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Culture-independent diagnostic approaches for invasive aspergillosis in solid organ transplant recipients

In Young Yoo, Yeon-Joon Park

Department of Laboratory Medicine, Seoul St. Mary's Hospital, College of Medicine, The Catholic University of Korea, Seoul, Korea

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Corresponding author: Yeon-Joon Park
Department of Laboratory Medicine, Seoul St. Mary's Hospital, College of Medicine, The Catholic University of Korea, 222 Banpo-daero, Seocho-gu, Seoul 06591, Korea
E-mail: yjpk@catholic.ac.kr

Prompt and accurate diagnosis of invasive aspergillosis (IA) is crucial for immunocompromised patients, including those who have received a solid organ transplant (SOT). Despite their low sensitivity, microscopic detection and conventional culture are considered the 'gold standard' methods. In conjunction with conventional culture, culture-independent assays such as serum galactomannan testing and *Aspergillus* polymerase chain reaction (PCR) have been incorporated into the diagnostic process for IA. The recently revised consensus definitions from the European Organization for Research and Treatment of Cancer and the Mycosis Study Group have adjusted the threshold for positive galactomannan testing based on the sample type, and have excluded 1,3-β-D-glucan testing as a mycological criterion. Following extensive standardization efforts, positive *Aspergillus* PCR tests using serum, plasma, or bronchoalveolar lavage fluid have been added. However, there are limited studies evaluating the clinical utility of these culture-independent assays for the early diagnosis of IA in SOT recipients. Therefore, further research is required to determine whether these assays could aid in the early diagnosis of IA in SOT recipients, particularly in relation to the organ transplanted. In this review, we examine the culture-independent diagnostic methods for IA in SOT recipients, as well as the clinical utility of these assays.

Keywords: Invasive aspergillosis; Galactomannan; Polymerase chain reaction; Transplant

INTRODUCTION

Invasive fungal disease (IFD) is associated with poor allograft outcomes and increased mortality in solid organ transplant (SOT) recipients who are on immunosuppressive medications. The primary risk factors for IFD development in SOT recipients include: (1) technical and anatomical abnormalities, such as the proficiency in operative and perioperative management, the use of vascular access devices, drainage catheters, and endotracheal tubes; (2) the extent of environmental exposures, whether community-based or hospital-acquired; and (3) the overall state of immunosuppression, which can be influenced by cytomegalovirus and other herpesviruses, steroid treatment for rejection, immunosuppressive medications or monoclonal antibodies, and renal failure [1]. The most common IFDs in SOT recipients are invasive candidiasis (IC) and invasive aspergillosis (IA) [2]. The incidence of fungal infections varies depending on the type of SOT. IC is most commonly reported in liver transplant recipients, while IA is most prevalent in lung transplant recipients [3,4]. A recent study reported the overall incidence of IA

HIGHLIGHTS

- Solid organ transplantation (SOT) was newly included as one of the host factors in the revised 2020 EORTC/MSG criteria for probable invasive fungal disease.
- Given the limited data available on 1,3-β-D-glucan testing in SOT recipients, routine testing is not currently recommended for invasive aspergillosis disease.
- For SOT recipients, the galactomannan assay performs better in bronchoalveolar lavage fluid than in serum.
- Further study is needed to assess the clinical utility of *Aspergillus* polymerase chain reaction for diagnosing invasive aspergillosis in SOT recipients.
- Further studies are needed to evaluate the impact of the revised criteria on SOT recipients, along with the clinical usefulness of culture-independent assays.

by transplanted organ as follows: less than 1% for liver transplant recipients, between 0.7% and 4% for renal transplant recipients, between 4% and 23% for lung transplant recipients, and between 1% and 14% for heart transplant recipients [5]. Despite the overall low incidence of IA in SOT recipients, the 1-year mortality rate following IA diagnosis ranges from 19% to 40%, and the rates of graft loss are notably high [6-8].

A prompt and accurate diagnosis is crucial for the appropriate treatment and improved outcomes of IA. In 2008, the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) established criteria to classify potential IA cases into proven, probable, and possible categories. This classification was based on host factors, clinical criteria, and mycological criteria, and was intended for research purposes. The definition proposed for proven IA includes definitive evidence of *Aspergillus* spp. isolated from normally sterile body fluids or confirmed by histopathology. Unlike probable and possible IA, serology testing is not applicable for the diagnosis of proven IA [9]. In 2020, the EORTC/MSG Education and Research Consortium revised the previously suggested guidelines. The definition of probable IFD was expanded to include additional host factors such as SOT and hematologic malignancy, which were not included in the 2008 guidelines [10]. Significant changes were also made to the mycological criteria in the 2020 update. These changes included the introduction of culture-independent assays, the incorporation of polymerase chain reaction (PCR)-based assays for defining probable IA using various clinical samples, revised thresholds for the galactomannan assay, and the exclusion of 1,3-β-D-glucan (BDG) detection (Table 1). However, despite technological advances in IA diagnosis and revised guidelines, accurately diagnosing IA and determining the need for antifungal treatment remains challenging due to the difficulty in distinguishing simple colonization [11]. While direct microscopy and conventional culture methods are considered the gold standard, they are often time-consuming and have limited sensitivity and specificity. Currently, culture-independent assays, including the serum galactomannan assay for *Aspergillus* spp. and PCR-based assays, are incorporated into the IA diagnosis process. However, these methods have their own limitations, such as a wide range of sensitivity and specificity for the serology assay and a lack of standardization for PCR-based assays [12,13].

Here, we review the currently available culture-independent diagnostic approaches for IA in SOT recipients and highlight the clinical usefulness of antifungal susceptibility testing with *Aspergillus* PCR.

CULTURE INDEPENDENT DIAGNOSTIC ASSAYS FOR INVASIVE ASPERGILLOSIS

1,3 β-D-Glucan

Most fungi have BDG as a significant and characteristic constituent of their cell walls. Detecting BDG in the bloodstream can be useful in diagnosing IFD, including clinically important fungi like *Aspergillus* spp., *Candida* spp., *Fusarium* spp., and *Pneumocystis jirovecii* [14]. In particular, BDG testing has proven to be a sensitive marker of *P. jirovecii* pneumonia in patients with human immunodeficiency virus infection [15]. However, it is important to note that BDG testing may not be suitable for detecting *Mucorales* and certain basidiomycetous yeasts, such as *Cryptococcus* spp., as these fungal species do not possess BDG as a major component of their cell walls [16]. Several BDG assays have been developed, each with different cut-off values: the Fungitell Assay (cut-off value 60–80 pg/mL; Associates of Cape Cod, Inc.), the Wako β-glucan test (cut-off value 11 pg/mL; Fujifilm Wako Pure Chemical Corp.), and the Fungitec-G (cut-off value 20 pg/mL; Seikagaku) [16].

Limited data are available on the effectiveness of

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Table 1. Comparison of the 2008 EORTC/MSG and the updated 2020 EORTC/MSG guideline for defining probable/possible invasive pulmonary aspergillosis

Criteria	2008 EORTC/MSG [9]	2020 EORTC/MSG [10]
Definition of probable invasive pulmonary aspergillosis	Presence of a host factor, a clinical criterion, and a mycological criterion	
Definition of possible invasive pulmonary aspergillosis	Cases that meet the criteria for a host factor and a clinical criterion but for which mycological criteria are absent	
Host factor	Recent history of neutropenia, receipt of an allogeneic stem cell transplant, prolonged use of corticosteroids, treatment with other recognized T cell immunosuppressants, inherited severe immunodeficiency	Recent history of neutropenia, receipt of an allogeneic stem cell transplant, hematologic malignancy, receipt of a solid organ transplant, prolonged use of corticosteroid treatment with other recognized T cell immunosuppressants, treatment with recognized B cell immunosuppressants, inherited severe immunodeficiency, acute graft-versus-host disease grade III or IV
Clinical feature	For lower respiratory tract fungal disease The presence of 1 of the following 3 signs on CT: 1) Dense, well-circumscribed lesions(s) with or without a halo sign 2) Air crescent sign 3) Cavity	For pulmonary aspergillosis The presence of 1 of the following 4 patterns on CT: 1) Dense, well-circumscribed lesions(s) with or without a halo sign 2) Air crescent sign 3) Cavity 4) Wedge-shaped and segmental or lobar consolidation
Mycological evidence	1. Direct test (cytology, direct microscopy, or culture) Mold in sputum, BAL fluid, bronchial brush, or sinus aspirate samples, indicated by 1 of the following: 1-1) Presence of fungal elements indicating a mold 1-2) Recovery by culture of a mold 2. Non–culture-based diagnostic tests 2-1) Aspergillosis Galactomannan antigen detected in plasma, serum, BAL fluid, or CSF ≥0.5 2-2) 1,3 β-D-glucan detected in serum	Aspergillosis only 1. Galactomannan antigen Antigen detected in plasma, serum, BAL, or CSF Any 1 of the following: 1-1) Single serum or plasma: ≥1.0 1-2) BAL fluid: ≥1.0 1-3) Single serum or plasma: ≥0.7 and BAL fluid ≥0.8 1-4) 1 CSF: ≥1.0 2. *Aspergillus* PCR Any 1 of the following: 2-1) Plasma, serum, or whole blood 2 or more consecutive PCR tests positive 2-2) BAL fluid 2 or more duplicate PCR tests positive 2-3) At least 1 PCR test positive in plasma, serum, or whole blood and 1 PCR test positive in BAL fluid 3. *Aspergillus* species recovered by culture from sputum, BAL, bronchial brush, or aspirate

EORTC/MSG, European Organization for Research and Treatment of Cancer-Invasive Fungal Infections Cooperative Group/National Institute of Allergy and Infectious Diseases Mycosis Study Group; CT, computed tomography; BAL, bronchoalveolar lavage; CSF, cerebrospinal fluid; PCR, polymerase chain reaction.

BDG testing in diagnosing IFD in SOT recipients. Several studies have shown that the sensitivity and specificity of BDG testing using serum in SOT recipients range from 58% to 100% and from 9% to 83%, respectively [17-21] (Table 2). The precise cut-offs for optimal accuracy of BDG testing in diagnosing IFD may vary depending on the host and the pathogen [22]. Furthermore, a number of studies have evaluated the use of BDG testing as a diag-

nostic tool using bronchoalveolar lavage (BAL) fluid, but found it to have poor specificity [20,23]. Several factors have been identified that can interfere with the results of the BDG test. These include bloodstream infections with microorganisms such as *Pseudomonas aeruginosa* and *Streptococcus* species, the utilization of certain β-lactam antibiotics, specific anti-tumor chemotherapy agents, various fractionated blood components, and hemodialysis membranes [24]. Currently, the revised EORTC/MSG criteria have been modified to exclude BDG detection as a microbiologic criterion for IFD (Table 1) [10]. Due to the limited data on BDG testing in SOT recipients, aside from those with hematologic cancer, and the moderate performance of the test in these settings, routine testing is not currently recommended for SOT recipients. However, BDG detection is considered suitable for diagnosing probable IFD in specific clinical contexts. These include patients with hematologic malignancies (with or without neutropenia), posthematopoietic stem cell transplantation neutropenia, and select high-risk intensive care unit patients experiencing complications from gastrointestinal surgery with a clinical suspicion of infection [10]. Despite this, BDG testing can be considered as a method for excluding IFD in patients due to its relatively high negative predictive value [16].

Galactomannan Assay

Galactomannan, a key component of *Aspergillus* spp. cell walls, is secreted by the fungus during its growth phase. This makes it a useful biomarker for detecting the presence of actively growing *Aspergillus* within the human body [25]. In the 2002 consensus definition, the detection of an *Aspergillus* antigen, such as galactomannan, was considered a mycologic criterion for categorizing probable cases of IA [26]. Nevertheless, a commercial assay specifically designed for *Aspergillus* antigen detection was not widely available at that time. In May 2003, the Platelia *Aspergillus* EIA (Bio-Rad) was first approved by the United States Food and Drug Administration, enabling the detection of serum galactomannan [27]. This assay uses the monoclonal antibody EB-A2 for detecting galactofuranose side chains linked to the mannan backbone [28]. Galactofuranose, a six-member ring form of galactose, is found in various glycoconjugates and is produced by nonmammalian eukaryotes and some prokaryotic pathogens [29]. Originally considered specific to *Aspergillus*, it is now understood that galactofuranose is also present in other fungi and certain other substances, causing false-positive results [30-32]. This diagnostic test is particularly valuable when used in conjunction with other diagnostic approaches, such as microbiologic cultures, histologic biopsy examination, and radiological findings, for diagnosing IA [9]. In 2011, this test was further cleared for use with BAL fluid, but it can also be applied to cerebrospinal fluid (CSF), pleural fluid, and urine [33].

At present, the manufacturer suggests utilizing a cut-

Table 2. Performance of serum BDG testing for the diagnosis of invasive fungal disease in solid organ transplant recipients

Study	Type of transplantation	BDG testing method	BDG cut-off (pg/mL)	No. of total enrolled patients	No. of patients with invasive fungal disease (IC/IA)	Invasive fungal disease according to[a)]	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Akamatsu et al. (2007) [17]	Liver	Fungitec G test	40	180	7/5	EORTC/MSG 2002 criteria [26]	58	83	35	93
Alexander et al. (2010) [18]	Lung	Fungitell	60	73	6/6	EORTC/MSG 2008 criteria [9]	64	9	14	50
Levesque et al. (2015) [19]	Liver	Fungitell	146	52	4/0	EORTC/MSG 2008 criteria [9]	100	61	25	100
Mutschlechner et al. (2015) [20]	Various[b)]	Fungitell	100	135	0/23	EORTC/MSG 2008 criteria [9]	79.2	81.8	69.2	83.1
Levesque et al. (2017) [21]	Liver	Fungitell	80	271	11/7	EORTC/MSG 2008 criteria [9]	75	65	17	96

BDG, 1,3 β-D-glucan; IC, invasive candidiasis; IA, invasive aspergillosis; PPV, positive predictive value; NPV, negative predictive value; EORTC/MSG, European Organization for Research and Treatment of Cancer/Mycosis Study Group consensus.

[a)]Only patients with proven or probable invasive fungal disease; [b)]Including double lung transplantation, single lung transplantation, kidney transplantation, liver transplantation, combined heart and lung transplantation, heart transplantation, and combined heart and kidney transplantation.

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off optical density index (ODI) value of 0.5 for both serum and BAL fluid in galactomannan testing. To mitigate the issue of false positives, the updated version of the EORTC/MSG criteria suggests considering higher cut-offs of 1.0 for a single plasma, serum, BAL fluid, or CSF sample. If both serum and BAL fluid yield positive galactomannan results, the diagnostic criteria can be applied using cut-off values of ≥0.7 for serum and ≥0.8 for BAL fluid to support the diagnosis [10].

While serum and BAL galactomannan testing have proven to be valuable diagnostic tools for IA in patients with hematologic malignancies [34], their role in SOT recipients lacks established evidence due to the considerable variation in their performance in such individuals [35,36]. According to a meta-analysis, among SOT recipients, serum galactomannan testing exhibited low sensitivity (0.22; 95% confidence interval [CI], 0.03–0.60) and comparable specificity (0.84; 95% CI, 0.78–0.88). In contrast, bone marrow transplant recipients exhibited higher sensitivity and specificity, with values of 0.82 (95% CI, 0.70–0.90) and 0.86 (95% CI, 0.83–0.88), respectively [35]. For SOT recipients, the limited number of studies made it difficult to estimate overall accuracy (Table 3) [37-41]. In this context, the sensitivity in SOT recipients is notably low, possibly due to limited angioinvasion in these patients who have better immune capabilities compared to those with neutropenia in hematologic malignancy patients [42]. The lack of neutropenia in patients may lead to a reduced fungal burden, potentially due to their intact ability to clear fungal mannan from the bloodstream via macrophages [43,44]. In hematologic patients, galactomannan testing with BAL fluid showed higher sensitivity and specificity compared to serum [45-47]. According to a Cochrane review evaluating galactomannan testing with BAL in immunocompromised patients, an ODI cut-off value of 0.5 demonstrated a diagnostic sensitivity of 0.88 (95% CI, 0.75–1.00) and specificity of 0.81 (95% CI, 0.71–0.91) in 12 studies, while an ODI cut-off value of 1.0 showed a sensitivity of 0.78 (95% CI, 0.61–0.95) and specificity of 0.93 (95% CI, 0.87–0.98) in 11 studies [48]. For SOT recipients, several studies have demonstrated the diagnostic accuracy of galactomannan testing with BAL fluid for the diagnosis of IA [40,41,49]. Although galactomannan testing with BAL fluid offers greater sensitivity than serum, the optimal cut-off value for galactomannan testing in BAL fluid remains a topic of ongoing debate.

In lung transplant recipients, where *Aspergillus* colonization is common, the ability to accurately distinguish between colonization and IA remains unproven [40,50]. When using BAL fluid for testing, the rate of false-positive results was significantly higher among lung transplant recipients, due to the high prevalence of airway colonization in this group [40]. In addition, concomitant administration of piperacillin-tazobactam [30], colonization of other fungal antigens including *Penicillium, Paecilomyces*, and *Alternaria* species [31], and intravenous injection of Plasma-Lyte (Baxter) [32] have been identified as major causes of false-positive galactomannan results.

Aspergillus Polymerase Chain Reaction

Several researchers have investigated the diagnostic performance of *Aspergillus* PCR from serum or BAL fluid for IA. According to several meta-analyses, serum *Aspergillus* PCR displayed moderate diagnostic accuracy when used as a diagnostic test for invasive pulmonary aspergillosis in immunocompromised patients [51-53]. A Cochrane review including 29 primary studies estimated the sensitivity and specificity of a single positive test result

Table 3. Performance of the galactomannan assay for the diagnosis of IA in solid organ transplant recipients

Study	Type of transplantation	Type of sample	Galactomannan cut-off (ODI)	No. of total enrolled patients/no. of samples	No. of patients with proven/probable IA/ no. of samples	Sensitivity (%)	Specificity (%)
Fortun et al. (2001) [38]	Liver	Serum	1.0	42/126	9/45	55.6	93.9
Husain et al. (2004) [37]	Lung	Serum	0.5	70/891	12/201	30	93
Kwak et al. (2004) [39]	Liver	Serum	0.5	154/1,594	1/31	NA[a]	87
Clancy et al. (2007) [40]	Various[b]	BAL	1.0	81/NA	5/NA	100	90.8
Husain et al. (2007) [41]	Lung	BAL	0.5	116/333	6/28	60	95
Pasqualotto et al. (2010) [49]	Lung	BAL	1.5	60/60	8/8	100	90.4

IA, invasive aspergillosis; ODI, optical density index; NA, not available; BAL, bronchoalveolar lavage.

[a]The sensitivity could not be meaningfully assessed due to the fact that there was only a single case of IA; [b]Including heart transplantation (n=24), kidney transplantation (n=22), liver transplantation (n=19), and lung transplantation (n=16).

as 0.79 (95% CI, 0.71–0.86) and 0.80 (95% CI, 0.70–0.87), respectively. For two consecutive positive test results, the summary estimates were 0.60 (95% CI, 0.41–0.76) for sensitivity and 0.95 (95% CI, 0.87–0.98) for specificity [51]. Two positive PCR results are required to confirm the diagnosis because the specificity is higher than that attained from a single positive test [52]. In addition to serum *Aspergillus* PCR testing, *Aspergillus* PCR from BAL fluid is recommended for diagnosing IA in severely immunocompromised patients, including SOT recipients, according to the latest guidelines from the American Thoracic Society and the EORTC/MSG consensus definition [10,54]. A systematic meta-analysis of BAL fluid *Aspergillus* PCR assay for the diagnosis of IA showed that the overall sensitivity and specificity were 0.75 and 0.94, respectively, based on 41 studies among heterogeneous groups [55].

Most studies have focused on patients with hematologic malignancies, leaving a gap in the literature regarding the evaluation of *Aspergillus* PCR performance in SOT recipients. Botterel et al. [56] proposed that performing concurrent *Aspergillus* PCR on the initial galactomannan test-positive serum sample could enhance the specificity of the galactomannan assay result, as evidenced in a study of 484 liver transplant recipients. In a separate study involving 150 BAL fluid samples from lung transplant recipients, Luong et al. [57] found that the sensitivity and specificity of pan-*Aspergillus* PCR, which targets the 18S ribosomal DNA region of all *Aspergillus* species, were 1.0 (95% CI, 0.79–1.00) and 0.88 (95% CI, 0.78–0.92), respectively. Furthermore, the sensitivity and specificity of *Aspergillus fumigatus*-specific real-time PCR assays were 0.85 (95% CI, 0.55–0.98) and 0.96 (95% CI, 0.92–0.99), respectively. The study noted a false positive rate of 12%, which could be attributed to procedural factors (e.g., environmental contamination with *Aspergillus* species, cross-reaction with sequences from other fungal species such as *Penicillium* species) or clinical factors such as respiratory tract colonization with *Aspergillus* species [57]. The primary limitations of *Aspergillus* PCR testing include the difficulty in differentiating between colonization and active infection, the lack of a standardized PCR methodology, and the inability to identify *Aspergillus* subspecies [58]. With recent advances in the standardization of laboratory procedures [59], various commercial PCR assays such as AsperGenius (target gene: 28S rDNA, PathoNostics) and MycAssay *Aspergillus* (target gene: 18S rDNA, Myconostica), have been developed and become available [60]. Montesinos et al. [61] evaluated the AsperGenius assay using BAL fluid from 100 patients, including 23 lung transplant recipients. The AsperGenius assay detected *Aspergillus* species in 27% (27/100) of patients using a standard cycle threshold value of <36. Importantly, 19 of these patients were diagnosed with proven or probable IA, and 47% were lung transplant recipients [61].

The extensive and prolonged use of broad-spectrum azoles has significantly contributed to the widespread emergence of azole-resistant *Aspergillus* spp. These resistant strains have been linked to therapeutic failure and high mortality rates [62,63]. A single-center study reported that the prevalence of azole-resistant *A. fumigatus* among lung transplant patients was 17% [61]. Traditional antifungal susceptibility testing is time-consuming and labor-intensive. As a result, molecular-based methods such as PCR have been developed to detect mutations in the *cyp51A* gene, which is the primary mechanism of triazole resistance in *A. fumigatus* [64]. The most common mutations in the *A. fumigatus cyp51A* gene conferring triazole resistance were TR34 (a 34-bp tandem repeat in the promoter region)/L98H, TR46/T289A/Y121F mutations, and mutations at codons 54 and 220 in *cyp51A* gene [65]. Several commercial assays and in-house assays have been developed to detect mutations in this region, using various strategies [66].

Aspergillus PCR testing may prove beneficial in differentiating between clinically significant *Aspergillus* species and identifying strains of *Aspergillus* spp. that are resistant to triazole. When judiciously applied in clinical settings where IA is suspected, this assay could serve as a crucial supplementary diagnostic tool.

CONCLUSION

Under the revised EORTC/MSG criteria, the introduction of two consecutive positive *Aspergillus* PCR tests using serum, plasma, or BAL fluid as a mycological criterion was combined with traditional fungal culture. Furthermore, the cut-off ODI value for positive galactomannan testing was increased to an ODI ≥1.0 for single serum/plasma or BAL fluid, while BDG testing was excluded from these revised criteria [10,67]. For patients with hematological malignancies, the application of these new criteria resulted in the identification of more episodes of possible and probable IA, with the most significant increase seen in probable IA cases, primarily due to *Aspergillus* PCR. Although there

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was no significant difference in mortality rates between the redefined possible and probable IA groups, probable cases with lower cycle threshold values showed higher mortality compared to those with higher cycle threshold values [68]. Since that study was not conducted among SOT recipients, there is insufficient evidence to conclude the usefulness of *Aspergillus* PCR for these SOT recipients. In particular, *Aspergillus* PCR testing using BAL fluid in lung transplantation patients has the problem of causing false positive results due to colonization. In addition, the ODI cut-off value of the galactomannan assay, which is currently designated as 1.0 by EORTC/MSG, has also been a matter of debate.

By comprehensively implementing these culture-independent assays in routine diagnosis, we can potentially optimize the management of IA, which could ultimately lead to improved treatment outcomes for SOT recipients.

ARTICLE INFORMATION

Conflict of Interest
No potential conflict of interest relevant to this article was reported.

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ORCID
In Young Yoo https://orcid.org/0000-0003-1505-846X
Yeon-Joon Park https://orcid.org/0000-0003-2182-5821

Author Contributions
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