



## Influence of the Amount of Fresh Specimen on the Isolation of Tumor Mesenchymal Stem-Like Cells from High-Grade Glioma

Soon Haeng Kong<sup>1\*</sup>, Jihwan Yoo<sup>1,2\*</sup>, Dongkyu Lee<sup>1,2</sup>, Sohyung Moon<sup>2</sup>, Kyoung Su Sung<sup>3</sup>, So Hee Park<sup>4</sup>, Jin-Kyoung Shim<sup>2</sup>, Ran Joo Choi<sup>2</sup>, Seon Jin Yoon<sup>5,6</sup>, Ju Hyung Moon<sup>2</sup>, Eui-Hyun Kim<sup>2</sup>, Su Jae Lee<sup>7</sup>, Jong Hee Chang<sup>2</sup>, and Seok-Gu Kang<sup>1,2</sup>

<sup>1</sup>Yonsei University College of Medicine, Seoul;

<sup>2</sup>Department of Neurosurgery, Brain Tumor Center, Severance Hospital, Yonsei University College of Medicine, Seoul;

<sup>3</sup>Department of Neurosurgery, Dong-A University College of Medicine, Busan;

<sup>4</sup>Department of Biostatistics, Graduate School of Public Health, Yonsei University, Seoul;

<sup>5</sup>Department of Biochemistry and Molecular Biology, Institute of Genetic Science, Integrated Genomic Research Center for Metabolic Regulation, Yonsei University College of Medicine, Seoul;

<sup>6</sup>Brain Korea 21 PLUS Project for Medical Science, Yonsei University College of Medicine, Seoul;

<sup>7</sup>Department of Life Science, Research Institute for Natural Sciences, Hanyang University, Seoul, Korea.

**Purpose:** A critical indicator of the overall survival of patients with high-grade glioma is the successful isolation of tumor mesenchymal stem-like cells (tMSLCs), which play important roles in glioma progression. However, attempts to isolate tMSLCs from surgical specimens have not always been successful, and the reasons for this remain unclear. Considering that the amount of surgical high-grade glioma specimens varies, we hypothesized that larger surgical specimens would be better for tMSLC isolation.

**Materials and Methods:** We assessed 51 fresh, high-grade glioma specimens and divided them into two groups according to the success or failure of tMSLC isolation. The success of tMSLC isolation was confirmed by plastic adherence, presenting antigens, tri-lineage differentiation, and non-tumorigenicity. Differences in characteristics between the two groups were tested using independent two sample t-tests, chi-square tests, or Kaplan-Meier survival analysis.

**Results:** The mean specimen weights of the groups differed from each other (tMSLC-negative group: 469.9±341.9 mg, tMSLC positive group: 546.7±618.9 mg), but the difference was not statistically significant. The optimal cut-off value of specimen weight was 180 mg, and the area under the curve value was 0.599.

**Conclusion:** Our results suggested a minimum criterion for specimen collection, and found that the specimen amount was not deeply related to tMSLC detection. Collectively, our findings imply that the ability to isolate tMSLCs is determined by factors other than the specimen amount.

**Key Words:** Glioblastoma, high-grade glioma, isolation rate, specimen weight, tumor mesenchymal stem-like cell

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**Corresponding author:** Seok-Gu Kang, MD, PhD, Department of Neurosurgery, Brain Tumor Center, Severance Hospital, Yonsei University College of Medicine, 50-1 Yonsei-ro, Seodaemun-gu, Seoul 03722, Korea.

Tel: 82-2-2228-0882, Fax: 82-2-393-9979, E-mail: seokgu9@gmail.com

\*Soon Haeng Kong and Jihwan Yoo contributed equally to this work.

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## INTRODUCTION

Mesenchymal stem cells (MSCs) have been successfully isolated from healthy brains,<sup>1</sup> and were first isolated from human glioma specimens by Lang, et al.<sup>2</sup> and Hossain, et al.<sup>3</sup> Tumor mesenchymal stem-like cells (tMSLCs) have also been isolated from some brain tumors.<sup>4-7</sup> These studies suggested that tMSLCs enhance glioma progression by influencing the stroma of the glioma microenvironment.<sup>3,8-11</sup> Moreover, a recent study found that the success of tMSLC isolation is a negative prognostic factor of the overall survival of patients with grade

IV gliomas, as classified based on the World Health Organization (WHO) criteria.<sup>12</sup> Notably, however, a previous study by our laboratory found that when tMSLCs were cultured in vitro from high-grade glioma (WHO grade III, IV) specimens under appropriate conditions, not all tMSLC isolations were successful.<sup>5</sup> Therefore, understanding the factors that determine the success or failure of tMSLC isolation is important for ensuring proper sampling.

Previously, we performed an analysis of the success or failure of tumor sphere isolation according to specimen weight.<sup>13</sup> We hypothesized that heavier specimens would yield higher tumor sphere isolation success rates; however, our data did not support this hypothesis, as no relationship was identified between the specimen amount and the tumor sphere isolation rate. Moreover, whether the specimen amount is associated with the rate of tMSLC isolation remains unclear. Since the amount of surgical specimens varies, it is possible that the tMSLC isolation rate is higher for heavier surgical specimens. Although

several prior studies reported the success rate of isolating MSCs and tMSLCs in high-grade gliomas and other tumors,<sup>1,3-7,14-18</sup> the optimal specimen weight for the successful isolation of tMSLCs has not yet been established.

Therefore, the present study aimed to determine the optimal weight of surgical specimens for maximizing the isolation rate of tMSLCs. Specifically, we tested the hypothesis that heavier specimens would have a higher success rate for tMSLC isolation.

## MATERIALS AND METHODS

### Patient information

Fifty-one patients with high-grade glioma (WHO grade III, IV gliomas) who underwent resection surgery at our institution (Severance Hospital, Yonsei University College of Medicine) from October 2014 to December 2015 were enrolled in this study (Table 1). Patients were included if they had histological-

**Table 1.** Demographic and Clinical Characteristics of Patients with High-Grade Glioma

Characteristics	tMSLC-negative (n=23)	tMSLC-positive (n=28)	p value
Sex			0.793
Male	14 (60.9)	16 (57.1)	
Female	9 (39.1)	12 (42.9)	
Age (yr)	50.9±17.3	58.5±12.2	0.083
Pathologic diagnosis			0.327
Anaplastic astrocytoma, IDH-mutant	1 (4.3)	0 (0.0)	
Anaplastic astrocytoma, IDH-wildtype	5 (21.7)	3 (10.7)	
Anaplastic oligodendroglioma, IDH-mutant, and 1p19q-codeleted	2 (8.7)	1 (3.6)	
Glioblastoma, IDH-wildtype	15 (65.2)	24 (85.7)	
Origin			0.132
Primary	18 (78.3)	26 (92.9)	
Recurrent	5 (21.7)	2 (7.1)	
Molecular markers			
IDH-1			0.211
Negative	20 (87.0)	27 (96.4)	
Positive	3 (13.0)	1 (3.6)	
EGFR			0.254
Mutant by FISH	12 (52.2)	19 (67.9)	
Wildtype	11 (47.8)	9 (32.1)	
p53			0.785
Mutant by immunohistochemistry	18 (78.3)	21 (75.0)	
Wildtype	5 (21.7)	7 (25.0)	
MGMT			0.254
Methylation	11 (47.8)	9 (32.1)	
Unmethylation	12 (52.2)	19 (67.9)	
1p19q codeletion			>0.999
No	18 (78.3)	23 (82.1)	
Yes	5 (21.7)	5 (17.9)	
Ki-67 (%)	20.9±12.4	22.2±19.3	0.765

tMSLC, tumor mesenchymal stem-like cell; IDH, isocitrate dehydrogenase; EGFR, epidermal growth factor receptor; FISH, fluorescence in situ hybridization; MGMT, O-6-methylguanine-DNA methyltransferase.

Data are presented as n (%) or mean±standard deviation.

ly confirmed WHO grade III, IV gliomas according to the 2016 WHO criteria.<sup>19-21</sup> Patients' demographic characteristics, survival time, the extent of resection, specimen weight, and results of tMSLC isolation were investigated and analyzed statistically. Molecular markers in the surgical specimens were examined by neuropathologists as follows: isocitrate dehydrogenase-1 (IDH-1) mutation was assessed by or immunohistochemistry using the R132H mouse monoclonal antibody (clone H09 L, Dianova; 1:80 dilution). O-6-methylguanine-DNA methyltransferase (MGMT) promoter methylation was evaluated by polymerase chain reaction. The loss of heterozygosity at chromosomes 1p and 19q and the epidermal growth factor receptor (EGFR) mutation status were assessed by fluorescence in situ hybridization, and the mutational status of the tumor-suppressor protein p53 was characterized by immunohistochemistry.<sup>22,23</sup> All patients provided written informed consent, and permission for specimen sampling and evaluation was obtained from the Institutional Review Boards of our institution (4-2012-0212, 4-2014-0649), as specified in the Declaration of Helsinki.

### Surgical specimen sampling

Fifty-one tumor specimens from 51 patients were freshly obtained from 5-aminolevulinic acid fluorescent-guided surgery. After obtaining the tumor tissue, half or a third of the tissue was removed and sampled for tMSLC culture. The areas that were stained brightly by fluorescence were excised with the help of a microscope so that high fluorescence uptake specimens could be included. Every fresh specimen was placed into a sterile centrifuge tube (SPL Life Sciences Co., Pocheon, Korea) on ice and weighed on the same electronic precision balance (Sartorius TE4101-L; Sartorius Weighing Technology GmbH, Göttingen, Germany) within 1 h.<sup>24-31</sup>

### Single-cell isolation and culture of tMSLCs

Fresh specimen samples were minced with a scalpel in minimal essential medium- $\alpha$  (MEM $\alpha$ ; Mediatech, Herndon, VA, USA) and passed through a series of 100- $\mu$ m nylon mesh cell strainers (BD Falcon, Franklin Lakes, NJ, USA). The resulting cell suspensions were combined and washed twice in serum-free MEM $\alpha$ , and then cultured in complete MSC medium consisting of MEM $\alpha$ , 10% fetal bovine serum (Gibco, Invitrogen, Carlsbad, CA, USA), 2-mM L-glutamine (Mediatech), and 1X antibiotic-antimycotic solution (Invitrogen, Carlsbad, CA, USA). After 24 h, nonadherent cells were removed by washing twice with phosphate-buffered saline (Mediatech) and cultured until they reached confluence. Thereafter, cells were trypsinized (0.25% trypsin with 0.1% ethylenediaminetetraacetic acid) and sub-cultured at a density of 5000 cells/cm<sup>2</sup>. The obtained isolated cells were assessed for several mesenchymal features, including adhesion to plastic, tri-lineage differentiation, and the presence of typical MSC surface markers (positive for CD105, CD90, and CD72; negative for CD45, CD31, and NG2).

### Statistical analysis

The patients' demographic characteristics and surgical specimen weights were statistically evaluated using independent two sample t-tests for continuous variables and chi-squared tests for categorical variables. Area under the curve (AUC) values and the Youden index, in conjunction with receiver-operating characteristic analyses, were used to determine the optimal cut-off weight of surgical specimens for isolating tMSLCs that maximized sensitivity and specificity. Survival curves for the two groups were calculated using the Kaplan-Meier method. All statistical analyses were performed using SPSS version 21.0 (IBM Corp., Armonk, NY, USA), and statistical significance was set at  $p < 0.05$ . Values are presented as the means  $\pm$  standard deviation or count (%).

## RESULTS

### Characteristics of the patients and surgical specimens

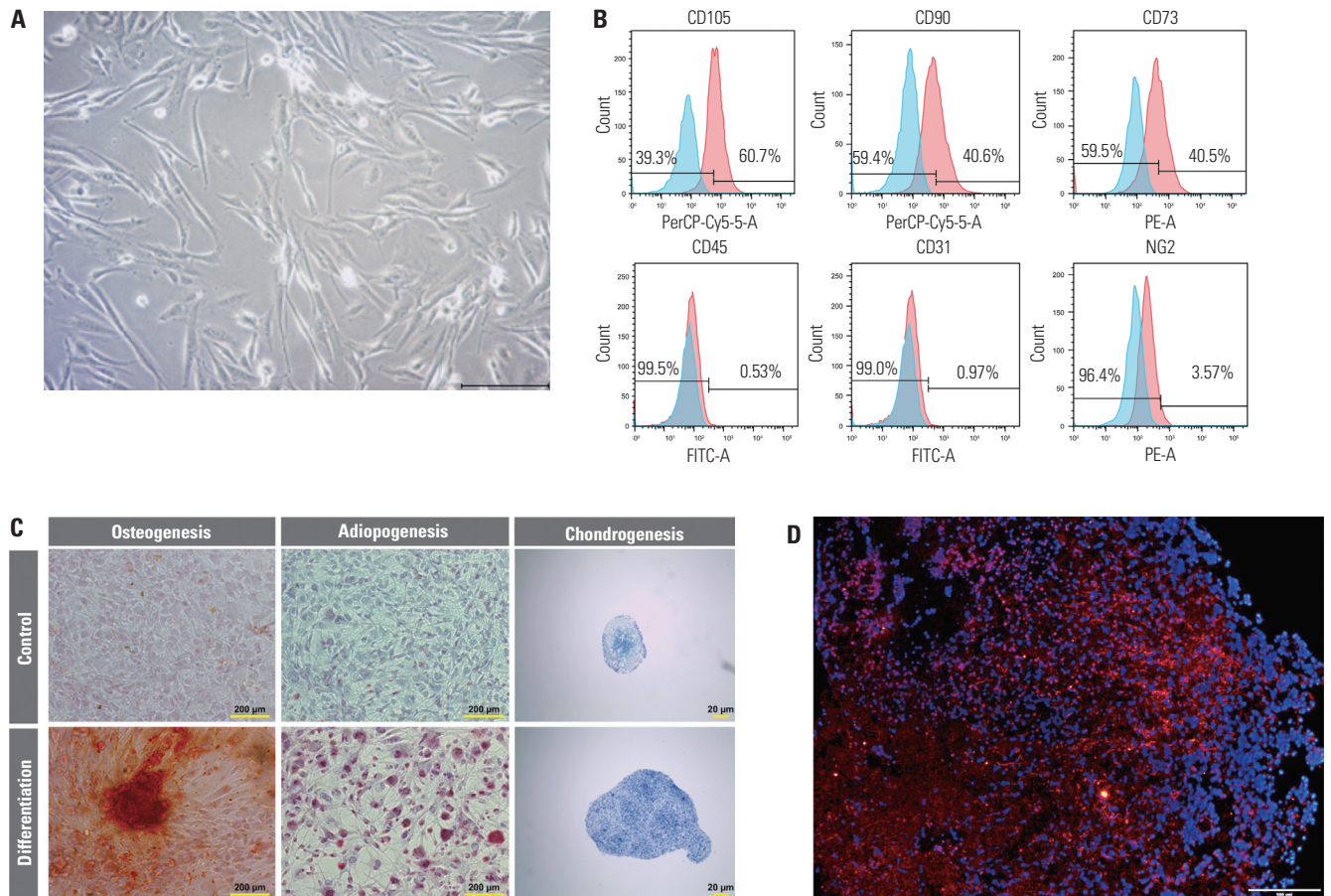
Among the 51 collected specimens, 30 were from male and 21 were from female, and the patients' age ranged from 21 to 77 years. Of the 51 specimens, 44 were primary tumors, none was secondary, and 7 were recurrent types. Pathological diagnoses included glioblastoma, IDH-wildtype (n=39); anaplastic astrocytoma, IDH-mutant (n=1); anaplastic astrocytoma, IDH-wildtype (n=8); and anaplastic oligodendroglioma, IDH-mutant and 1p19q-codeleted (n=3). Success of tMSLC was assessed by adherence to plastic (Fig. 1A), the presence of typical MSC surface markers (positive for CD105, CD90, and CD72; negative for CD45, CD31, and NG2) (Fig. 1B), and tri-lineage differentiation (Fig. 1C). Immunofluorescent labeling of CD105 revealed dispersed existence of the tMSLC (Fig. 1D). The tMSLCs were successfully isolated from 28 of the 51 specimens (positive group) (Table 1), whereas 23 specimens failed to yield tMSLCs (negative group) (Table 1). There were no statistically significant differences in age, sex, pathological diagnosis, tumor origin, IDH-1 mutation, 1p19q codeletion, MGMT promoter methylation, Ki-67 index, p53, or EGFR mutation status between the tMSLC isolation-positive and tMSLC isolation-negative groups (Table 1).

### Specimen weight distribution

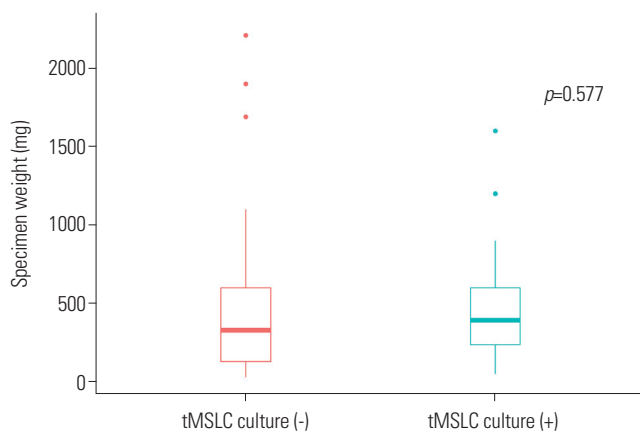
The weights of the glioma specimens ranged from 30 to 2210 mg. There was no statistically significant difference between the mean surgical specimen weights in the tMSLC isolation-positive and tMSLC isolation-negative groups (469.9 $\pm$ 341.9 mg vs. 546.7 $\pm$ 618.9 mg, respectively,  $p=0.577$ ) (Fig. 2).

### Minimum amount of specimens for tMSLC isolation

Through receiver operating characteristic (ROC) curve, measured AUC was 0.599 and the corresponding Youden index was 2.197. This value failed to suggest the optimal cut-off weight of fresh specimens for isolating tMSLC (Fig. 3). However, the val-



**Fig. 1.** Representative example of tMSLCs characterization (tMSLC 15-88). (A) The spindle-shaped morphology of the tMSLCs was detected by phase-contrast microscopy ( $\times 100$ ). (B) Flow cytometry was used to detect mesenchymal stem cells surface antigen markers (CD105, CD90, CD73, CD45, CD31, and NG2). (C) Differentiation of tMSLCs into osteogenic cells, adipogenic cells, and chondrogenic cells was evaluated by Alizarin Red, Oil Red O, and toluidine blue staining, respectively. (D) Immunofluorescent labeling in a glioblastoma specimen showing a disperse pattern of tMSLCs. Red fluorescence labeling-marked CD105+ cells (tMSLC) were counterstained with DAPI (blue fluorescence) ( $\times 200$ ). tMSLCs, tumor mesenchymal stem-like cells.



**Fig. 2.** Weight distributions of the fresh specimens in the tMSLCs isolation-positive and -negative groups. A comparison of the mean specimen weights of the groups ( $469.9 \pm 341.9$  mg vs.  $546.7 \pm 618.9$  mg) did not reveal a statistically significant difference. tMSLCs, tumor mesenchymal stem-like cells.

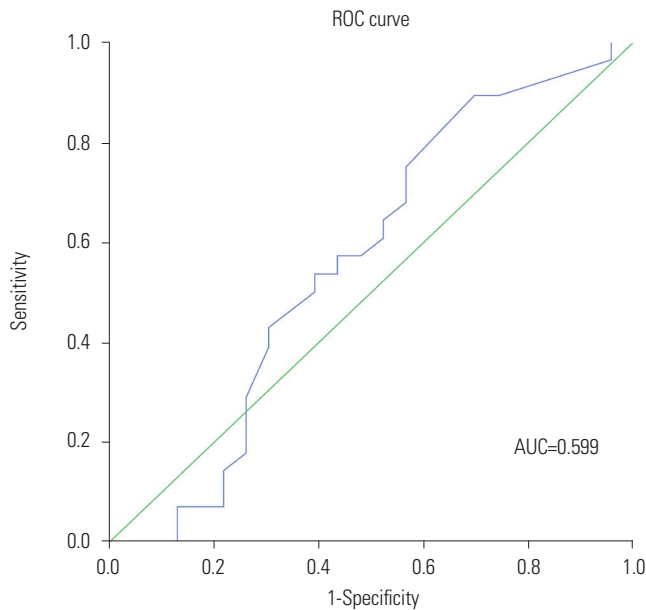
ue that maximizes sensitivity and specificity was 180 mg, and the smallest specimen value in the group in which tMSLC culture was successful was 100 mg.

### Kaplan-Meier survival analysis

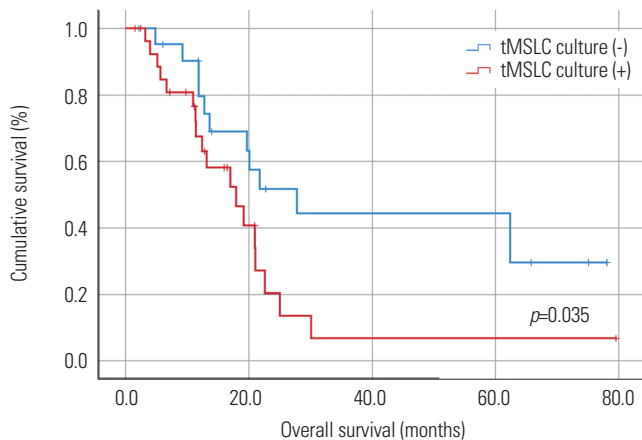
The mean follow-up period was 65.8 months with a range of 1.5 to 79.5 months. The overall survival of the tMSLC-positive group was 17.9 months, and that of the tMSLC-negative group was 27.8 months. Our analysis revealed that the isolation-positive group had a lower survival rate compared to the isolation-negative group ( $p=0.035$ ) (Fig. 4).

## DISCUSSION

The ability to isolate tMSLCs from high-grade gliomas is negatively associated with long-term survival rates.<sup>12</sup> We were interested in determining whether certain physical properties of tumors, specifically their weight reflecting the amount of specimen, influence the success rate of tMSLC isolation. Therefore, we examined the relationship between surgical specimen weight and the tMSLC isolation rate from patients with high-grade glioma, with the presumption that isolation would be more successful from larger specimens. Through this study, we



**Fig. 3.** Receiver-operating characteristic (ROC) curve for the isolation of tMSLCs. The area under the curve (AUC) value of 0.599 indicates a poor capacity for discrimination, showing that the specimen weight is unrelated to the success of tMSLC isolation. tMSLCs, tumor mesenchymal stem-like cells.



**Fig. 4.** Kaplan-Meier curve of the overall survival of the two tMSLCs isolation groups. The tMSLC isolation-positive group had a lower survival rate than the tMSLC isolation-negative group, as calculated by the log-rank test ( $p=0.035$ ). tMSLCs, tumor mesenchymal stem-like cells.

suggest that the success of tMSLC isolation is highly associated with poor prognosis and the minimum value for tMSLC isolation. However, the optimal cut-off value did not reach statistical significance through the ROC curve. Furthermore, no significant differences between the patients' clinical characteristics, such as age, sex, pathological diagnosis, tumor type, and molecular markers, were found between the two groups.

Although several previous studies have isolated MSCs and tMSLCs from high-grade gliomas and other tumors,<sup>1,3-7,14-18,32</sup> no ideal sample weight for successful isolation has been established to date. It was reported that the ability to isolate tu-

mor spheres from grade IV gliomas was not correlated with the specimen weight.<sup>13</sup> Another study suggested that the optimal volume of tumor specimens for glioma cancer stem cell isolation and culture was at least 0.5 cm<sup>3</sup>.<sup>33</sup> However, this study also reported that larger specimens might be better than smaller specimens for cell isolation, owing to the differences among fresh specimens in cell density, tissue texture, and necrosis status. This implies that the technical success rate of tMSLC isolation from larger specimens may be higher than from smaller samples, regardless of the actual presence of tMSLCs in the sample, leading to a false prediction of longer survival rates in patients with small "tMSLC-negative" tumors. Herein, we tried to clarify the relationship between the specimen weight and the successful isolation of tMSLCs affecting the patients' prognosis.

The MSC isolation procedure can be difficult to perform, as it requires deft technical skills and close attention to detail. Therefore, some studies have proposed protocols that are putatively more effective for MSC isolation,<sup>34-37</sup> although they acknowledge that tMSLCs may not be obtained from all fresh tumor specimens using these protocols. In our study, tMSLCs were obtained from 28 of the 51 (54.9%) WHO grade III/IV glioma specimens, and from 24 of the 39 (61.5%) WHO grade IV gliomas under optimal conditions. Due to the technical difficulties associated with tMSLC isolation, researchers may suspect their low success rates. To alleviate some of these concerns, we showed that the amount of the specimens is not critical for tMSLC isolation. Furthermore, the AUC value of the ROC curve (0.599) indicated a poor capacity for discrimination, showing that the specimen amount appears to be less related to the success of tMSLC isolation. Instead, we suggested the minimum specimen weight (100 mg) for which tMSLC isolation was successful, and this value could be useful as a reference for future studies on tMSLC.

Consistent with previous reports, we found that the isolation-positive group had lower patient survival rates compared to the isolation-negative group (Fig. 4).<sup>12</sup> By dividing the patients into two groups based on tMSLC isolation, we were able to determine whether other factors, such as specimen weight, were associated with the success of isolation. However, no difference was found in any patient characteristics. Based on the fact that human MSCs inhibit the immune response by blocking allorecognition and interfering with dendritic cell or T-cell function,<sup>38</sup> we speculate that the poor prognosis of the tMSLC isolation-positive group is related to tMSLCs downregulating the body's immune reaction against the tumor, helping the tumor to flourish.

However, the conclusions of this study must be interpreted carefully, as this study had several limitations. First, the number of specimens used was not enough to obtain conclusive results. Since type 2 error cannot be ruled out, a larger study would reveal a clearer relationship between the amount of specimen and the success of tMSLC isolation. Second, we failed to deter-

mine a plausible reason for being unable to isolate the tMSLCs. While some tumors may be tMSLC-negative, technical difficulties in isolating tMSLCs imply that the false-negative rate is likely high. Third, although all samples were weighed after removing blood clots and debris as much as possible, it was not possible to completely dry up the samples to perform tMSLC isolation, which could cause some bias. The present study suggests the minimum weight of specimen for tMSLC isolation, which is highly associated with poor prognosis. However, due to low AUC value, a successful tMSLC isolation does not depend on the weight of the specimen only, or on other properties of the tumor. The results of this study will alleviate surgeons' concerns about whether specimen weight impacts the successful isolation of tMSLCs.

## DATA AVAILABILITY

All data supporting the conclusions of this article are available from the corresponding authors upon reasonable request.

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## AUTHOR CONTRIBUTIONS

**Conceptualization:** Soon Haeng Kong and Seok-Gu Kang. **Data curation:** Jihwan Yoo. **Formal analysis:** Jihwan Yoo. **Funding acquisition:** Seok-Gu Kang. **Investigation:** Dongkyu Lee, Jin-Kyoung Shim, Ran Joo Choi, and Seon Jin Yoon. **Methodology:** Sohyung Moon. **Project administration:** Seok-Gu Kang. **Resources:** Kyoung Su Sung, Ju Hyung Moon, Eui-Hyun Kim, and Jong Hee Chang. **Software:** So Hee Park. **Supervision:** Su Jae Lee and Seok-Gu Kang. **Validation:** Dongkyu Lee. **Visualization:** Soon Haeng Kong and Jihwan Yoo. **Writing—original draft:** Soon Haeng Kong. **Writing—review & editing:** Jihwan Yoo. **Approval of final manuscript:** all authors.

## ORCID iDs

Soon Haeng Kong <https://orcid.org/0000-0002-2851-6282>  
 Jihwan Yoo <https://orcid.org/0000-0001-8746-1245>  
 Dongkyu Lee <https://orcid.org/0000-0002-0241-1554>  
 Sohyung Moon <https://orcid.org/0000-0002-3904-2289>  
 Kyoung Su Sung <https://orcid.org/0000-0003-3289-0143>  
 So Hee Park <https://orcid.org/0000-0001-8513-5163>  
 Jin-Kyoung Shim <https://orcid.org/0000-0003-3819-0180>  
 Ran Joo Choi <https://orcid.org/0000-0001-5024-8221>  
 Seon Jin Yoon <https://orcid.org/0000-0002-3255-5081>  
 Ju Hyung Moon <https://orcid.org/0000-0002-8925-5821>

Eui-Hyun Kim <https://orcid.org/0000-0002-2523-7122>  
 Su Jae Lee <https://orcid.org/0000-0002-4023-4819>  
 Jong Hee Chang <https://orcid.org/0000-0003-1509-9800>  
 Seok-Gu Kang <https://orcid.org/0000-0001-5676-2037>

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