Early postoperative urinary MCP-1 as a potential biomarker predicting acute rejection in living donor kidney transplantation: a prospective cohort study

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We investigated the clinical relevance of urinary cytokines/chemokines reflecting intrarenal immunologic micromilieu as prognostic markers and the optimal measurement timing after living donor kidney transplantation (LDKT). This prospective cohort study included 77 LDKT patients who were followed for ≥ 5 years. Patients were divided into control (n = 42) or acute rejection (AR, n = 35) group. Early AR was defined as AR occurring within 3 months. Serum and urine cytokines/chemokines were measured serially as follows: intraoperative, 8/24/72 h, 1 week, 3 months, and 1 year after LDKT. Intrarenal total leukocytes, T cells, and B cells were analyzed with immunohistochemistry followed by tissueFAXS. Urinary MCP-1 and fractalkine were also analyzed in a validation cohort. Urinary MCP-1 after one week was higher in the AR group. Urinary MCP-1, fractalkine, TNF-α, RANTES, and IL-6 after one week were significantly higher in the early AR group. Intrarenal total leukocytes and T cells were elevated in the AR group compared with the control group. Urinary fractalkine, MCP-1, and IL-10 showed positive correlation with intrarenal leukocyte infiltration. Post-KT 1 week urinary MCP-1 showed predictive value in the validation cohort. One-week post-KT urinary MCP-1 may be used as a noninvasive diagnostic marker for predicting AR after LDKT.

Abbreviations
AR  Acute rejection
AUROC  Area under the receiver operating characteristic
CKD  Chronic kidney disease
CI  Confidence interval
CV  Coefficient of variation
DSA  Donor specific antibody
eGFR  Estimated glomerular filtration rate
ESKD  End-stage kidney disease
ICC  Intraclass correlation coefficient
KT  Kidney transplantation

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Kidney transplantation (KT) is the final and definitive treatment option for end-stage kidney disease (ESKD) patients. Early prediction of acute rejection (AR) and overall renal allograft function is crucial for appropriate management considering long-term outcome of KT. Although kidney biopsy is the gold standard to diagnose rejection in KT patients, it is not convenient or feasible as a follow-up diagnostic tool because of its invasiveness, especially during the immediate postoperative period after KT. Therefore, non-invasive surrogate markers for early diagnosis and treatment of AR are required.

With the advance of molecular biology, a number of biomarkers have been evaluated for prediction, diagnosis, and risk stratification in KT patients. However, there is no consensus regarding specific types of noninvasive biomarkers and timing of sample acquisition in KT patients. Urinary cytokines and chemokines have been reported as promising biomarkers substituting kidney biopsy because urine was expected to contain inflammatory mediators involved in alloimmune responses leading to AR or interstitial fibrosis/tubular atrophy after KT. Since the association of pretransplant serum levels of CXCL9 and AR was reported, urinary CXCL9 at 6 months post-KT was suggested as a promising biomarker in KT patients. If specific urine cytokines/chemokines can be used as surrogate markers substituting kidney biopsy, especially during the early postoperative period, it would be very helpful for both KT patients and physicians.

In this study, we aimed to evaluate the diagnostic and prognostic potential of urinary cytokines/chemokines at different time points after KT and to identify specific cytokines/chemokines reflecting intrarenal immunologic micromilieu as well as the optimal sample acquisition time for early diagnosis of AR.

### Results

#### Baseline characteristics.

Table 1 shows patient baseline characteristics according to the group. Patients’ age ranged from 18 to 69 years (mean: 42.4 years) and 52 patients were male. Three patients received a second KT. A total of 35 patients experienced AR after KT. There was no difference in age or sex between the control and AR groups. Hypertension was more prevalent in the control group while lupus nephritis, obstructive nephropathy, and Alport syndrome were more prevalent in the AR group. There were no differences in proportions of induction therapy agents and ABO-incompatible KT. The proportions of patients with ABO incompatibility, PRA positivity, and DSA positivity were comparable between the groups.

Changes in renal function for 5 years after KT. Changes in renal function were assessed as serial changes in eGFR over 5 years. Renal function of the AR group was comparable with the control group until
3 months after KT. The mean eGFR from 2 years after KT was significantly lower in the AR group compared with the control group. (P < 0.05) (Fig. 1).

**Postoperative serial follow-ups of urinary cytokines/chemokines.** Urinary level of MCP-1 at 1 week after KT was higher in the AR group (Fig. 2a). To compare the nearest previous value of urinary cytokines/chemokines from when AR was diagnosed, urinary samples at 1 week after KT (1–9 weeks prior to biopsy) from 13 patients who experienced early AR within 3 months and the control group were compared. Urinary fractalkine, MCP-1, RANTES, TNF-α, and IL-6 at 1 week after KT were significantly higher in patients who were diagnosed with early AR within 3 months compared with the control group (Fig. 2b).

**Postoperative serial follow-ups of serum cytokines/chemokines.** Serial changes in serum cytokines/chemokines according to AR are shown in Fig. 3. Serum cytokines/chemokines at all time points were comparable between the control and AR groups. Serum cytokines/chemokines at each time point were not correlated with urinary cytokines/chemokines.

**Correlations between urinary cytokines/chemokines and intrarenal leukocyte infiltration.** Intrarenal infiltration of total leukocytes, T cells, and B cells was analyzed in renal allograft biopsy tissues using immunohistochemistry with anti-CD45, anti-CD3, and anti-CD20. Total leukocytes, T cells, and B cells were significantly elevated in the AR group (Fig. 4).

Urinary fractalkine, MCP-1, and IL-10 showed positive correlation with total leukocyte infiltration. Urinary excretion of fractalkine, MCP-1, and IL-10 was positively correlated with intrarenal T cells and B cells (Fig. 5).

**Potential value of urinary MCP-1 and fractalkine for predicting AR and early AR.** Potential predictive value of urinary MCP-1 and fractalkine was further analyzed using sensitivity analysis. The best thresholds for predicting early AR and AR were 1100 pg/mg for urinary MCP-1/creatinine and 300 pg/mg for urinary fractalkine/creatinine. The results of the sensitivity analysis are summarized in Table 2.
a Fractalkine

RANTES

IL-4

IL-10

VEGF

MCP-1

TNF-α

IL-6

IL-10

Figure 2. Urinary cytokines/chemokines of the control and AR groups. (a) Serial changes in urinary cytokines/chemokines of the control and AR groups. Urinary MCP-1 at 1 week post-KT was significantly higher in the AR group. (b) Post-KT 1-week urinary cytokines/chemokines of the control and early AR (AR within 3 months) groups. The early AR group showed higher urinary fractalkine, MCP-1, RANTES, TNF-α, and IL-6 compared with the control group. *P < 0.05 compared with the control group. AR acute rejection, Cr creatinine, IL interleukin, KT kidney transplantation, MCP-1 monocyte chemoattractant protein-1, RANTES regulated on activation, normal T cell expressed and secreted, TNF-α tumor necrosis factor-α, VEGF vascular endothelial growth factor.
Figure 2. (continued)
Validation of urinary MCP-1 and fractalkine. Internal validation was performed using bootstrapping (bootstrap B = 1000). The area under the receiver operating characteristic (AUROC) of urinary MCP-1 predicting early AR and AR was 0.795 (95% confidence interval (CI) 0.659–0.931) and 0.691 (95% CI 0.563–0.818), respectively (Fig. 6a). AUROC of urinary fractalkine predicting early AR and AR was 0.670 (95% CI 0.485–0.854) and 0.643 (95% CI 0.511–0.776), respectively (Fig. 6b).

Urinary MCP-1 and fractalkine were further validated using ELISA in an independent cohort (Table 3). Time separate independent cohort included a total of 79 patients with 38 patients in the control group and 41 patients in the AR group. In the AR group, 28 patients had early AR. A logistic regression of AR and early AR on a continuous, normally distributed variable (X) with a sample size of 79 observations achieves 80% power at a 0.05 significance level to detect an odds ratio of 1.96 and 2.03, respectively, when the prevalence of AR and early AR in the population is 0.52 and 0.35, respectively 11. In this independent cohort, post-KT 1 week urinary
MCP-1 was significantly higher in the early AR and AR groups than in the control group (Fig. 7a). However, post-KT 1 week urinary fractalkine was comparable in all groups (Fig. 7b).

Reliability of measurements for triplicates of each sample was analyzed with both variability and absolute agreement using coefficient of variation (CV) and intraclass correlation coefficient (ICC), respectively. MCP-1 and fractalkine showed low variability (CV = 0.181 and 0.091), and high agreement (ICC = 0.972 [0.959, 0.981] and 0.956 [0.932, 0.972]) \(^{12,13}\), showing good reliability of measurements for both MCP-1 and fractalkine.

**Discussion**

In this study, the diagnostic and prognostic potentials of several serum and urinary cytokines/chemokines were analyzed with serially collected urine samples after LDKT. Urinary MCP-1 at 1 week after KT was identified as an important predictive factor of AR development. Urinary fractalkine, TNF-α, RANTES, and IL-6 at 1 week after KT showed predictive potential for early AR within 3 months post-KT. Urinary fractalkine, MCP-1, and IL-10 exhibited positive correlation with intrarenal total leukocytes, T cells, and B cells. These results suggest clinical usefulness of urinary cytokines/chemokines for early diagnosis of AR in LDKT patients and support the potential role of urinary cytokines/chemokines as surrogate markers of intrarenal immunologic micromilieu.
AR is a serious problem after KT and exerts significant impacts on both allograft and patient survival. Early diagnosis and treatment of AR are crucial for long-term allograft survival. Although kidney biopsy is the gold standard for assessing kidney injury, timely detection of early alterations in allograft immunologic micromilieu has not been feasible with current techniques. Therefore, new biomarkers for predicting changes in intrarenal immunologic micromilieu after KT are required. Ideal biomarkers should be measured reproducibly in noninvasively collected samples. Urine biomarkers are ideal for immune monitoring after KT because they are directly produced by the allograft and can be collected noninvasively and repeatedly. Cytokines and chemokines

**Figure 5.** Correlation between intrarenal leukocytes infiltration and urinary cytokines/chemokines. (a) Urinary fractalkine, MCP-1, and IL-10 showed positive correlation with infiltration of total leukocytes expressing CD45. (b) Urinary fractalkine, MCP-1, and IL-10 showed positive correlation with intrarenal T cells expressing CD3. (c) Urinary fractalkine, MCP-1, and IL-10 showed positive correlation with intrarenal B cells expressing CD20. CD clusters of differentiation, Cr creatinine, IL interleukin, MCP-1 monocyte chemoattractant protein-1.

**Table 2.** The sensitivity analyses for post-KT 1 week urinary MCP-1 and fractalkine. AR acute rejection, KT kidney transplantation, MCP-1 monocyte chemoattractant protein-1, NPV negative predictive value, PPV positive predictive value.
have been reported to play crucial roles in controlling immune responses within renal allografts\textsuperscript{16}. After KT, proinflammatory cytokines are released from allografts and subsequently induce local chemokine secretion\textsuperscript{17,18}. Based on urinary cytokines/chemokines can reflect overall changes in intrarenal cytokines/chemokines, our study aimed to find the most appropriate timing and types of urinary cytokines/chemokines during the early postoperative period for predicting AR.

In our study, urinary fractalkine, MCP-1, RANTES, TNF-\(\alpha\), and IL-6 at 1 week after KT were significantly higher in patients who developed early AR within 3 months post-KT. Urinary MCP-1 at 1 week post-KT showed the most prominent increment in early AR patients compared with the control group. Elevation of serum proinflammatory cytokines and chemokines depending on allograft status was previously reported in KT patients\textsuperscript{19,20}. MCP-1 and RANTES, important members of the C–C chemokine family, are known to substantially contribute to glomerular and tubulointerstitial damage in diabetic nephropathy through a positive feedback loop of inflammation\textsuperscript{21}. Blocking of the MCP-1/CCR2 chemokine pathway was reported to improve survival of islet
Urinary MCP-1 was also reported to be elevated in patients with AR or polyomavirus urinary protein excretion per se already includes urinary cytokines/chemokines. We also protocolized the protein-to-creatinine ratio as a denominator can cause other confounding effects because the total amount of procedure for urine collection, storage, and analysis since standardized management of urine samples is critical for consistent results. Third, some discrepancies in the types of cytokines/chemokines showing correlations with creatinuria have been reported in KT patients. Although our findings were consistent with previous reports and further revealed that excretion of these proinflammatory cytokines/chemokines increased early 1 week after KT in patients at risk for AR, external validation of urinary fractalkine using an independent cohort did not show reproducible results. Further studies in a larger cohort may be required to evaluate the clinical value of urinary fractalkine as a biomarker in LDKT patients. The activity and subsets of intrarenal leukocytes are known to change actively within renal allografts and create a specific intrarenal immune environment such as renal tissue memory T cells and B cells. A previous study analyzing the mechanisms of AR in a baboon KT model reported a significant correlation between urinary expression of interferon-γ inducible protein-10 and monokine. Our study supports the clinical usefulness of urinary cytokines/chemokines, especially urinary fractalkine and MCP-1, as surrogate biomarkers that reflect the intrarenal immunologic micromilieu.

A few limitations of this study require consideration. First, there were some time differences in the collection of urine samples and kidney biopsies, ranging from 5 days to 1 month, which was inevitable given the prospective study design. Our results support these previous findings and further reveal the diagnostic potential of urinary MCP-1 as a surrogate biomarker for predicting AR via serial measurement. Although our findings were consistent with previous reports and further revealed that excretion of these proinflammatory cytokines/chemokines increased early 1 week after KT in patients at risk for AR, external validation of urinary fractalkine using an independent cohort did not show reproducible results. Further studies in a larger cohort may be required to evaluate the clinical value of urinary fractalkine as a biomarker in LDKT patients.

Table 3. Baseline characteristics, induction therapy for kidney transplantation, and immunologic risk factors of the validation cohort. Continuous variables are expressed as mean (standard deviation) and categorical variables are expressed as number (percentage). Others for cause of ESKD included autosomal dominant polycystic kidney disease, lupus nephritis, obstructive nephropathy, and Alport syndrome. % in patients with PRA, mean ± standard deviation. AR acute rejection, ATG anti-thymocyte globulin, DSA donor specific antibodies, ESKD end-stage kidney disease, KT kidney transplantation, PRA panel reactive antibodies, SD standard deviation.

| Total (n = 79) | Control (n = 38) | AR (n = 41) | P value |
|---------------|----------------|------------|---------|
| Age, mean (SD) (years) | 46.9 (11.2) | 47.5 (10.7) | 46.4 (11.7) | 0.655 |
| Male, number (%) | 43 (54.4) | 18 (47.4) | 25 (70.0) | 0.263 |
| Cause of ESKD, number (%) | 0.039 |
| Diabetes mellitus | 23 (29.1) | 14 (36.8) | 9 (22.0) | 0.145 |
| Hypertension | 7 (8.9) | 6 (15.8) | 1 (2.4) | 0.051 |
| Glomerulonephritis | 33 (41.7) | 10 (26.4) | 23 (56.1) | 0.007 |
| Others | 7 (8.9) | 4 (10.5) | 3 (7.3) | 0.705 |
| Unknown | 9 (11.4) | 4 (10.5) | 5 (12.2) | 1.000 |
| Induction therapy, number (%) | > 0.999 |
| Basiliximab and steroid | 43 (54.4) | 19 (50.0) | 24 (58.5) | |
| ATG and steroid | 12 (15.2) | 7 (18.4) | 5 (12.2) | |
| Rituximab and steroid | 24 (30.4) | 12 (31.6) | 12 (29.3) | |
| ABO-incompatible KT, number (%) | 0.393 |
| PRA, class I (+), number (%) | 19 (23.1) | 9 (23.7) | 10 (24.4) | > 0.999 |
| % in patients with PRA, class I | 32.3 ± 29.41 | 51.3 ± 29.60 | |
| PRA, class II (+), number (%) | 0.772 |
| % in patients with PRA, class II | 14 (17.7) | 6 (15.8) | 8 (19.5) | 0.3145 |
| DSA (+), number (%) | 0.145 |
| % in patients with DSA | 10 (12.7) | 3 (7.9) | 7 (17.1) | 0.3145 |

allografts. Intrarenal tissue expression of RANTES and MCP-1 was elevated in KT patients with chronic allograft dysfunction. Urine MCP-1 was also reported to be elevated in patients with AR or polyomavirus nephropathy. However, most previous studies investigated these cytokines/chemokines using a cross-sectional study design. Our results support these previous findings and further reveal the diagnostic potential of urinary MCP-1 as a surrogate biomarker for predicting AR via serial measurement.
AR or intrarenal leukocytes were found. Further studies including a large cohort of KT patients are required to confirm the types of urinary cytokines/chemokines that more precisely reflect both cellular and humoral factors of intrarenal immunologic micromilieu. Fourth, a power study estimation could not be performed in our original cohort because of the prospective design and serial measurements of multiple serum and urine samples. The validation cohort was designed with a power study estimation to overcome this limitation.

In conclusion, urinary MCP-1 at 1 week after KT can be used as a noninvasive surrogate marker for predicting early AR in LDKT patients. Moreover, positive correlation with intrarenal leukocytes infiltration and urinary fractalkine, MCP-1, and IL-10 suggests the clinical usefulness of these urinary cytokines/chemokines as biomarkers that reflect the intrarenal immunologic micromilieu.

Methods

Study design and participants. This prospective cohort study recruited 77 adult patients who underwent living donor KT (LDKT) from April 2011 to August 2013 at Samsung Medical Center and were followed-up for more than 5 years. Adult (≥ 18 years) LDKT patients with no history of cancer whose urine samples were collected at each time point were included in the study. Exclusion criteria were (1) serum samples missing at more than 3 time points, (2) patients with autosomal-dominant polycystic kidney disease who received simultaneous bilateral nephrectomy of native kidneys with LDKT, and (3) significant bleeding or infection during the immediate postoperative period.

Validation cohort for post-KT 1 week urinary MCP-1 and fractalkine included 79 adult patients who underwent LDKT from September 2013 to December 2015. Sample size was determined to achieve 80% power at a 0.05 significance level considering the incidence of AR and early AR.*

This study was approved by the Institutional Review Board of Samsung Medical Center in compliance with the Declaration of Helsinki (IRB number: 2011-06-117). Informed consent was obtained from all participants before enrollment.

Data collection. Clinical data including baseline characteristics, induction therapy for KT, immunologic risk factors, and serial changes in renal function were collected from the electronic medical record system. Immunologic risk factors included the presence of ABO incompatibility, panel reactive antibodies (PRA), and donor specific antibody (DSA).

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*Figure 7. Post-KT 1 week urinary MCP-1 and fractalkine in a validation cohort. (a) In the validation cohort including 79 patients, both early AR and AR groups showed significantly higher urinary MCP-1 at 1 week after KT than the control group. (b) Post-KT 1 week urinary fractalkine was comparable in both control and AR groups. AR acute rejection, KT kidney transplantation, MCP-1 monocyte chemoattractant protein-1.
Patients who did not experience AR were classified into the control group and those who developed acute cellular rejection were classified as the AR group. Renal allograft biopsy was used as the diagnostic standard and AR was scored according to the Banff criteria. There were no cases of antibody-mediated rejection. Early AR was defined as AR within 3 months. Renal function was compared with the estimated glomerular filtrate rate (eGFR), which was calculated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation.

Sample collection and cytokine/chemokine assays. Both urine and serum samples were serially collected at the following time points to measure cytokines/chemokines: intraoperative (during KT) and postoperative 8 h, 24 h, 72 h, 1 week, 3 months, and 1 year. The collected urine samples were centrifuged at 2500 rpm for 15 min under −4 °C. The supernatant of each sample was aliquoted with 1.4 mL of urine and 1.0 mL of serum. After adding 1/100 volume of protease inhibitor and 0.1 M PMSF (Sigma Aldrich, St. Louis, MO, USA) to each sample, all samples were stored at −70 °C until analysis. A total of 8 cytokines/chemokines were measured in each sample as follows: regulated on activation, normal T cell expressed and secreted (RANTES), fractalkine, interleukin (IL)-4, IL-6, IL-10, monococyte chemoattractant protein (MCP)-1, tumor necrosis factor (TNF)-α, and vascular endothelial growth factor (VEGF). These 8 cytokines/chemokines were measured using a Milliplex MAP human cytokine/chemokine kit (Merck Millipore, Burlington, MA, USA) according to the manufacturer’s instructions. In the validation cohort, urinary MCP-1 and fractalkine were measured using Quantikine ELISA Kits (R&D Systems, Inc. Minneapolis, MN, USA) according to the manufacturer's instructions. The concentrations of urinary cytokines/chemokines (pg/mL) were normalized by dividing the raw value by urinary creatinine concentration (mg/dL). Therefore, final values of urinary cytokines/chemokines are expressed as pg/mg.

Quantification of infiltrating immune cells into renal allografts. Renal allograft biopsy was performed between 12 and 14 days after KT as protocol biopsies in 37 patients or when AR was suspected in 9 patients. Immunohistochemical staining with CD45, CD3, and CD20 was performed on formalin-fixed kidney tissues. Sections (4 μm) were deparaffinized with xylene, rehydrated in a graded alcohol series, and then placed in citrate buffer solution (pH 6.0). Slides were placed in a pressure cooker and heated with microwaves for 10 min to enhance antigen retrieval. After cooling, sections were immersed in hydrogen peroxide solution (DAKO, Carpinteria, CA, USA) for 30 min to block endogenous peroxidase and then treated with serum-free protein block (DAKO) at 4 °C overnight. The next day, slides were incubated for 1 h at room temperature with a 1:100 dilution of monoclonal antibody to CD45, CD3, and CD20. After incubation of slides with a mixed solution containing dextran coupled with peroxidase molecules and goat secondary antibody molecules (DAKO) for 30 min at room temperature, 3,3′-diaminobenzidine tetrahydrochloride (DAKO) was applied to the slides to develop a brown color. Then, slides were counterstained with Mayer’s hematoxylin (DAKO). To calculate the percentage of CD45-, CD3-, and CD20-positive cells among total nucleated cells in renal allografts, the whole field of the slide was scanned and analyzed with TissueFAXS. (TissueGnostics, Vienna, Austria).

Statistical methods. All results were expressed as mean ± standard deviation (SD) or standard error of the mean (SEM) as appropriate. SPSS 24.0 statistical software (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 9 (GraphPad Software, La Jolla, CA, USA) were used for statistical analyses. Group means were compared using the Mann–Whitney test. The Chi-square test or Fisher’s exact test was used for comparing baseline characteristics between the control and AR groups. The correlations between each cytokine/chemokine and intrarenal leukocytes, and post-KT 1 week urinary and serum MCP-1 or fractalkine were assessed using the Pearson R correlation. Internal validation of post-KT urinary MCP-1 and fractalkine was performed using bootstrapping (bootstrap B = 1000) by calculating AUROC.

Power analysis was performed using PASS software (PASS 2021: v21.0.2, NCSS Statistical Software, Kaysville, UT, USA). Reliability of measurements was evaluated with coefficient of variation (CV) and intraclass correlation coefficient (ICC) [1, 2]. Statistical significance was determined when the P value was <0.05.

Data availability
The data that support the findings of this study are available on reasonable requests from the corresponding author.

Received: 16 May 2021; Accepted: 1 September 2021
Published online: 22 September 2021

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Acknowledgements

We thank the staff of the organ transplantation center and the nursing team of Samsung Medical Center, Sungkyunkwan University School of Medicine. We deeply appreciate the great technical support from Ji Hyun Kang, Kyungyi Choi, Ji Woo Kim, and Wu Hyun Lee at the Samsung Biomedical Research Institute, Samsung Medical Center, Sungkyunkwan University School of Medicine. Hye Ryoun Jang was supported by a grant from the Korea
Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare, Republic of Korea (Grant number: HC20C0085). Kyungho Lee was supported by a grant from the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare, Republic of Korea (Grant number: HI19C1337). Wooseong Huh was supported by the Samsung Biomedical Research Institute (Grant number: OTC 1600781) and the National Research Foundation of Republic of Korea (Grant number: 2017R1D1A1B04032172).

**Author contributions**

H.R.J. and W.H. conceived and designed the experiments. H.R.J. and M.K. performed the experiments and wrote the manuscript. H.R.J., M.K., S.H., K.L., M.Y.P., K.E.Y., C.J.L., J.J., K.K., and G.Y.K. analyzed the data. K.W.L., J.E.L., J.B.P., Y.G.K., and D.J.K. contributed reagents/materials/analytical tool. All named authors have read the journal's authorship agreement and reviewed the manuscript.

**Funding**

This study was supported by the Samsung Biomedical Research Institute (Grant number: OTC 1600781) and a grant from the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare, Republic of Korea (Grant number: HC20C0085).

**Competing interests**

The authors declare no competing interests.

**Additional information**

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