Terahertz reflectometry imaging for low and high grade gliomas

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Gross total resection (GTR) of glioma is critical for improving the survival rate of glioma patients. One of the greatest challenges for achieving GTR is the difficulty in discriminating low grade tumor or peritumor regions that have an intact blood brain barrier (BBB) from normal brain tissues and delineating glioma margins during surgery. Here we present a highly sensitive, label-free terahertz reflectometry imaging (TRI) that overcomes current key limitations for intraoperative detection of World Health Organization (WHO) grade II (low grade), and grade III and IV (high grade) gliomas. We demonstrate that TRI provides tumor discrimination and delineation of tumor margins in brain tissues with high sensitivity on the basis of Hematoxylin and eosin (H&E) stained image. TRI may help neurosurgeons to remove gliomas completely by providing visualization of tumor margins in WHO grade II, III, and IV gliomas without contrast agents, and hence, improve patient outcomes.

Around 28% of all primary brain tumors and 80% of primary malignant brain tumors are gliomas. Many malignant glioma types, especially glioblastoma (GBM), have a poor prognosis even though aggressive therapy including surgery, radiotherapy, and chemotherapy are performed. Several prognostic factors for better outcomes in glioma patients have been reported, which include age, genetic mutations, achievement of gross total resection (GTR). Among these factors, achievement of GTR was found to be crucial for the better prognosis. Incomplete resection, even in low grade gliomas, leads to higher probability of recurrence and shorter survival by residual tumor. The most serious obstacle to achieving GTR is the difficulty in tumor discrimination and margin delineation during surgery. Various techniques have been employed to distinguish gliomas and demarcate accurate tumor margins. For example, a neuronavigation system based on preoperative magnetic resonance imaging (MRI) has been widely used to perform glioma surgery. However, the method often fails to trace tumor margins during the operation due to brain-shift, and consequently GTR rates are lower as compared with advanced methods such as intraoperative MRI and fluorescence imaging with 5-aminolevulinic acid (5-ALA) or fluorescein sodium. Intraoperative MRI has been used to increase GTR rates, but involves long operation time owing to additional scanning and requires repetitive administration of contrast media such as gadolinium (Gd) chelates. Fluorescence-guided surgery with 5-ALA is a recently adopted method to enable real-time tumor margin discrimination in surgery by means of emission of protoporphyrin IX (ppIX) fluorescence induced by 5-ALA. However, low grade and some grade III glioma tissues do not exhibit visible ppIX fluorescence due to an intact blood brain barrier (BBB) or high ferrochelatase activity. Therefore, this method has not been utilized for World Health Organization (WHO) grade II and III glioma surgeries. Recently, some investigators introduced surface enhanced Raman scattering (SERS) imaging and photoacoustic imaging with contrast media for high grade glioma surgery. However, these methods also do not enable detection of low grade and some grade III gliomas, as an intact BBB prevents such contrast media from extravasating into the tumor.

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Most up-to-date intraoperative technologies to discriminate and delineate tumor margins for glioma surgery have been suffered from and limited by the followings: (i) brain-shift, (ii) use of contrast agent, or (iii) low sensitivity for low grade glioma and WHO grade III glioma with intact BBB.

Here we present a terahertz reflectometry imaging (TRI) method to tackle the aforementioned limitations. TRI has been applied to brain diseases and cancer diagnosis for over a decade, since it is highly sensitive to water molecules and tissue components. First of all, TRI intrinsically avoids the brain-shift problems of the neuronavigation system because real-time measurement can be performed with portable TRI devices. Secondly, TRI, a label-free imaging method, does not use contrast media. Lastly, TRI is capable of detecting low grade and grade III tumor with intact BBB for the successful GTR.

**Results**

**TRI of *ex vivo* human GBM tumorsphere (TS) bearing mouse.** We conducted TRI of *ex vivo* GBM bearing mouse samples (n = 4). It has been noted that as malignant gliomas grow, they are manifested by a significant reduction of the total lipids and increase in water content. We therefore hypothesized that TRI may be capable of discriminating glioma from normal brain owing to its high sensitivity to water and lipid content. We used an orthotopic xenograft model in which enhanced green fluorescent protein (eGFP)-transfected human GBM TS (eGFP + GSC-11) were implanted into BALB/c nude mice (Materials and Methods). The tumor growth was screened with a 9.4T animal MRI, and the mice were sacrificed at 42 days after injection of tumor cells (Fig. 1a and Materials and Methods). 5-ALA solution was injected 2 h before the sacrifice to obtain ppIX fluorescence images, which were compared with TRI images as an existing advanced intraoperative tumor discrimination method. The extracted brain tumor samples were placed on a quartz sample window, and we sequentially obtained white light, TRI, optical coherence tomography (OCT), ppIX fluorescence, GFP fluorescence and Hematoxylin and eosin (H&E) stained images (Materials and Methods). The tumors were invisible in white light images (Fig. 1b), whereas they were clearly visualized in GFP fluorescence and H&E stained images.
normal brain tissues. The threshold value 1 (TH1) was set to 0.8542 AU, which was determined by mean and diffuse tumors or normal gray matter. Nonetheless, neurosurgeons can judge whether the surgical region is neurologically. Our results indicate that the green regions in TRI images may represent either low cellularity tumors (Fig. 2c–D,G), even though these green and red regions (Fig. 2c–C–G) represented WHO grade II gliomas histologically. The red regions denote regions exceeding the TH1 values that correspond to either high grade glioma or a dense tumor region of low grade glioma (Fig. 2a–d). Meanwhile, some regions with TP values smaller than TH1 were pathologically confirmed as grade II gliomas infiltrating into the white matter. It was speculated that when the tumor cells infiltrated to peripheral white matter around dense tumor, the water and lipid contents of diffused tumor region may become similar or lower than that of normal gray matter. The threshold value 2 (TH2) was thus set to be 0.7852 AU, which corresponds to mean S.D. of normal white matter (dashed red line in Fig. 2b). The red regions were better correlated with tumor regions in GFP fluorescence, and H&E stained images, as compared with ppIX images (Fig. 1g). Especially in mouse #4, the fluorescence of tumor regions was not positive in the ppIX fluorescence image due to weak tumor development, which was inferred from the H&E stained image and the low intensity of the GFP fluorescence image; however, the tumor region was well visualized in the TRI image (bottom of Fig. 1).

**TRI for WHO grade II, III, and IV glioma specimens obtained from patients.** We performed TRI for human glioma specimens to validate its capability of tumor discrimination from normal brain tissues. Glioma specimens were obtained from surgeries of 14 glioma patients. Tumor grades of the specimen were confirmed as four grade IV, four grade III, and six grade II gliomas by routine pathological examinations (Table 1, Materials and Methods). A neuropathologist diagnosed these tumors according to WHO classification. All TRI images were characterized by the time-domain THz reflectometry system.

| Case | Age | Sex | Pathology         | WHO grade | 5-ALA | IDH1 | MGMT | 1p/19q co-deletion | Ki-67 L.I. |
|------|-----|-----|-------------------|-----------|-------|------|------|-------------------|------------|
| 1    | 57  | F   | Glioblastoma      | IV        | +     | Wild | Unmethylated | No               | 50%        |
| 2    | 55  | M   | Glioblastoma      | IV        | +     | Wild | Methylated  | No               | 5–7%       |
| 3    | 67  | M   | Glioblastoma      | IV        | +     | Wild | Methylated  | Yes              | 15–20%     |
| 4    | 62  | F   | Glioblastoma      | IV        | +     | Wild | Unmethylated | No               | 15–20%     |
| 5    | 46  | M   | Anaplastic oligodendroglioma | III | + | Mutant | Methylated | No | 3–4% |
| 6    | 34  | F   | Mixed oligoastrocytoma | II | NA | Mutant | Unmethylated | No | 3–4% |
| 7    | 51  | F   | Oligodendroglioma  | II        | NA   | Mutant | Methylated  | Yes              | 3–4%       |
| 8    | 40  | F   | Diffuse astrocytoma | II        | NA   | Wild | Unmethylated | No               | 2–3%       |
| 9    | 56  | F   | Anaplastic astrocytoma | III | + | Wild | Unmethylated | No | 5–6% |
| 10   | 41  | F   | Anaplastic astrocytoma | III | − | Wild | Unmethylated | No | 50% |
| 11   | 36  | M   | Anaplastic astrocytoma | III | NA | Mutant | Methylated | Yes | 20–25% |
| 12   | 51  | F   | Oligoastrocytoma   | II        | NA   | Mutant | Methylated  | No               | 2–3%       |
| 13   | 38  | M   | Oligoastrocytoma   | II        | NA   | Mutant | Unmethylated | No | 5% |
| 14   | 41  | M   | Oligodendroglioma  | II        | NA   | Mutant | Methylated  | Yes              | 3–4%       |

**Table 1. Summary of patient pathological information.** WHO: World Health Organization; 5-ALA: 5-aminolevulinic acid; IDH1: isocitrate dehydrogenase 1; MGMT: O6-methylguanine-methyltransferase; 1p/19q: chromosomal co-deletions that are characteristic of oligodendrogliomas; Ki-67 L.I.: labeling index associated with proliferation; NA: not available.
gliomas, where ppIX fluorescence has been scarcely expressed, could be well discriminated by TRI. Although the four tumor specimens including ROIs B-E in Fig. 2c were obtained from the same patient, 5-ALA-induced ppIX fluorescence was only expressed at specimens including ROIs B and C, but not in other specimens; in contrast, all tumors were discriminated in TRI images (Fig. 2c–d). We assume that our TRI image results reflect a compositional change associated with increased water content and decreased lipid content and this change accompanied not only high grade but also low grade gliomas. As a result, we could find the presence of tumor in all cases by TRI images.

We then validated the tumor margin delineation ability of TRI. The wavelength of terahertz electromagnetic waves is a few hundred-μm, so TRI may be more suitable as a macroscopic imaging tool of gliomas. While TRI may not be able to delineate tumor margin at the cellular level, it has the potential to macroscopically delineate foci-glioma margins. Indeed, our macroscopic delineation of the tumor margins were correlated with the pathologically determined tumor margins with the H&E stained image. Red and green tumor regions over TH1 and TH2 in the TRI image of low grade glioma specimen (case 6) were well correlated with the tumor margin pathologically determined with the H&E stained image (Fig. 3a–c). However, a few tumor cells existed outside of the

**Figure 2.** Discrimination of low and high grade of human gliomas with TRI. (a) Terahertz parameter (TP) values from regions of interest (ROIs) in tumors (n = 14), normal gray matters (n = 4), and normal white matters (n = 4). (b) Quantification of threshold value 1 (TH1) and TH2 (dashed red and green lines, respectively) for tumor discrimination using the data shown in (a). Data represent mean ± SD. ***P < 0.001 (Kruskal-Wallis test.) Representative cases of grade IV, III and II gliomas, characterized by (c) TRI images and (d) H&E stained image. Red regions indicate regions with TP value over TH1. The capitals A–G shown in (d) correspond to the ROIs in (c).
margin of the foci-tumor region, and the diffused tumor cells were confirmed in an immunochemistry stained image (isocitrate dehydrogenase 1 [IDH1, 1/80, clone H09, Dianova, Germany] stain, Materials and Methods) and not in a H&E stained image (Fig. 3c–f). The precise tumor margins determined with the immunochemistry stained image was found to be further extended than those determined with the TRI image and the H&E image (Fig. 3g). Therefore, when a neurosurgeon practically uses this method during glioma surgery, the neurosurgeon would need to apply safety margin that could be determined by further investigations or other complementary microscopic imaging tools to complete the glioma resection.

**Discussion**

We describe a highly sensitive TRI for low and high grade glioma identification without exogenous contrast agents. TRI is an imaging technique, implementable to the biomedical field. Because TRI signal is affected by the molecular dynamics of water and the differences in the refractive indices between water and lipids, the resultant images reflect high sensitivity to water and lipid content in tissues. Glioma is characterized by an increased level of water and smaller lipid content in contrast to the adjacent normal brain. Therefore, TRI may serve as a high-sensitivity detector for glioma, allowing neurosurgeons to ‘see’ tumor regions without the use of contrast agents during glioma surgery. Several papers reported on the possibility of glioma detection using TRI. However, they used only fresh rat or paraffin-embedded samples. In this paper, we performed TRI imaging in not only fresh mouse samples but also fresh human specimens. We further carried out in vivo TRI imaging of a tumor-bearing mouse model. Through the experiments, we validated the feasibility of TRI for glioma detection with preclinical, clinical and in vivo settings in only an article.

TRI exhibits distinct advantages compared with conventional intraoperative surgical methods. Firstly, TRI, in a form of handheld device, would be intrinsically obviated from brain-shift challenges that surgeons face frequently in using the brain neuronavigation system. Handheld TRI devices have been already developed, so the neurosurgeons could utilize these tools to locate tumors during operation. Secondly, this method has the potential to provide a real-time display for the tumor detection at surgery. Thirdly, TRI is a label-free method, so it is unrestrained from the various adverse effects of contrast agent: photo-bleaching, restricted photo retention time, patient hypersensitivity to light at pPIX fluorescence-guided surgery method with 5-ALA, and the adverse effects produced by repeated gadolinium contrast administration at intraoperative MRI. Finally, this method...
could discriminate not only high grade gliomas but also low grade gliomas from normal brain tissues, whereas ppIX fluorescence imaging is only effective in high-grade gliomas.

Recent advances in Raman spectroscopy made it possible to discriminate high and low grade gliomas with a sensitivity and a specificity of 93% and 91% respectively. Raman methods also use the intrinsic optical properties of tissues to characterize the decreased lipid content in gliomas. Therefore, the results from the Raman technique could indirectly imply TRI’s high feasibility in the clinical glioma surgery application.

Though Raman imaging has remarkable capability to detect gliomas, it has several obstacles to practically be employed in glioma surgery such as low signal to noise ratio and long measurement time. It is difficult to compare TRI directly with Raman imaging, because there are differences in operating principles and implementations. With regard to spatial resolution, TRI is generally macroscopic, whereas Raman imaging is microscopic. These features may be combined to provide complementary information for the complete resection of gliomas. For example, neurosurgeons may preferentially remove most of the glioma through TRI-validated images where the macroscopic tumor margins are visualized, and then they can completely remove residual glioma tissue in the periphery of the tumor through Raman-validated images where diffusely invasive brain cancer cells are identified at cellular resolution.

The penetration depth of THz waves in tissues is rather limited (generally under 500–μm) due to the high absorption by water. As such, TRI could detect tumor only when the tumor is exposed on the outer surface of the brain. However, we do not consider this as a serious limitation because ppIX fluorescence-guided surgery is also limited by a short penetration depth but is very helpful to neurosurgeons.

In some regions of our three human glioma cases (cases 8, 11 and 13), false-positives over TH1 were identified although the regions did not possess tumor tissue on histological examination. High-reflection THz signals larger than the TH1 should be considered abnormal despite scarce or absent tumor cells. Further investigation on this observation will be performed with a larger set of clinical data. As previously mentioned, TRI is a macroscopic method, so diffused tumor or peripheral foci-tumor may be expressed as low-signal regions below TH2. This limitation may be overcome by simultaneous use of TRI with a complementary microscopic method such as Raman imaging.

Today, neurosurgeons mobilize various intraoperative technologies such as the neuronavigation system, intraoperative MRI, 5-ALA-induced ppIX fluorescence imaging, and ultrasonic systems for complete tumor resection. Our TRI method enables the neurosurgeon to detect during surgery not only high grade gliomas, but also low grade gliomas with intact BBB involving increased water and decreased lipid content. In the foreseeable future, TRI can mitigate the bottleneck of incomplete tumor resection rate in low grade gliomas that are underestimated by ppIX fluorescence imaging. We expect this method will be able to substantially contribute to successful glioma resections and lead to better outcomes for glioma patients in the future.

Materials and Methods

Study design. Conventional imaging methods for glioma surgery have been limited by the following: (i) brain-shift, (ii) use of contrast agent, or (iii) low sensitivity to low grade glioma. The main aim of this study was to introduce and demonstrate the potential feasibility of TRI for glioma surgery that enables to overcome current key limitations for intraoperative detection of high and low grade gliomas. It has been known that malignant...
gliomas are manifested by a significant reduction of the total lipids and increase in water content. We therefore hypothesized that TRI may be capable of discriminating glioma from normal brain due to its high sensitivity to water and lipid content. First, we conducted preclinical experiments with ex vivo xenotransplantation brain tumor models (n = 4) to verify our hypothesis. We compared TRI images with multi-modality images including standard H&E images. Next, we evaluated TRI of fresh WHO grade II, III, and IV glioma specimens obtained from human patients (n = 14). Finally, we demonstrated that TRI is a viable technique for in vivo glioma detection using an in vivo xenotransplantation brain tumor model (n = 1).

**Lentiviral vector transduction and expression.** Green fluorescent protein (GFP) stably expressing GSC11 cells (GSC11-GFP) were generated by growing GSC11 cells in complete medium and then applying GFP-expressing lentiviral supernatants. Polybrene (Sigma, Dorset, UK) was added to a final concentration of 8 μg/ml and incubated with cells for 18h. After infection, the cells were placed in fresh growth medium and cultured in a standard manner. Cells were treated with 1 mg/ml puromycin (Life Technologies Korea, Seoul, Republic of Korea) to eliminate uninfected cells and generate stable GSC11-GFP. GSC11-GFP was isolated for use in further experiments by fluorescence activated cell sorting (FACS).

**Mouse care and information.** Five-week-old male athymic Balb/c nude mice (Orient Bio, Seongnam-Si, Republic of Korea) were used for tumor xenograft experiments. Mice were retained in micro-isolator cages under sterile conditions and observed for at least 1 week before study initiation to ensure proper health. Temperature, lighting and humidity were controlled centrally. All experimental procedures were carried out in accordance with the guidelines Institutional Animal Care and Use Committees (IACUC) which were approved by Yonsei University College of Medicine Institutional Animal Care and Use Committee. The mice were anesthetized with a solution of Zoletil (30 mg/kg; Virbac Korea, Seoul, Republic of Korea) and xylazine (10 mg/kg; Bayer Korea, Seoul, Republic of Korea) delivered intraperitoneally. After the holes were drilled on the mouse skull using a 26-gauge needle, GSC11-GFP cells (1 × 10⁶) were implanted directly into right frontal lobe of nude mice using Hamilton syringe (Dongwoo Science Co., Seoul, Republic of Korea) at the depth of 4.5 mm. Cells were simultaneously injected into five mice using a multiple microinfusion syringe pump (Harvard Apparatus, Holliston, MA, USA) at a rate of 0.5 μl/min as previously described.⁴⁷,⁴⁸

**9.4T animal MRI.** We performed in vivo MR imaging experiments with a 9.4T animal MRI instrument with a Bruker animal coil (RF SUC 400 1H M-BR-LIN ROAD, Bruker Medical Systems, Germany). The sequence parameters were adopted as follows: Echo = 1, TR = 2500 ms, TE = 22.2 ms, FA = 180 deg, TA = Oh2m5s0ms, NEX = 1, FOV = 2.50/1.80 cm.

**TRI system.** To measure the THz signal, we used a homemade THz reflectometric imaging system with photography antennas that were driven by femto-second laser pulses to generate and detect terahertz pulses. We used four off-axis parabolic reflectors to collimate and focus THz pulses on ex vivo and in vivo samples and guide reflected THz pulses to the detector. The extracted mouse tumor samples and glioma specimens from patients were placed on a quartz sample window, and then we mounted the sample window on a computer-controlled x-y translation stage which was used to raster scan. The focused beam diameter of THz pulses was 0.8-mm at 0.5 THz, and THz signals were measured with 20 Hz and 250-μm scanning resolution. A more detailed description is presented in our previous report.⁴⁵ We obtained TRI images for ex vivo and in vivo mouse samples using peak-to-peak amplitude extracted from reflection THz signals.

**Combined Optical Coherence Tomography (OCT) and ppIX fluorescence imaging system.** Our OCT system was implemented based on optical frequency domain imaging as described in [Yun et al. Opt. Express, 11: 2953–2963 (2003); Yun et al. Nature Medicine, 12: 1429–1433 (2006)]. A 1.31-μm wavelength-swept laser (SS-1310, Axsun Technologies Inc., USA) was employed as a light source, which provided a sweep range of 110 nm, repetition rate of 50 kHz, and average power of ~20 mW. Light from the laser was first directed to a narrowband fixed-wavelength filter, serving as a trigger for signal acquisition. The k-clock from the laser was directly connected to a digitizer for signal sampling. Ninety percent of the remaining light was directed to the OCT interferometer.

In the OCT sample arm, we combined ppIX fluorescence imaging setup to perform a direct comparison of the tumor regions measured by TRI and OCT against the regions by ppIX fluorescence imaging. A 405-nm laser diode (FL laser) was employed as a fluorescence excitation light source. The light from the laser was combined with the 1.3-μm OCT probe light through the dichroic mirrors DM1 (47267, Edmund Optics, USA) and DM2. The combined light was then directed to a specimen through the same galvametric scanners (GM) and illumination optics described above. This arrangement enabled both OCT and fluorescence images to share the same field of view, facilitating direct image comparison. The beam size of the 405-nm excitation light on the specimen plane was measured to be 80 μm. The backscattered OCT light from the sample was re-coupled to the fiber and its interference with reflection from a reference mirror was detected by a high-speed balanced detector (Thorlabs, Inc., PDB410C, New Jersey, USA). For the fluorescence signal, the fluorescence emission from the specimen was collected by the focusing lens, reflected by the dichroic mirror DM3 (NFD-01-633, Semrock, USA), and subsequently detected with a large-area photodetector (PDA100A-EC-Si, Thorlabs, USA) through a lens.

**GFP fluorescence imaging.** We performed ex vivo fluorescence imaging using green fluorescent protein. The fluorescence recovery profiles in eGFP+ GSC-11 tumor-bearing mice were imaged by positioning mice on an animal plate, heated to 37°C, in the IVIS spectrum system (Caliper Life Science, Hopkinton, MA, USA) as per the manufacturer’s instructions. Excitation and emission spots were raster-scanned over the selected region.
of interest (ROI) to generate emission wavelength scans, wavelength of fluorescence were 488 nm and 520 nm respectively for ex vivo samples. All data, including whole body and two-dimensional (2D) slice images, were calculated using the ROI function of the Analysis Workstation software.

**Patient population.** Fourteen patients with WHO grade II, III, and IV glioma, including 4 grade IV, 4 grade III, and 6 grade II patients, treated at our institution between February 2014 and December 2014 were included in this study (Table 1). All patients were histologically diagnosed and graded by neuro-pathologists according to 2007 WHO classification criteria. Informed consent was provided according to the Declaration of Helsinki. All patients provided written informed consent, and the study was approved by the Institutional Review Boards of Severance Hospital, Yonsei University College of Medicine. All experiments were conducted in accordance with principles expressed in the Declaration of Helsinki or other relevant guidelines and regulations.

O-6-methylguanine-DNA methyltransferase (MGMT) promotor methylation and isocitrate dehydrogenase (IDH)-1 mutations were assessed by polymerase chain reaction (PCR) and immunohistochemistry (IHC). Epidermal growth factor receptor (EGFR) amplification and loss of heterozygosity (LOH) at chromosomes 1p and 19q were evaluated by fluorescent in situ hybridization (FISH). P53 and ki-67 were examined by IHC.

**IDH1 Immunohistochemistry (IHC).** With a representative section, we performed IHC with a VentanaBenchMark XT autostainer (Ventana Medical System, Inc. Tucson, AZ) according to the protocol. The antibody used was anti-human IDH1 R132H mouse monoclonal (Clone H09L, 1:80 dilution, Dianova, Hamburg, Germany). A neuropathologist (S.H. Kim) reviewed the immunohistochemical and histologic findings without information of IDH1 mutation status assessed by other methods. When the cytoplasmic expression of the IDH1 R132H was identified in glioma cells, we regarded those cases as “mutant” or “positive”.

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Author Contributions
J.-S.S., Y.B.J., S.J.O., C.J., J.H., S.-G.K. and J.H.C. conceived the project; J.-S.S. managed the study; Y.B.J., S.J.O., S.-H.K., Y.M.H., S.J.H., C.J., S.-G.K., J.H.C. and S.-H.K. contributed to experimental design; Y.B.J., S.-H.K., Y.C., J.H. and S.S. performed the experiment and obtained images; Y.M.H., H.Y.S., Y.C. and S.-G.K. created tumor models; J.H.C. provided surgical specimens; J.H.L and S.-G.K. performed the statistical analysis; S.-S.-H.K., Y.C., J.H. and S.S. performed the experiment and obtained images; Y.M.H., H.Y.S., Y.C. and S.-G.K. contributed to experimental design; Y.B.J., S.J.O., C.J., S.-G.K. and J.H.C. wrote the manuscript; All authors reviewed and edited the manuscript.

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