (14, 30). Deletion of 11q and 17p were overrepresented within patients with proneural glioblastoma–bearing PDGFRα amplification (P ≤ 0.05) and deletion of 11p and 19q were overrepresented within patients with proneural glioblastoma–bearing IDH1 mutations (P ≤ 0.01; Supplementary Table S5), which in the case of 19q, resembles the scenario described in oligodendrogliomas (30, 31).

**Proneural phenotype precedes the accumulation of genetic deletions in murine gliomas**

PTEN tumors show malignant progression, including appearance of malignant histology and genetic alterations, but there is a clearly identifiable early stage in which these lesions are lacking such features. This allowed for direct investigation of the temporal relationship between genetic alterations and proneural phenotype during tumor progression. To evaluate whether the proneural phenotype precedes deletion of these genes, we performed RNA-Seq analysis of early- and late-stage tumors and used these data to classify tumor phenotype according to the Verhaak and colleagues, gene set (14). We found that at 21 dpi, a time point that precedes the presence of highly recurrent gene deletions, murine gliomas already had higher resemblance to proneural glioblastoma than to any other subgroup (Fig. 3A). As shown previously (13), end-stage PTEN mouse in mouse gliomas are localized to six different loci on chromosomes 11, 12, 14, 17, and 19 of the human genome that are found deleted in proneural glioblastoma. Each of these regions is represented on a separate column, and the limits of the region are framed in a red box at chromosome diagram (top). The four rows represent the four subtypes of glioblastoma. Red lines represent amplifications and blue lines represent deletions.

**Figure 2. Cross-species comparison identifies gene deletions common to mouse and human proneural glioblastoma**. A, bar chart showing the number of genes deletions in specific subtypes of human glioblastoma (Supplementary Table S4). B, cross-species comparison shows that the 92 proneural-specific gene deletions (P ≤ 0.05; FDR ≤ 0.1) seen in 75% to 100% of PTEN mouse in mouse gliomas are localized to six different loci on chromosomes 11, 12, 14, 17, and 19 of the human genome that are found deleted in proneural glioblastoma. Each of these regions is represented on a separate column, and the limits of the region are framed in a red box at chromosome diagram (top). The four rows represent the four subtypes of glioblastoma. Red lines represent amplifications and blue lines represent deletions.
murine model, expression of a proneural signature precedes accumulation and selection of highly recurrent gene deletions, including those genes that are specifically deleted in human proneural glioblastoma. This suggests that the proneural phenotype, which is characterized by the expression of OPC genes, provides the cellular context in which these specific genetic alterations are selected.

**p53 is a master regulator of proneural transcriptional network**

To further characterize the proneural phenotype in which relevant deletions are selected, we used the MARINa algorithm (25). This analysis predicts transcription factors that are master regulators of the genes differentially expressed between two sample sets. Specifically, we used the algorithm to identify transcription factors in which ARACNe-inferred transcriptional targets are highly enriched in genes that are differentially expressed in 21 dpi, or end-stage PTEN tumors compared with normal brain (Supplementary Table S7). Among these master regulators, a few (proneural master regulators) have ARACNe-inferred targets that are enriched in proneural signature genes compared with any other transcription factor in the genome ($P < 0.05$; Fig. 4A). Moreover, the proneural master regulators form a densely connected transcriptional regulatory module among themselves and with other master regulators identified by MARINa (Fig. 4B). Notably, p53 was identified as a proneural master regulator of both early- and late-stage tumor progression. As such, it was found to interact with many other master regulators in the transcriptional regulatory network (Fig. 4).
To compare master regulators of murine proneural progression with those driving human proneural glioblastoma, we also used the MARINa algorithm to identify master regulators in which targets are enriched in genes differentially expressed in human proneural glioblastoma samples from TCGA versus normal brain (Supplementary Table S7). Findings from TCGA were highly consistent with those obtained by the MARINa analysis in an independent glioblastoma dataset (Rembrandt, NCI; P ≤ 1 × 10^{-25} by the Fisher exact test). Cross-species comparison of murine and human master regulators of proneural glioblastoma showed a highly statistically significant overlap (P ≤ 1 × 10^{-25} by the Fisher exact test), as well as a significant overlap between the ARACNe-inferred targets of the human and mouse master regulators (Supplementary Fig. S3 and Supplementary Table S7). Notably, p53 is also identified as a master regulator of human proneural glioblastoma. Thus, these analyses provide a putative regulatory context for the study of proneural gliomas, in which p53 and other transcription factors serve as master regulators during different stages of tumor development.

An independent analysis was performed to identify potential upstream regulators of the genes differentially expressed between normal brain and end-stage *PTEN gliomas using the Ingenuity upstream regulator analysis (http://www.ingenuity.com/). This analysis also identified p53 as the most significant upstream transcriptional regulator (P ≤ 1 × 10^{-25}). Thus,
several independent analyses, across both murine and human gliomas, suggest that p53 plays an important role in the transcriptional regulation of the proneural subtype (Fig. 4; Supplementary Table S7).

**Upfront deletion of Trp53 obviates subsequent proneural-specific deletions during glioma progression**

As discussed, regulatory analysis revealed p53 as a master regulator of the proneural transcriptional signature, at both early and late stages of tumor progression, in *PTEN* gliomas. Moreover, *TP53* mutations are enriched within the proneural subtype of human glioblastoma (14). Considering the well-established function of p53 as a tumor suppressor, these findings suggest p53 plays a central role in regulating the growth of cells with a proneural phenotype. Furthermore, inactivation of p53, via direct mutations and deletions of the *TP53* gene or via genetic alterations of other genes that are part of the p53 transcriptional network, will likely provide a powerful selective advantage to proneural glioma cells. If gene deletions in *PTEN* end-stage tumors are functionally redundant to loss of p53, then upfront ablation of *Trp53* should reduce the selective pressures to accumulate these deletions. To investigate this hypothesis, we analyzed the genome of a cohort of PDGF*PTEN*"/"p53"/" (*PTEN*/p53) mouse tumors. These tumors have Trp53 loss as one of the initiating genetic alterations because these are induced by intracranial injection of PIC into *PTENfox/fox;gluc/fox* mice (13). The *PTEN*/p53 tumors have a proneural expression pattern similar to that of *PTEN* tumors; however, the *PTEN*/p53 tumors more rapidly progress to glioblastoma (13). At 17 dpi the *PTEN*/p53 tumors were highly infiltrative and showed marked nuclear atypia and numerous mitotic figures (Supplementary Fig. S4). By 21 days the tumors show the histologic feature of glioblastoma, including vascular proliferation and necrosis (Supplementary Fig. S4).

Array CGH analysis across mouse tumor groups showed that end-stage *PTEN* and *PTEN*/p53 tumors had similar levels of Cre-mediated *Pten* exon 5 deletion, indicating that both tumor models were composed of a similar proportion of retrovirus-infected cells (Supplementary Methods and Supplementary Fig. S1). Gene copy number analysis revealed that *PTEN*/p53 tumors accumulated gene deletions with a significantly lower frequency than *PTEN* end-stage tumors (Fig. 5), whereas gene amplifications occurred with a low frequency in both models (Fig. 5; Supplementary Table S2).

To rule out the possibility that differences in gene copy number alterations between end-stage *PTEN* and *PTEN*/p53 merely reflect the additional time for acquisition of these deletions, due to the difference in median survival between these two groups, CGH results were compared between end-stage *PTEN*/p53 tumors and *PTEN* tumors at 35 dpi, a time point that matched the end of the survival curve for mice bearing *PTEN*/p53 tumors (n = 8; Fig. 6A). Similar to the comparison with end-stage *PTEN*, at 35 dpi *PTEN* tumors had significantly higher frequency of gene deletions than *PTEN*/p53 (P ≤ 0.05; Fig. 5). Thus, time alone does not explain the difference in copy number alterations seen between these two models.

**Murine proneural gliomas acquire alterations in the p53 pathway during tumor progression**

*PTEN*/p53 tumors had a significantly shorter survival than *PTEN* lesions (log-rank *P* ≤ 0.01; Fig. 6A; ref. 13). This difference in survival might reflect an inherently less aggressive phenotype for *PTEN* tumors compared with *PTEN*/p53. However, an alternative hypothesis is that the difference in survival may be due to the additional time required for *PTEN* tumors to accumulate the additional genetic alterations needed for tumor progression, after which, *PTEN* tumors grow as aggressively as *PTEN*/p53 tumors. To directly compare the growth dynamics of end-stage lesions of both tumor models, we transplanted 2 × 10⁵ cells from end-stage *PTEN* or *PTEN*/p53 into the brains of adult naïve mice, and in this case there was no significant difference in survival (Fig. 6A). These findings, and the similar Ki-67 labeling (13), suggest that *PTEN* tumor cells evolve over time into a more aggressive and proliferative phenotype, which, in the end-stage tumors, is comparable to that seen in *PTEN*/p53 tumors.

Given the prominent transcriptional activity of p53 at different stages of *PTEN* tumor development (Fig. 4), we investigated the expression of transcriptional targets of p53 that are known to play a role in tumor suppression (34). Consistent with previous reports (13), a series of p53 targets had a significantly higher expression in *PTEN* end-stage than on *PTEN*/p53 tumors. Furthermore, our analysis of tumor progression in the *PTEN* model shows that these transcriptional targets of p53 were highly upregulated at early stages of tumor formation and then were significantly downregulated as the tumors progressed to end-stage (Fig. 6B). These downregulations coincided with the accumulation of deletions seen in *PTEN* tumors, suggesting a possible functional relationship between the deleted genes and the expression of p53 and its targets. In support of this idea, ingenuity-based analysis identified numerous previously described functional interactions between the gene deletions encountered in 75% to 100% of *PTEN* tumors with p53 and/or its transcriptional targets that are established tumor suppressors (Fig. 6C).

*PTEN* tumors also acquired nonsynonymous mutations in *Trp53*. Sanger sequencing analysis of *Trp53* (exons 5 through 9) revealed that one third of the end-stage *PTEN* tumors (n = 5/15) had acquired missense mutations leading to a single amino acid substitution within the DNA-binding domain of p53. Of note, all of these mutations were located in mutational "hotspots" in the *p53* gene, which correspond to the most common mutations seen in human glioblastoma (Fig. 6D; Supplementary Fig. S5; refs. 35, 36). Previous studies have shown that these mutated alleles are expressed, but have abnormal transcriptional activity and loss of p53 tumor suppressor function (36). Examination of the RNA-Seq data of *PTEN* end-stage tumors confirmed the transcription of the mutated allele in two cases that had paired RNA/DNA sequencing data. Sanger sequencing of two cell lines derived from *PTEN* tumors also showed *Trp53* mutations, with the presence of a single peak, indicative of loss of heterozygosity with loss of the wild-type allele. Immunohistochemical analysis of one of these cells lines, which harbors a single mutant allele (R175H), showed nuclear staining for p53 (Supplementary Fig. S5).
Discussion

A key question within the field of cancer biology is the cause-effect and temporal relationship that exists between tumor phenotype and genotype, especially in cases in which a particular genetic alteration is associated with a specific phenotype. Here, we showed that in a mouse model of proneural glioma, the proneural phenotype preceded the acquisition of highly specific gene deletions during tumor progression. Therefore, these genetic alterations occurred, and were selected for, within the context of a preexisting proneural phenotype. The fact that these genetic deletions were also commonly seen in human proneural glioblastoma, suggests a functional link between genetic alterations and tumor phenotype that is conserved across species. Furthermore, we showed that in a highly controlled experimental setting, tumor evolution can be modeled with remarkable consistency, with spontaneous acquisition of some gene deletions in 75% to 100%. Our results also suggest that the selection of specific genetic alterations was influenced by initial conditions of gliomagenesis, which include the initiating genetic alteration and the cellular context in which these occur.

Deletion of Trp53 provided an experimental link between the proneural regulatory network and genetic alterations encountered in our model. Given that the proneural phenotype preceded the appearance of these gene deletions, the transcriptional regulatory network that drives this phenotype must be independent of these deletions. Nevertheless, this regulatory network had a strong influence on the selective pressure for these deletions during tumor progression. This point was illustrated by the fact that upfront deletion of Trp53, a highly interconnected master regulator transcription factor identified in the proneural regulatory network, accelerated tumor progression while obviating the selection of the highly recurrent deletions seen on the "PTEN" tumor model. This finding suggests that there is a functional interplay between these genetic alterations and the intrinsic regulatory network.

p53 is highly active as a transcription factor at early and late stages of proneural glioma development, as evidenced by the elevated expression of p53 and its transcriptional targets in "PTEN"21 dpi, as well as in "PTEN" end-stage lesions and human proneural glioblastoma. These targets include the genes that constitute the proneural signature, as well as other proneural master regulator transcription factors (Fig. 4). Notably, a subset of p53 targets that are implicated in tumor suppression is significantly downregulated as the "PTEN" tumors progress from 21 dpi to end-stage, and this is coincident with the accumulation of recurrent gene deletions observed during malignant progression. Moreover, these gene deletions did not occur when Trp53 was deleted as an initiating genetic lesion, suggesting functional redundancy.

Several previous studies have shown that deleting p53, in combination with other genetic alterations, will facilitate the formation of brain tumors (33, 37, 38). Similarly, we found that injecting PIC retrovirus into p53lox/lox mice induces tumors...
with the histologic characteristics of glioblastoma with 100% penetrance. The survival curve for the PDGF/+/p53−/− ("p53" model is significantly longer than that seen on "PTEN/p53" tumors, and significantly shorter than that seen on "PTEN" tumors (Supplementary Fig. S6). Similar to the "PTEN and "PTEN/p53" tumors (13), the "p53" tumors express high levels of glial progenitor markers and have a proneural phenotype (Supplementary Fig. S6 and Supplementary Table S6).

In addition to p53, several other master regulators identified in our analysis are known to play a role in glial progenitor...
biology and cancer. TCF3 inhibits the differentiation along the oligodendrocyte lineage, and regulates the cellular response to p53 in multiple cancers (39, 40). YY1, plays an essential role in oligodendrocyte progenitor differentiation, and has previously been identified as a regulator of the proneural-specific expression pattern in glioma (41, 42). E2F1 and E2F3, both identified as master regulators, promote cell-cycle progression, and were previously found amplified in transgenic mouse models of glioma (38). MYC, a master regulator in both early and late stages of proneural glioma development, was previously implicated in self-renewal capacity and inhibition of differentiation in mouse glioma models, specifically in the context of loss of function of Pten and p53 (37).

In addition to this murine model, other groups have found that a variety models using different genetic alterations can induce the formation of tumors that resemble proneural gliomas (13, 33, 38, 43). Chow and colleagues, reported the induction of tumors that resemble proneural function of Pten and p53 (37). Pdgfra was found as a regulator of the proneural-specific expression, and this may obviate the fact that in our model, glioma growth is driven by oncogenes that provide additional growth-promoting signals to the tumor cells. Interestingly, Chow and colleagues found large scale deletions of chromosomes 12 and 14 that overlapped with some of the deletions that were identified in our studies.

These studies demonstrate how cross-species comparison of cancer genomics provides a powerful approach to identify genes involved in tumorigenesis. This approach takes advantage of the phenotypic and genetic similarities between the mouse tumor models and human cancer, as well as the species-specific differences in genomic architecture and size of the copy number alterations. By doing this, we refined the list of thousands of genes deleted in human proneural glioblastoma, to less than 100 genes that were specifically deleted in both mouse and human proneural tumors. Furthermore, some genes contained within a single deletion in human gliomas were distributed into multiple deletions on separate chromosomes in mouse gliomas, suggesting independent selection pressure for their loss (Supplementary Fig. S2).

Our findings reveal nonrandom relationships between different genetic alterations in proneural glioblastoma. These relationships included correlations of some of these deletions with established proneural-specific oncogenic genetic alterations (14), such as in the case of 11q and 17p with PDGFR amplification, or 11p and 19q with IDH1.R132H mutation, as well as correlations among the deletions that we encountered. These associations suggest a possible cooperation between tumor-promoting genetic alterations, with subsequent selection for their combination.

Understanding the forces and constraints that influence tumor evolution raises the possibility of new therapeutic interventions to manipulate this process toward a more indolent path. Such considerations are particularly relevant in setting of low-grade gliomas, which have a strong tendency for malignant progression and which are mainly classified as proneural tumors (16, 30).

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References
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The Transcriptional Regulatory Network of Proneural Glioma Determines the Genetic Alterations Selected during Tumor Progression

Adam M. Sonabend, Mukesh Bansal, Paolo Guarnieri, et al.


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