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**Corresponding Author:** Jeong Ho Lee

**Authors:** Joo Ho Lee<sup>1</sup>, Jeong Ho Lee<sup>2,\*</sup>

**Institution:** <sup>1</sup>Department of Radiation Oncology, Seoul National University Hospital, Seoul National University College of Medicine, Seoul, Korea,

<sup>2</sup>Graduate School of Medical Science and Engineering, KAIST, Daejeon, Korea,

# **The origin-of-cell harboring cancer-driving mutations in human glioblastoma**

Joo Ho Lee<sup>1</sup>, Jeong Ho Lee<sup>2</sup> \*

<sup>1</sup>Department of Radiation Oncology, Seoul National University Hospital, Seoul National University College of Medicine, Seoul, Korea

<sup>2</sup>Graduate School of Medical Science and Engineering, KAIST, Daejeon, Korea

Keyword: Glioblastoma, subventricular zone, neural stem cell, somatic mutation

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Glioblastoma (GBM) is the most common and aggressive form of human adult brain malignancy. The identification of the cell of origin harboring cancer-driver mutations is the fundamental issue for understanding the nature of GBM and developing the effective therapeutic target. It has been a long-term hypothesis that neural stem cells in the subventricular zone (SVZ) might be the origin-of-cells in human glioblastoma since they are known to have life-long proliferative activity and acquire somatic mutations. However, the cell of origin for GBM remains controversial due to lack of direct evidence thereof in human GBM. Our recent study using various sequencing techniques in triple matched samples such as tumor-free SVZ, tumor, and normal tissues from human patients identified the clonal relationship of driver mutations between GBM and tumor-free SVZ harboring neural stem cells (NSCs). Tumor-free SVZ tissue away from the tumor contained low-level GBM driver mutations (as low as 1% allelic frequency) that were found in the dominant clones in its matching tumors. Moreover, via single-cell sequencing and microdissection, it was discovered that astrocyte-like NSCs accumulating driver mutations evolved into GBM with clonal expansion. Furthermore, mutagenesis of cancer-driving genes of NSCs in mice leads to migration of mutant cells from SVZ to distant brain and development of high-grade glioma through the aberrant growth of oligodendrocyte precursor lineage. Altogether,

the present study provides the first direct evidence that NSCs in human SVZ is the cell of origin that develops the driver mutations of GBM.

The current standard treatment for GBM is surgical removal with maximal extent followed by radio- and chemotherapy. However, with the standard treatment, the cure rate of GBM remains poor with a median survival of 15 months. Although extensive and comprehensive genetic analysis of GBM has been undertaken to identify the druggable target, the advances in the targeted therapy of GBM and their clinical outcomes have been very limited, compared to other cancers. Especially, to overcome the treatment resistance and high recurrence rate of GBM, it is necessary to identify the cell of origin as a clonal ancestor of GBM and understand the molecular pathogenesis of GBM evolution.

According to the World Health Organization classification of tumors, GBM can be subdivided into two types, based on the mutation in codon 132 of isocitrate dehydrogenase 1 (IDH1). The majority of GBMs (>90%) are IDH1-wildtype that arise *de novo* without any previous low-grade tumor; whereas GBM, IDH1-mutant is rare and secondary progressed from the precursor low-grade glioma. The cell of origin in GBM, IDH-wildtype arising without any precursor disease has been a controversial issue, unlike GBM, IDH-mutant. Considering the necessity of multiple somatic mutations for gliomagenesis, the self-renewal and proliferative property of neural stem cells (NSCs) might ensure appropriate conditions for endogenous accumulation of somatic mutations. Moreover, it is reported that nearly all the driver mutations in cancers of the brain are attributable to DNA replicative errors, which are correlated with the total number of divisions of stem cells. Thus, somatic mutations caused by endogenous processes such as DNA replicative errors can be accumulated mainly in the NSCs of the human brain. Interestingly, NSCs in the adult human brain are limited in the SVZ and hippocampus. Thus, it is hypothesized that SVZ can be the main source for *de novo* somatic mutations other than other regions of the brain over a lifetime. Also, SVZ-originated progenitor cells have the migratory potential, thereby implying that driver mutations carrying stem cells in the SVZ can be away from the tumor mass of primary GBM. Thus, over the decade, the hypothesis about the cell of origin in GBM states that NSCs in the SVZ may be the origin of cell harboring driver

mutations in GBM in IDH-wildtype patients. However, the mouse model studies and neuroimaging studies have provided the inconsistent reports without any direct molecular evidence in human and mouse. Moreover, it is not tested whether the ancestor clone for GBM distant from SVZ is also originated and migrated from SVZ. Based on the hypothesis, we deduce that the clonal ancestors harboring cancer-driver mutations might occur with low-frequency in the SVZ and migrate and expand to the dominant clones with high-frequency in the tumor since no tumor mass may be radiographically or histologically observed in the SVZ.

To systematically analyze low-level mutations in the SVZ away from the tumor mass, we first performed deep whole exome or targeted sequencing of triple-matched samples consisting of i) normal-appearing SVZ away from the tumor mass, ii) tumor and iii) unaffected cortical tissue (or blood) from GBM and other brain tumor patients after the supra-total resection accessible to the radiologically and histologically tumor-free SVZ distant from the primary tumor (Fig. 1). Deep sequencing revealed that 42.3% of the patients with GBM, IDH-wildtype (11 out of 26) contained somatic mutations with low-level (down to ~1%) in the tumor-free SVZ, which was also shared with the matched tumor as high allelic frequency. Interestingly, low-level driver mutations in TERT promoter or cancer-driving genes were discovered as the shared mutations between two tissues in the patients with GBM, IDH-wildtype, whereas it was not the case in the patients with other types of brain tumor. In addition to the shared driving mutations in the matched tumor, most of the somatic mutations (75.3%) were accumulated as tumor-private mutations during the clonal evolution of tumor. We also discovered the clue of clonal expansion in copy number variation (CNV) of EGFR utilizing the real-time quantitative PCR of tumor-free SVZ and tumor tissues.

Subsequently, we aimed to decide the temporospatial direction of clonal expansion between tumor-free SVZs and tumor. The distribution of mutations in the single clone can indicate the temporal order between the two tissues. For example, if the tumor-private mutations were acquired additionally after the occurrence of shared mutations in SVZ, the tumor-private mutations will exist in the same clone with tumor-free SVZ, and it can indicate that the clones harboring shared driver mutations in tumor-free SVZ are the clonal ancestor of the tumor. In single cell cloning and sequencing, we observed that none of the single clones possessed only shared mutations without tumor-private mutations. Furthermore, we clarified the histologically distinct three

layers of tumor-free SVZs in GBM, IDH-wildtype patients through immunohistochemistry staining as the ependymal layer, hypocellular layer, and astrocytic ribbon. After dissecting the three layers individually via laser microdissection, we found that the driver mutations recognized through deep WES were enriched only in the astrocytic ribbon. The astrocytic ribbon layer has been reported to be the histological source for astrocyte-like stem cells in the previous literature. Taken together, these results suggested that the astrocyte-like stem cells from the astrocytic ribbon of the SVZ harbor driver mutations and clonally evolve into tumor away from the SVZ in GBM, IDH-wildtype patients.

Subsequently, we sought to find the etiology and process of mutation accumulation of stem cells in the SVZ, necessary for the acquisition of driver mutations. We analyzed the genetic signature of the mutational process, including intrinsic DNA replicative errors, exogenous or endogenous mutagens exposure, etc. In the analysis of the genetic signature of the tumor-free SVZ and the tumor, signature 1 and 5 were found as the two dominant mutation signatures suggesting a clock-like process of mutation accumulation. Meanwhile, the TERT promoter mutations were found in all the patients with IDH-wildtype GBM that had driver mutations in tumor-free SVZ tissue. Mutation-driven activation of TERT prevents the shortening of telomere and enables the extension of proliferation without senescence. To understand the frequency of occurrence of TERT promoter mutations in the NSCs niche of 'non-cancer' aged brain, we performed deep sequencing of TERT promoter mutation sites in the non-cancer aged hippocampus (another NSC niche). The sequencing of non-cancer aged NSC niche revealed that 3 of 49 aged brains harbored TERT promoter mutations leading to TERT over-activation. Overall, the endogenous mutagenesis occurred during NSCs' time-dependent activity and replication might cause the mutation accumulation in the SVZ.

Furthermore, to test the cancer-driving potential of mutations arising in the NSCs of SVZ, we introduced the cancer-driving mutations of *Trp53*, *Pten* and *Egfr* in the mouse NSCs through genome editing. These mutations were recurrent driver mutations found in the tumor-free SVZ tissues collected from the patients with GBM. We found the mutation-harboring cells to be initially located in NSCs of SVZ and subsequently migrated to the distant region of the brain through the aberrant growth of oligodendrocyte progenitor lineage. The mutant cells migrating to distant regions

developed high-grade glioma in the distant cortex whereas the mutation-arising SVZ still retained normal cytoarchitecture, similar to the results from the human patients. Meanwhile, when injecting the mutations in the cortex, we could observe neither spreading of cells to the SVZ nor the significant proliferation of cells, compared to the mouse model carrying driver mutations in SVZ. These results strongly suggested that NSCs harboring low-level driver mutations migrate from the SVZ and lead to the development of high-grade malignant gliomas in distant brain regions.

The present study identified the astrocyte-like NSCs in SVZ as the cell of origin harboring driver mutations in GBM with direct genetic and molecular evidence from human patients and genetically engineered mouse model. Compared to previous studies dealing with only SVZ-contacted GBM, the present study states that the GBM even distant to SVZ can be originated from the NSCs of SVZ through the migrating and proliferative potential of NSCs. Furthermore, this study provides new target regions and specifies the cell types that can be the origin of GBM. Future studies should focus on the new target regions and the cell types to prevent the development and recurrence of GBM. Through the direct analysis of mutations in NSCs of human, the etiology and process of mutation accumulation highlight the potential role of somatic mutations in the brain related to various neurological disorders. However, in future studies, the detailed mechanisms driving NSCs to tumor should be investigated with a precise mouse model and human tissue analysis. Nevertheless, our findings will serve as the foundation for further studies on NSCs and brain disease.

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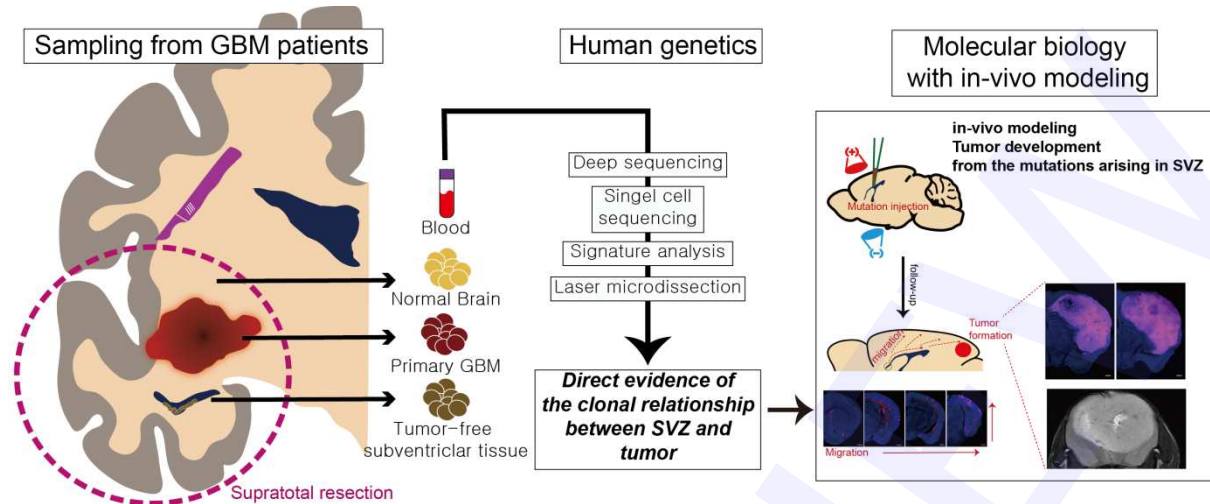


Fig. 1. Schematic representation of the experimental design. After collecting triple matched tissues (tumor, tumor-free SVZ, and normal paired tissue), we performed deep sequencing to define the clonal relationship between tumor and tumor-free SVZ. Subsequently, we identified the clonal evolution from tumor-free SVZ into the tumor via single-cell sequencing, signature analysis, and laser microdissection. Finally, we validated the migrating and cancer-driving potential of mutation arising in the NSCs of SVZ through genetically engineered mouse model.