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Targeted Genomic Sequencing Reveals Different Evolutionary Patterns Between Locally and Distally Recurrent Glioblastomas

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Abstract. *Background/Aim: Glioblastoma is the most malignant form of astrocytoma. The purpose of this study was to analyze the genetic characteristics of primary and recurrent glioblastomas using targeted sequencing and investigate the differences in mutational profiles between the locations of tumor recurrence. Materials and Methods: Fourteen glioblastoma patients who developed local (n=10) or distal (n=4) recurrence were included in the study. Targeted sequencing analysis was performed using the primary (n=14) and corresponding recurrent (n=14) tumor tissue samples. Results: The local and distal recurrence groups showed different genetic evolutionary patterns. Most of the locally recurrent glioblastomas demonstrated concordant mutational profiles between the primary and recurrent tumors, suggesting a linear evolution. In contrast, all cases of distally recurrent glioblastomas showed changes in mutational profiles with newly acquired mutations when compared to the corresponding primary tumors, suggesting a branching evolution. Conclusion: Locally and distally recurrent glioblastomas exhibit different evolutionary patterns.*

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Glioblastoma is the most common malignant tumor of the central nervous system (1-9). The current standard treatment for glioblastoma involves surgical resection, followed by concurrent chemoradiation therapy and adjuvant chemotherapy using temozolomide (8-12). Due to the invasive nature of glioblastoma, surgical resection rarely eliminates all tumor cells, and postoperative treatment is usually necessary to prevent disease recurrence. Despite the advances made in therapeutic strategies, the prognosis of patients with glioblastoma remains very poor, with an average survival of 15 months (13-15) and disease recurrence being the major cause of mortality (16-18). Recurrent glioblastomas tend to be more invasive and resistant to chemotherapy than primary tumors. An understanding of the genetic characteristics of recurrent glioblastoma is crucial for identifying potential targets for drug discovery, stratifying patients for diagnosis, and optimizing an effective treatment strategy.

Previous studies have shown the molecular features of recurrent glioblastomas. In particular, the mutational profiles observed in recurrent glioblastomas were compared to those of the corresponding primary tumors (19-21). However, there is a lack of studies exploring the differences in mutational profiles between recurrent glioblastomas at different sites (21, 22). In this study, we investigated the mutational profiles of primary and recurrent glioblastomas using

targeted sequencing analysis. We then compared the sequencing results between the locations of recurrent tumors, *i.e.* local and distal recurrences. We anticipate that our comprehensive analyses will extend the current knowledge in relation to the clonal evolution of recurrent glioblastoma by describing its genetic characteristics obtained from targeted sequencing analysis.

Materials and Methods

Case selection. This study (OC18TESI0052) was reviewed and approved by the Institutional Review Board of Incheon St. Mary's Hospital. Fourteen patients with glioblastoma were included in this study. Twenty-eight tumor tissue samples, including the primary (n=14) and corresponding recurrent (n=14) glioblastomas, were obtained from the surgical pathology archives of the Incheon St. Mary's Hospital. Ten of the fourteen patients developed recurrence at the same site or a location closely adjacent to that of the primary tumor and were classified as the local recurrence group (Figure 1A-B). The remaining four patients developed recurrence at different anatomical locations from that of the primary tumor and were classified as the distal recurrence group (Figure 1C-D). Clinical information was obtained from the electronic medical record system. All patients received postoperative concurrent chemoradiation therapy, with subsequent adjuvant chemotherapy using temozolomide. None of the patients received preoperative radiation therapy or chemotherapy.

Pathological examination. Two board-certified pathologists specializing in neuro-oncology reviewed all available hematoxylin and eosin-stained slides using light microscopy and analyzed the following histological characteristics of primary and recurrent glioblastoma tissues: cellularity, nuclear pleomorphism, microvascular proliferation, and coagulative tumor cell necrosis (23). They chose representative slides for each case to perform targeted sequencing.

Targeted sequencing. The tissue samples from tumors were obtained by manual microdissection and were then subjected to DNA and RNA extraction for library preparation. The normal tissues of each case were obtained from the adjacent non-neoplastic areas. DNA and RNA were isolated from 10-μm thick slices of formalin-fixed, paraffin-embedded tissues using a sterile 26-gauge needle and RecoverAll™ Multi-Sample DNA/RNA Isolation Workflow (Thermo Fisher Scientific, Waltham, MA, USA). DNA and RNA were quantified using the Qubit 2.0 Fluorometer (Thermo Fisher Scientific). DNA and RNA libraries were prepared as previously described (24-35). These DNA libraries were generated from 20 ng of DNA per sample using an Ion AmpliSeq Library Kit 2.0 (Thermo Fisher Scientific) and the Oncomine Comprehensive Assay v1 (OCA v1) panel (Thermo Fisher Scientific). RNA libraries were generated from 15 ng of RNA per sample using Ion AmpliSeq RNA Library Kit (Thermo Fisher Scientific) and OCA v1 panel. Libraries were quantified using the Ion Library Universal Quantification Kit (Thermo Fisher Scientific). The OCA v1 panel (Thermo Fisher Scientific) included 143 genes, of which 73 oncogenes were examined for mutational hotspots, and 26 tumor suppressor genes were examined for the presence of all of the exons. The panel was used to detect copy number alterations (CNAs) in 49 genes and fusion drivers in 22 genes. The gene list is available at https://www.thermofisher.com/kr/ko/home/clinical/preclinical-companion-diagnostic-development/oncomine-oncology/oncomine-cancer-research-panel-workflow.html. A pool of DNA library (60 pmol/l) was used to prepare a template Ion Sphere Particle (Thermo Fisher Scientific). DNA sequencing was performed using the Ion 540 Kit-Chef (Thermo Fisher Scientific) and Ion S5 system (Thermo Fisher Scientific). Sequencing data of approximately 200 bp reads were generated after 500 flow runs.

Analysis of the sequencing data was performed using the Torrent Suite Software v5.2.2 (Thermo Fisher Scientific). This workflow was created by adding a custom hotspot Browser Extensible Data file to report mutations of interest and a custom CNA baseline (described below) using the manufacturer's default workflow, as described previously (24, 25, 35). The pipeline used included signaling processing, base calling, quality score assignment, adapter trimming, read mapping to the human genome assembly GRCh37, quality control of mapping, coverage analysis with downsampling, and variant calling. The identification of variants was performed using the Torrent Variant Caller plug-in and Ion Reporter Software v5.2 (Thermo Fisher Scientific). Coverage maps were generated using the Coverage Analysis plug-in (Thermo Fisher Scientific). Additionally, ANNOVAR (http://annovar.openbioinformatics.org/) was used for functional annotation of identified single nucleotide variants (SNVs) to investigate their genomic locations (36). To eliminate artifacts, sequence data were visually confirmed using the Integrative Genomics Viewer software (Broad Institute, Cambridge, MA, USA). This workflow was able to report SNVs and insertions/deletions (indels) in as low as 1% of the variant allele fraction. Based on the results of a feasibility study, the variant allele fraction threshold was established at 2%. Copy number analysis was performed using the copy number module within the aforementioned workflow of the Ion Reporter Software v5.2. Copy numbers of four or greater were considered concordant if the orthogonal assay also reported a copy number of four or greater for target genes. Gene fusions were detected using the fusion detection module within the Ion Reporter Software (Thermo Fisher Scientific) workflow. This pipeline only reported fusions that were annotated previously, as defined in a reference file preloaded into the workflow (24, 25, 35).

Results

Histological features of primary and recurrent glioblastomas. All examined tumor tissue samples displayed the typical histological features of high-grade astrocytoma (hypercellularity, severe nuclear pleomorphism with anaplastic and bizarre nuclei and occasional multinucleation, and increased mitotic activity with atypical mitotic figures), coagulative tumor cell necrosis, and microvascular proliferation (capillary wall thickening due to endothelial cell hyperplasia and hypertrophy or formation of multiple lumina). None of the cases showed foci of giant cell glioblastomas, gliosarcomas, or epithelioid glioblastomas. Representative photomicrographs showing the histological features of glioblastomas are shown in Figure 2. No significant morphological difference was observed between the primary (Figure 2A) and recurrent (Figure 2B) glioblastomas. Additionally, there was no significant difference in the histological features observed between the local and distal recurrence groups.

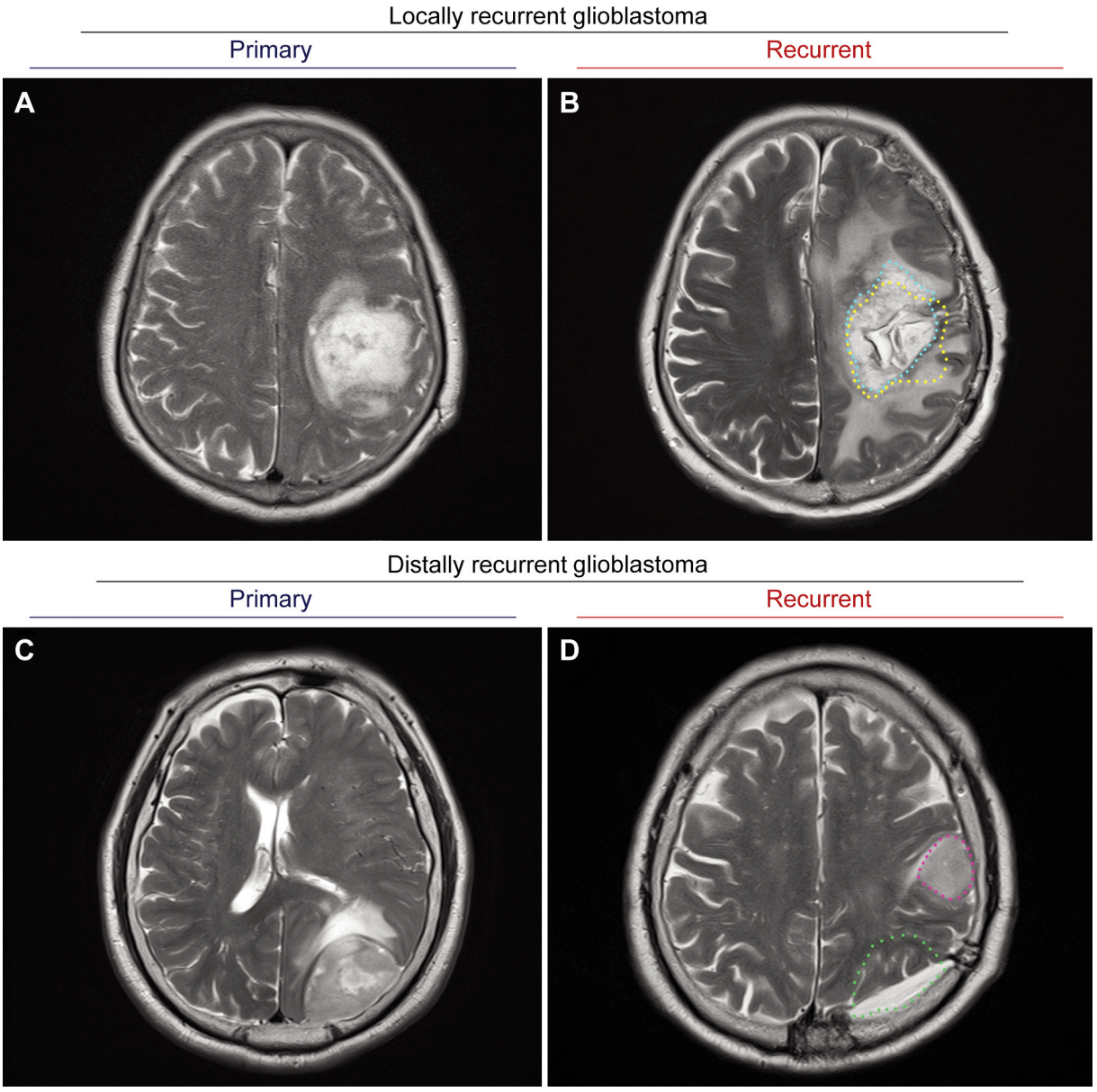

Figure 1. *Representative magnetic resonance images showing the locations of recurrent glioblastomas. (A-B) Locally recurrent glioblastoma. The location of primary tumor (outlined by yellow dots) is nearly identical to that of the recurrent tumor (blue dots). (C-D) Distally recurrent glioblastoma. The location of recurrent tumor (purple dots) is different from that of primary tumor (green dots).*

Targeted sequencing results. Sequence data were successfully obtained from all tissue samples. Figure 3 summarizes the targeted sequencing results. In 9 cases of primary glioblastoma, 19 SNVs/indels were identified in 7 genes, and they consisted of 14 missense mutations, 1 frameshift deletion mutation, and 4 nonsense mutations. The most commonly detected SNV/indel in the sequences of primary glioblastoma samples was tumor protein 53 (*TP53*) mutation (7/14); these mutations included p.Cys135Tyr (1/7), p.Arg158His (1/7), p.Tyr220Cys (1/7), p.Arg267Trp (1/7), p.Arg273Cys (1/7), p.Pro278Thr (1/7), and p.Asp281Tyr (1/7). The second most common detected SNV/indel in sequences of primary glioblastoma samples was the phosphatase and tensin homolog deleted in chromosome 10 (*PTEN*) mutation (5/14); these mutations included p.Tyr68Ter (1/5), p.Gln110Ter (1/5), p.Arg130Gly (1/5), p.Gly132Arg (1/5), and p.Lys254fs (1/5). Additionally, mutations in neurofibromin 1 (*NF1*; 1/14), platelet-derived growth factor receptor-alpha (*PDGFRA*), adenomatous polyposis coli (*APC*),

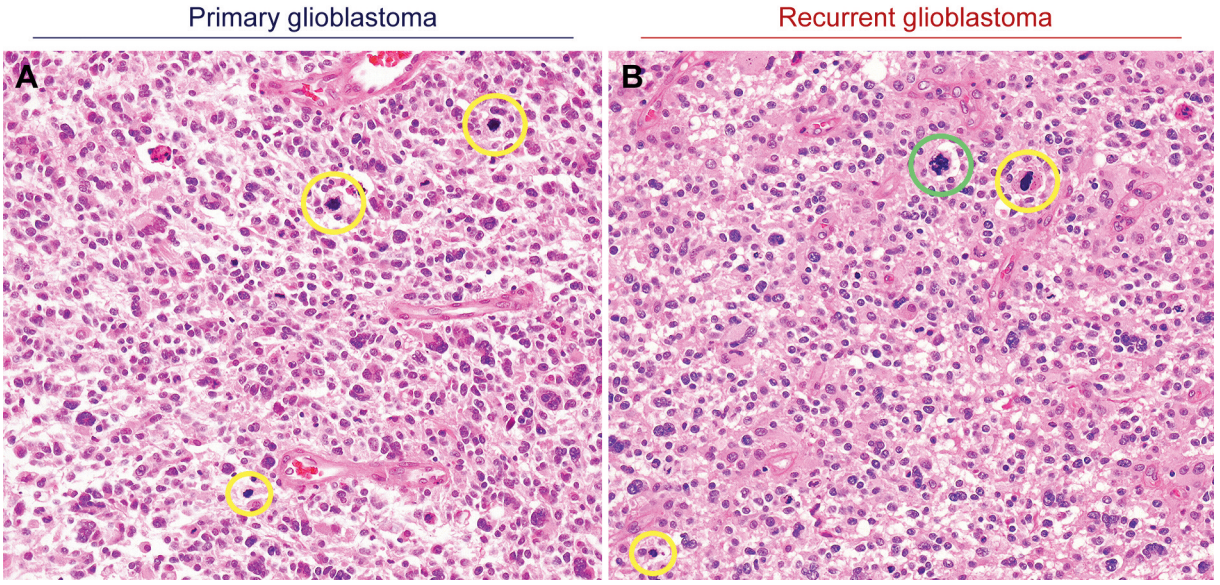

Figure 2. *Representative photomicrographs of glioblastoma showing histological concordance between primary and recurrent tumors. No significant histological difference is observed between primary and recurrent glioblastomas. Yellow and green circles indicate typical and atypical mitotic figures, respectively. Staining method, hematoxylin and eosin staining. Original magnification of A and B, 200×.*

and v-raf murine sarcoma viral oncogene homolog B1 (*BRAF*; 1/14), were detected in four different primary glioblastomas. Ten primary glioblastomas harbored CNAs, that were detected in four genomic regions and included seven gains and seven losses. The most common CNAs in primary glioblastomas were epidermal growth factor receptor (*EGFR*) amplification (6/14) and cyclin-dependent kinase inhibitor 2A (*CDKN2A*) deletion (6/14). Additionally, an *NF1* deletion (1/14) and *PDGFRA* amplification (1/14) were detected in two cases of primary glioblastoma.

Ten recurrent glioblastomas harbored SNVs/indels, which consisted of 14 missense mutations, 1 frameshift deletion mutation, and 1 nonsense mutation. Seven recurrent glioblastoma tissue samples most commonly harbored *TP53* mutations, all of which showed the same mutation type as their corresponding primary tumor tissue samples. The second most common SNVs/indels in the DNA of recurrent glioblastoma samples were *PTEN* (3/14) and *EGFR* mutations (3/14). Mutations in the F-box and WD repeat domain-containing 7 (*FBXW7*; p.Arg505His) and *EGFR* (p.Pro596Leu and p.Gly598Val) that were not identified in any of the primary glioblastoma cases were detected in three different recurrent glioblastoma samples. In eight cases of recurrent glioblastoma, CNAs were detected in four genomic regions and included six gains and six losses. The most common CNAs in recurrent glioblastoma were *EGFR* amplification (5/14) and *CDKN2A* deletion (5/14). *CDKN2A* deletion and *PDGFRA* amplification, both of which were not observed in any of the primary glioblastoma cases, were detected in one recurrent glioblastoma case. No gene fusion was detected, and no pathogenic mutation was identified in the normal brain tissue.

Differences in mutational profiles between the locations of tumor recurrence. We compared the targeted sequencing results between the local and distal recurrence groups (Figure 4). The location of SNVs/indels and type of CNAs were completely concordant in 6 of the 10 locally recurrent glioblastoma cases (cases 1-6; Table I). In contrast, four cases (cases 7-10; Table II) displayed mutations in the primary tumors, including nonsense *PTEN* mutation, *CDKN2A* deletion, and amplifications of *EGFR* and *PDGFRA*, all of which were not detected in the recurrent tumors. Two of the four cases showed a partial concordance in the missense *TP53* mutation (case 7) and *NF1* deletion (case 8), respectively.

None of the distally recurrent glioblastoma cases (cases 11-14) showed concordance in the mutational profiles when compared to primary glioblastoma cases. Acquired mutations were detected in all cases of distally recurrent glioblastoma and included missense *FBXW7* mutation (case 12), missense *EGFR* mutations (cases 13 and 14), *CDKN2A* deletion (case 11), and *PDGFRA* amplification (case 11). In three cases, mutations observed in the primary tumors, including mutations or CNAs in *CDKN2A*, *EGFR*, *PDGFRA*, *PTEN*, and *APC*, were not detected in the recurrent tumors.

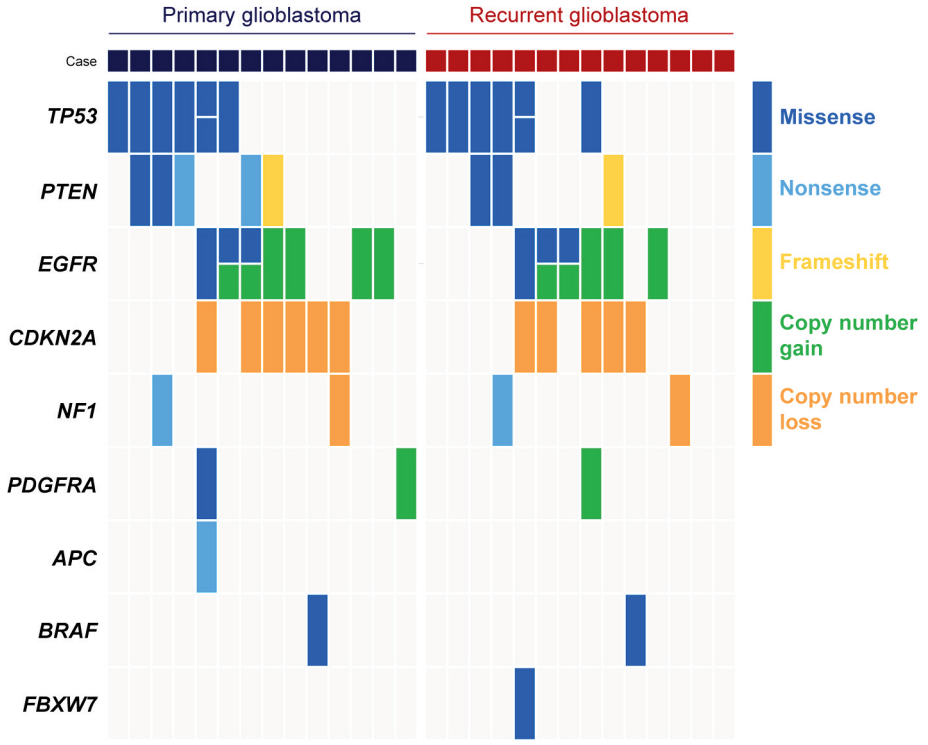

Figure 3. *Targeted sequencing results of the primary (n=14) and corresponding recurrent (n=14) glioblastomas.*

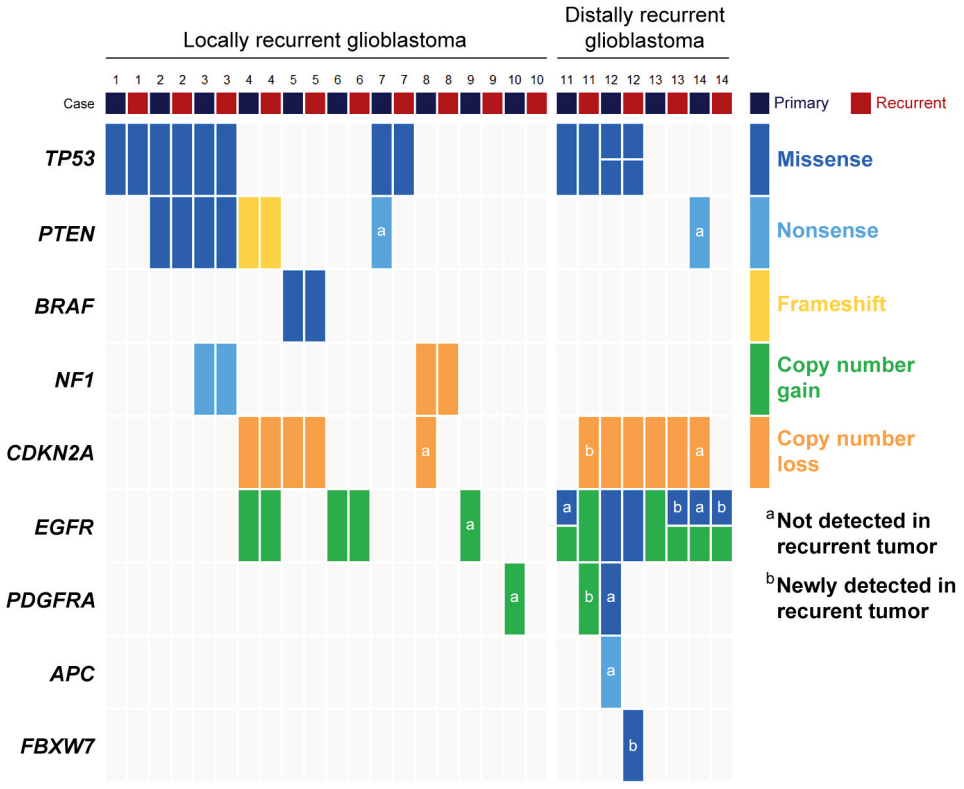

Figure 4. *Comparison of targeted sequencing results between locally (n=10) and distally (n=4) recurrent glioblastomas.*

Table I. *Targeted sequencing results of 10 cases of locally recurrent glioblastoma.*

Case no.	Sample type	Gene	Mutation type	Nucleotide change	Amino acid change	Allele frequency	Copy number
1	Primary	*TP53*	Missense	c.799C>T	p.Arg267Trp	25.0%	
	Recurrent	*TP53*	Missense	c.799C>T	p.Arg267Trp	39.3%	
2	Primary	*TP53*	Missense	c.404G>A	p.Cys135Tyr	72.8%	
		PTEN	Missense	c.388C>G	p.Arg130Gly	32.0%	
	Recurrent	*TP53*	Missense	c.404G>A	p.Cys135Tyr	13.0%	
		PTEN	Missense	c.388C>G	p.Arg130Gly	2.8%	
3	Primary	*TP53*	Missense	c.841G>T	p.Asp281Tyr	61.3%	
		PTEN	Missense	c.394G>C	p.Gly132Arg	56.3%	
		NF1	Nonsense	c.5959C>T	p.Gln1987Ter	60.4%	
	Recurrent	*TP53*	Missense	c.841G>T	p.Asp281Tyr	42.4%	
		PTEN	Missense	c.394G>C	p.Gly132Arg	39.7%	
		NF1	Nonsense	c.5959C>T	p.Gln1987Ter	39.6%	
4	Primary	*PTEN*	Frameshift	c.761_765delAAGTA	p.Lys254fs	65.1%	
		EGFR	CNA				98.26 (amplification)
		CDKN2A	CNA				0 (deletion)
	Recurrent	*PTEN*	Frameshift	c.761_765delAAGTA	p.Lys254fs	35.7%	
		EGFR	CNA				71.59 (amplification)
		CDKN2A	CNA				0.8 (deletion)
5	Primary	BRAF	Missense	c.1799T>A	p.Val600Glu	41.0%	
		CDKN2A	CNA				0.34 (deletion)
	Recurrent	BRAF	Missense	c.1799T>A	p.Val600Glu	50.6%	
		CDKN2A	CNA				0.21 (deletion)
6	Primary	*EGFR*	CNA				18.91 (amplification)
	Recurrent	*EGFR*	CNA				43.35 (amplification)
7	Primary	*TP53*	Missense	c.817C>T	p.Arg273Cys	56.1%	
		PTEN	Nonsense	c.328C>T	p.Gln110Ter	36.7%	
	Recurrent	*TP53*	Missense	c.817C>T	p.Arg273Cys	40.5%	
8	Primary	*NF1*	CNA				0 (deletion)
		CDKN2A	CNA				0 (deletion)
	Recurrent	*NF1*	CNA				0 (deletion)
9	Primary	*EGFR*	CNA				24.81 (amplification)
	Recurrent	Not detected	Not applicable				
10	Primary	*PDGFRA*	CNA				27.25 (amplification)
	Recurrent	Not detected	Not applicable				

BRAF: v-raf murine sarcoma viral oncogene homolog B1, *CDKN2A*: cyclin-dependent kinase inhibitor 2A, CNA: copy number alteration, *EGFR*: epidermal growth factor receptor, *NF1*: neurofibromin 1, *PDGFRA*: platelet-derived growth factor receptor A, *PTEN*: phosphatase and tensin homolog deleted on chromosome 10, *TP53*: tumor protein 53.

Discussion

Previous studies have documented the genetic alterations in primary glioblastomas and their corresponding recurrent glioblastomas and have suggested two different patterns of evolution (18, 21, 37-39). The first pattern is a linear evolution demonstrating a high concordance between the mutation patterns of primary and recurrent tumors, suggestive of late evolutionary divergence. The second pattern is a branching evolution showing relatively distinct mutation patterns between the primary and recurrent tumors, suggestive of an early separation in the lineage. In a previous study reporting the genomic profile of glioblastomas (21), distally recurrent glioblastomas shared only a fraction of the primary tumor mutations, indicative of branching evolution. In contrast, locally recurrent glioblastomas shared the majority of mutations harbored by the primary tumors, indicating a linear evolution. These data suggest that the location of recurrent tumors may help predict the genomic evolutionary patterns in glioblastomas. In this study, the recurrent glioblastomas showed two different evolutionary patterns according to their location of recurrence. Eight of the ten local recurrent glioblastomas showed a linear evolutionary pattern, demonstrating a complete or partial concordance in the mutational profiles between primary and recurrent tumors, without any acquired pathogenic mutation. Four distal recurrent glioblastomas showed branching evolutionary patterns with changes in the mutational profiles between the primary and recurrent tumors, including losing and

Table II. *Targeted sequencing results of four cases of distally recurrent glioblastoma.*

Case no.	Sample type	Gene	Mutation type	Nucleotide change	Amino acid change	Allele frequency	Copy number
11	Primary	*TP53*	Missense	c.659A>G	p.Tyr220Cys	67.0%	
		EGFR	Missense	c.664C>T	p.Arg222Cys	25.0%	
		EGFR	CNA				27.97 (amplification)
	Recurrent	*TP53*	Missense	c.659A>G	p.Tyr220Cys	91.0%	
		EGFR	CNA				107.83 (amplification)
		CDKN2A	CNA				0 (deletion)
		PDGFRA	CNA				6.09 (amplification)
12	Primary	*TP53*	Missense	c.832C>A	p.Pro278Thr	47.2%	
		TP53	Missense	c.473G>A	p.Arg158His	42.9%	
		EGFR	Missense	c.2029C>T	p.Arg677Cys	51.3%	
		PDGFRA	Missense	c.2524G>T	p.Asp842Tyr	36.9%	
		APC	Nonsense	c.8446C>T	p.Arg2816Ter	38.6%	
		CDKN2A	CNA				0 (deletion)
	Recurrent	*TP53*	Missense	c.832C>A	p.Pro278Thr	48.1%	
		TP53	Missense	c.473G>A	p.Arg158His	45.1%	
		EGFR	Missense	c.2029C>T	p.Arg677Cys	51.0%	
		FBXW7	Missense	c.1514G>A	p.Arg505His	43.0%	
		CDKN2A	CNA				0.07 (deletion)
13	Primary	*EGFR*	CNA				128.13 (amplification)
		CDKN2A	CNA				0.04 (deletion)
	Recurrent	*EGFR*	Missense	c.1787C>T	p.Pro596Leu	92.1%	
		EGFR	CNA				36.53 (amplification)
		CDKN2A	CNA				0.98 (deletion)
14	Primary	*PTEN*	Nonsense	c.204C>A	p.Tyr68Ter	46.3%	
		EGFR	Missense	c.2369C>T	p.Thr790Met	7.3%	
		CDKN2A	CNA				0.71 (deletion)
		EGFR	CNA				68.26 (amplification)
	Recurrent	*EGFR*	Missense	c.1793G>T	p.Gly598Val	7.5%	
		EGFR	CNA				28.56 (amplification)

APC: Adenomatous polyposis coli, *CDKN2A*: cyclin-dependent kinase inhibitor 2A, CNA: copy number alteration, *EGFR*: epidermal growth factor receptor, *FBXW7*: F-box and WD repeat domain-containing 7, *PDGFRA*: platelet-derived growth factor receptor A, *PTEN*: phosphatase and tensin homolog, *TP53*: tumor protein 53.

acquiring mutations in single or multiple genes. Our observations support the previous notions that locally and distally recurrent glioblastomas exhibit different evolutionary patterns, in particular, linear and branching evolutions, respectively, and that the location of tumor recurrence helps predict the evolutionary pattern in glioblastoma.

Concurrent chemoradiation therapy exerts a powerful selective pressure, and chemo- and radio-resistant cell clones contribute to the development of glioblastoma recurrence (7, 40-44). Common ancestral cells are likely to pre-exist in the untreated primary glioblastoma and are positively selected by chemoradiation therapy (18, 41, 45-47). We observed that single or multiple pathogenic mutations that were not observed in any of the primary glioblastomas, were detected in all of the corresponding distally recurrent glioblastomas, raising the possibility that the residual tumor cells that survive initial treatment may acquire new mutations involving chemo- and radio-resistance, invasion, migration, and adaptation to the new microenvironment (21, 48). Further investigations are necessary to clarify the relationship between the development of distal recurrence of glioblastoma and genetic abnormalities in *CDKN2A*, *PDGFRA*, *EGFR*, or *FBXW7*.

Interestingly, missense mutations of the *EGFR* gene are only detected in distally recurrent glioblastoma cases, raising the possibility that EGFR mutation may be related to cellular migration and invasion. *EGFR* mutations have been reported to be involved in the stimulation of tumor angiogenesis, which is essential to metastasis (49, 50). In particular, *EGFR* mutations have shown to significantly increase cellular migration and invasion *via* promoting angiogenesis in lung carcinoma cells (50). Similarly, missense mutations affecting the extracellular domain of EGFR protein, which are detected in approximately 10% of glioblastomas, promote tumorigenesis, cellular proliferation, and invasiveness (9, 51, 52). Further investigations are needed to clarify the effects of missense *EGFR* mutations on angiogenesis and cellular migration in glioblastoma.

A limitation of this study involved the lack of analysis of the mutational status of the transcriptional regulator *ATRX* and telomerase reverse transcriptase promoter, which are frequently detected in glioblastomas, since they were not included in the targeted sequencing panel that we used. We excluded the possibility of *de novo* glioblastoma in the distally recurrent glioblastoma cases based on the histological similarities and a high concordance in mutational profiles between primary and recurrent tumors. Previous studies have reported that recurrent glioblastomas are derived from a population of cells that are resistant to chemotherapy and radiation therapy (9, 53), and glioblastomas contain a subpopulation of highly tumorigenetic glioblastoma stem cells, from which recurrent tumors are thought to derive (8, 47, 54).

In summary, we investigated the genetic characteristics of glioblastomas using primary and corresponding recurrent tumor tissue samples. We performed targeted sequencing analysis and compared the sequencing data between locally and distally recurrent glioblastoma cases to examine whether there is a difference in the mutational profiles between the two groups. We found that *TP53* is the most commonly mutated gene in both the primary and recurrent glioblastomas, followed by *PTEN* and *EGFR*. Additionally, we observed that the most common CNAs in primary and recurrent glioblastomas were *EGFR* amplification and *CDKN2A* deletion. More importantly, the mutational profiles were completely or partially concordant in 8 of the 10 locally recurrent glioblastoma cases. In contrast, none of the distally recurrent glioblastoma cases showed concordance in the mutational profiles between primary and recurrent tumors. Instead, acquired mutations were detected in all cases of distally recurrent glioblastoma. Our findings support previous notions that locally and distally recurrent glioblastomas exhibit different evolutionary patterns, in particular, linear and branching evolutions, respectively, and that the location of tumor recurrence may help predict the evolutionary patterns in glioblastoma.

Conflicts of Interest

The Authors have no conflicts of interest to declare.

Author' Contributions

All the Authors made substantial contributions to the conceptualization and design of the study. This included the acquisition, analysis, curation, and interpretation of the data; drafting, critical revision, and editing of the manuscript for important intellectual content; and the approval of the final version to be published.

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