A modified LC-MS/MS method for the detection of whole blood tacrolimus and its clinical value in Chinese kidney transplant patients

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ABSTRACT

Background: For patients who received tacrolimus after kidney transplant, therapeutic drug monitoring is essential to improve their prognosis. However, previous detection methods have limitations, such as the overestimation and unacceptable bias in the immunoassays. Precision medicine has been challenged. The liquid chromatography-tandem mass spectrometry (LC-MS/MS) method is recognized as the gold standard due to its accuracy and specificity, but lack of throughput and complex process limits its clinical application. Therefore, an accurate, simple and high throughput method for tacrolimus monitoring is needed for clinical practice.

Methods: A modified LC-MS/MS method was introduced and validated. Whole blood samples were prepared by a one-step protein precipitation method. Chromatographic separation was achieved using a Phenomenex Kinetex 2.6 μm XB-C18 2.1 × 50 mm column with a total run time of 3.5 min to avoid matrix effect. An electrospray ionization source (ESI) was used in positive ion multiple reaction monitoring (MRM) mode for mass spectrometric detection. In order to protect the mass spectrometer, only part of the sample after LC separation was allowed to enter the mass spectrum, through a two HPLC systems coupled one mass spectrometry design. In this way, the instrument throughput is also improved and realizing the detection of 2 samples within 3.5 min and carried out a shorter analyzing time for each sample of 1.75 min. Additionally, we calculated tacrolimus-intrapatient variant (Tac-IPV) based on this modified method and assessed the prognostic value of Tac-IPV in Chinese kidney transplant patients.

Results: The LC-MS/MS was modified by streamlining the procedure and increasing the throughput. The method proved to be accurate and reproducible with all performance parameters suitably meeting the clinical requirements over a calibration ranged from 0.37 to 42.90 ng/mL. Parameters such as linearity, limit of quantification (LoQ) and dilution integrity were validated with a clinical reportable range from 0.37 to 343.20 ng/mL, which was particularly useful for high drug concentrations patients (rare but very serious). Both cross-contamination and matrix effects were negligible. Clinical data of 83 patients showed that Tac-IPV was associated with poor kidney transplant outcome in Chinese (Hazard Ratio (HR) = 3.96, 4.75; 95% CI: 1.10–14.21, 1.23–18.16; P < 0.05).

Conclusions: This modified LC-MS/MS method possessed high throughput and simple sample preparation, allowing it to meet daily clinical needs. At the same time, Tac-IPV based on this modified LC-MS/MS had excellent prognostic value in kidney transplantation. These advantages have great significance for the individualized treatment of Chinese kidney transplant patients and broad application of Tac-IPV.

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1. Introduction

Tacrolimus is the most potent calcineurin inhibitors which is used almost universally as a part of triple immunosuppressive therapy after kidney transplantation to improve the patients’ quality of life and their survival. Considerable researches have shown that tacrolimus-intrapatent variant (Tac-IPV) is associated with the long term outcome for the kidney transplant recipients [1, 2, 3]. But the clinical indicator is less well-studied in Chinese patients. Due to the narrow therapeutic window and large individual variations of tacrolimus, precision medicine is largely limited [4, 5]. A low concentration results in insufficient immunosuppression and acute rejection, while a high concentration increases the risk of drug toxicity [6, 7, 8]. Therefore, routine monitoring of whole blood tacrolimus is a must for transplanted patients.

Currently, the most widespread methods for tacrolimus detection are immunoassays and LC-MS/MS. Immunoassays lacks of specificity and accuracy due to monoclonal antibodies cross-reactivity with drug metabolites, resulting in overestimation of the drug concentration with unacceptable bias [9, 10], thereby impacting the clinical decision making. Despite immunoassays having been optimized and improved in recent years, LC-MS/MS is still recognized as the gold standard. However, some LC-MS/MS described in the previous study have shortcomings too [11]. First, multi-step protein precipitation or liquid-liquid extraction increases the risk of drug toxicity [6, 7, 8]. Therefore, routine monitoring of whole blood tacrolimus is a must for transplanted patients.

In this study, a rapid, simple and high throughput tacrolimus detection method was developed and validated. Additionally, we applied the modified LC-MS/MS method to clinical practice and assessed the prognostic value of Tac-IPV in Chinese kidney transplant patients.

2. Subjects and methods

2.1. Tacrolimus assays

2.1.1. Reagents

Tacrolimus standard was purchased from LC Laboratories and calibration 6 PLUS 1 Multilevel Calibrator Set Immunosuppressants In Whole Blood was purchased from CHROMSYSTEMS. The quality control agents (QCs) Lyphocheck Whole Blood Control Immunosuppressant Control was purchased from Bio-Rad. Ascomycin (ASC) was purchased from Toronto Research Chemicals Inc, and was used for internal standard (IS). ASC has been proved to have no effect on the quantitation of tacrolimus [20]. Formic acid and zinc sulfate were from Sigma, and were of reagent grade. Other solvents and reagents were HPLC grade. Ultrapure water of 18.2 MΩ cm resistivity was obtained from a Milli-Q (Millipore) water purification system.

2.1.2. Sample preparation

30 μL of calibrators, quality controls, and well-mixed whole blood were added into 300 μL of internal standard extracting solution containing 3.0 ng/mL ASC, 50 mmol/L ZnSO4, and 50% methanol/water separately. Each centrifuge tube was vortexed for more than 30s and then mixed for 5 min (Vortex mixer V3, Essenscien). After 5 min centrifugation at 14,000 rpm at 4 °C, at least 100 μL of the supernatant was pipetted into a sample vial for LC-MS/MS analysis.

2.1.3. LC-MS/MS conditions

LC-MS/MS detection was performed on two LC-20AXR (Shimadzu, Japan) HPLC system-tandem mass spectrometry AB SCIEX API4000 PLUS. Two HPLC systems were paralleled via a six-port switching valve mounted on MPX driver. Mass spectrometry was equipped with an electrospray ionization (ESI) ion source. Quantitative analysis was completed using MultiQuant 2.1.1 software.

Multiple reaction monitoring (MRM) in positive mode was conducted by monitoring the m/z 821.6 > 768.6 (qualifier) and m/z 821.6 > 796.5 (qualifier) for tacrolimus, while the ASC internal standard was detected using the m/z 809.6 > 756.4 (qualifier) and m/z 809.6 > 774.6 (qualifier) transition. The optimized instrumental settings were declustering potential (60 V), entrance potential (6 V), collision energy (27.5 V), collision cell exit potential (20 V), ionspray voltage (5500 V), ionization source temperature (400 °C). The curtain gas, collision gas, nebulizer gas, and auxiliary gas were set at 25, 7, 50, and 60 pounds per square inch (psi), respectively.

A Kinetex XB-C18 column (100 Å, 2.1 × 50 mm, 2.6 μm, Phenomenex) was used for chromatographic separation by using 2 mmol/L ammonium acetate and 0.1% formic acid in water (solvent A) and methanol (solvent B) as the mobile phase under gradient elution as follows: initial, 60% B: 0.00–1.50 min, 60%–100% B; 1.50–2.50 min, 100% B; 2.50–2.51 min, 100%–60% B; 2.51–3.50 min, 60% B. The flow rate was 0.5 mL/min with an injection volume of 30 μL. The column temperature was set to 60 °C. Through the paralleled HPLC systems only during the interval 1.5 min, from 0.8 min to 2.3 min, the LC was set to the mass spectrometer, and another sample could be detected during the remaining time. By this way, within 3.5 min 2 samples were detected and carried out a shorter analyzing time for each sample of 1.75 min, which improved the instrument throughput finally.

2.2. Intrapatient variability in clinical practice

2.2.1. Patients and settings

Our study cohort involved 208 kidney transplant patients who were followed at Traditional Chinese Medicine Hospital of Guangdong Province between January 2017 and December 2019. The date of surgery, age, gender, treatment plan, and laboratory tests were recorded. Adult patients who treated with tacrolimus and mycophenolate mofetil (MMF) in the period between 6 and 12 months after kidney transplantation were included. The inclusion and exclusion criteria used for patient selection were shown in Table 1. All included patients had an estimated glomerular filtration rate (eGFR) of ≥25 mL/min at month 6 after transplantation.

2.2.2. End points

We set an endpoint that consisted of graft failure defined as the restart of dialysis, patient death because of renal graft, an eGFR <15 mL/min, histopathological examination confirming acute rejection, or at least

| Inclusion criteria | Exclusion criteria |
|--------------------|-------------------|
| Age > 18 | Patients were not followed up regularly and data was lost |
| Between 6 and 12 months after kidney transplant, tacrolimus trough concentrations were monitored by high-performance liquid chromatography. | Patients have received other organ transplants |
| Until May 23, 2021, patients who were still alive and had not met the end point survived at least two years. | Patients were not treated with Tac and mycophenolate mofetil (MMF) in the period between 6 and 12 months after the kidney transplant |
| Patients' tacrolimus trough concentrations were monitored at least 8 times between 6 and 12 months after kidney transplant | Patients met the end point before 12 months after the surgery |
three times double the serum creatinine concentration than the reference and lasted a month after the first 6 months.

### 2.2.3. IPV and outcome variables

IPV is defined as fluctuations in Tac blood concentrations in an individual patient over a certain time period in which the Tac dose was not changed [21]. The coefficient of variation (CV) is commonly used to quantify IPV. In statistics, the CV assesses the degree of variation represented by the ratio of the standard deviation (SD) (σ) to the mean value (μ):

\[
CV\% = \left( \frac{\sigma}{\mu} \right) \times 100
\]

In any given dataset, the percentage of CV (CV%) can be estimated:

\[
CV\% = \sqrt{\frac{\sum (X_i - \bar{X})^2}{n - 1}} \div \overline{X} \times 100
\]

Where \( X \) is the average of all Tac dose-corrected \( C_0 \) concentrations measured in time period, \( i \), \( X_i \) is an individual \( C_0 \) dose-corrected concentration, \( n \) and is the total number of available \( C_0 \) in time period \( i \). It is available for calculation when at least 3 predose Tac concentrations (\( C_0 \)) for an individual. A median of 6 (range: 3–15) Tac measurements were used to calculate Tac IPV. Only data on Tac exposure measured at outpatient clinic visits in the period of 6–12 months post-transplantation were collected, as RTRs (renal transplant recipients) are not on a stable outpatient clinic visits in the period of 6 months after transplantation and they often use interacting drugs (such as antibiotics and glucocorticoids) in this period. Tac concentration measurements obtained during hospitalization were not considered. As not all patients received a constant drug between months 6–12, the obtained were corrected for the corresponding daily Tac dose (\( C_0 \)/D, D is Tac dose) [22].

### 2.3. Statistical analysis

Data were analyzed for statistical significance by SPSS version 23 (IBM). Continuous variables are expressed as the mean ± SD with normal distribution and median without normal distribution. The t-test, chi-squared test, binary logistic analyses, and Kaplan–Meier (KM) analyses were applied to study the association between Tac-IPV, other clinical variables, and the composite endpoint. All tests were two-sided, and a \( P \)-value < 0.05 was considered statistically significant.

### 3. Results

#### 3.1. Method validation

Method validation results were shown in Table 2. Intra-assay precision was assessed by analysis of whole blood samples at three concentrations (low, medium and high) in one batch. 12 samples of each concentration are processed in parallel, and each sample is tested once. Inter-assay precision was assessed by analysis of two sets of QCs on ten different days. To assess the deviation at lower limit of quantification (LLOQ), blood sample at 0.39 ng/mL was tested twice per day for different days. To assess the deviation at lower limit of quantification (LLOQ), blood sample at 0.39 ng/mL was tested twice per day for different days (n = 10). Precision results were all within 4% except at LLOQ, where deviated by no more than 5.54%, which met the goal of ≤10% for all concentrations.

Linearity was assessed by the eight point calibrations at concentrations of 0.37, 1.51, 2.25, 6.05, 12.10, 17.70, 24.30 and 42.90 ng/mL. To fit the peak area ratio (Tac peak area to ASC peak area) vs analyte concentrations for linearity, a weighted least-squares method was used with a weighting factor of 1/\( x \). Linearity of six calibrations showed good correlation in range of 0.37–42.90 ng/mL (weighting 1/\( x \)) with a correlation coefficients (r) > 0.99 and the lower limit of quantification (LLOQ) was set to be 0.37 ng/mL.

The dilution performance was assessed by diluting a high concentration sample with homogeneous drug-free whole blood (EDTA anti-coagulant) for 2, 4, 8 times. Dilution recoveries were within the acceptance limits (85%–115%) and the deviation of five duplicates were less than 4.11%. Hence, sample could be quantitated by dilution when the concentration of the analyte exceeded the upper limit of quantification (ULoQ, 42.90 ng/mL), which mean that this method could obtain quantitative results of concentration in 0.37–343.20 ng/mL.

| Carryingover (n = 3) | ≤±5.8% |
|---------------------|--------|
| Accuracy (n = 3)    | 2.00%  |
|                    | 3.33%  |
| Patient sample (Low)| 1.05 ng/mL 107.47 ± 5.24% |
| Patient sample (Medium)| 10.51 ng/mL 112.77 ± 1.59% |
| Patient sample (High)| 20.82 ng/mL 101.20 ± 2.09% |

### Table 2. Validation results.

| Parameters                              | Validation results |
|-----------------------------------------|--------------------|
| Intra-assay precision (n = 12)          | 3.83 ± 0.07 ng/mL 1.94% |
|                                        | 7.42 ± 0.17 ng/mL 2.28% |
|                                        | 23.79 ± 0.43 ng/mL 1.82% |
| Inter-assay precision (n = 20)          | 4.25 ± 0.14 ng/mL 3.32% |
|                                        | 8.29 ± 0.33 ng/mL 3.99% |
|                                        | 15.41 ± 0.51 ng/mL 3.34% |
| Inter-assay precision of blood sample at 0.39 ng/mL (n = 10) | 0.39 ± 0.021 ng/mL 5.54% |
| Linear (n = 6)                          | 0.37–42.90 ng/mL |
|                                        | y = 0.03312x + 6.245e^-5 |
|                                        | r = 0.99707 (R^2 = 0.9941) |
| Dilution (n = 5)                        | 43.81 ± 1.80 ng/mL |
| 2 times:                                | 21.74 ± 0.22 ng/mL 99.24 ± 0.99% |
| 4 times:                                | 11.38 ± 0.36 ng/mL 103.90 ± 3.26% |
| 8 times:                                | 5.39 ± 0.07 ng/mL 98.46 ± 1.25% |
| Accuracy (n = 1)                        | 0.99%              |
|                                        | 3.33%              |
| Patient sample (Low)                    | 0.53 ng/mL 94.00 ± 4.33% |
|                                        | 1.05 ng/mL 107.47 ± 5.24% |
| Patient sample (Medium)                 | 5.28 ng/mL 90.40 ± 4.69% |
|                                        | 10.51 ng/mL 112.77 ± 1.59% |
| Patient sample (High)                   | 10.51 ng/mL 104.57 ± 3.33% |
|                                        | 20.82 ng/mL 101.20 ± 2.09% |

The Prince of Wales Hospital in Hong Kong under the ISD program (CICTAC-Tacroilimus) were detected [23]. The bias and Z scores were within ±14.3% and ±1.61, respectively, which met the acceptable range.
for the LC-MS/MS method provided by the organization institutions (bias of ±25% and Z scores of ±3).

Matrix effects occurred in the assay were validated by three replicates of standard solutions (QCs at low and high level; Calibrations at low, medium and high level; n = 5), patient samples (n = 6) and mixed samples (standard solutions mixed with six patient samples separately, n = 30). The deviation between the response of the mixed samples and the response average of the patient samples and standards was -4.2–18.7% (Figure 1), indicating no relative matrix effects (<±20%).

3.2. Tac-IPV based on the modified LC-MS/MS

3.2.1. Patient characteristics and groups

Between January 2017 and January 2019, a total of 208 adult patients received transplants and were started on Tac/MMF-based immunosuppression. Of these, 83 patients met the criteria and were enrolled in this study. Patient characteristics are presented in Table 3. The median follow-up was 785 days with a range of 371–2193 days after the transplant. A total of 14 events (16.9%) were documented at risk: 2 cases of graft loss, 4 cases of late biopsy acute rejection (BPAR), 2 cases of interstitial nephritis, 1 case of Henoch-Schönlein purpura nephritis, and 6 cases of doubled serum creatinine.

The median Tac-IPV was 23.71% (range: 5.50%–63.90%; Figure 2). Dividing the patients into two groups based on their variability, using the median as cutoff, resulted in 41 patients in the low-variability group with a mean variability of 16.20% (median = 15.65%, range: 5.50%–23.71%), and 42 patients with high variability with a mean IPV of 33.41% (median = 31.27%, range: 23.71%–63.90%).

3.2.2. The risk factors associated with kidney transplant outcome

To visualize the association between the composite endpoint and Tac-IPV, other clinical variables (t-test and chi-squared test) were performed to filter out statistically significant features (Table 4). As shown in Table 4, acute rejection in the first post-transplant year (P = 0.001), serum creatinine (mol/L) between 6 and 12 months (P = 0.003), and Tac-IPV (P = 0.038) were statistically significant. Then, univariable Cox proportional hazards analyses were done (Table 5), which showed that Tac-IPV, acute rejection in the first post-transplant year, serum creatinine between 6 and 12 months, and eGFR between 6 and 12 months were independent risk factors for renal graft dysfunction (HR = 3.96, 15.68%, range: 5.50%–23.71%), and 42 patients with high variability with a mean IPV of 33.41% (median = 31.27%, range: 23.71%–63.90%).

Figure 1. The deviation between the response of the mixed samples and the response average of the patient samples and standards was -4.2–18.7%.

3.2.3. Tac-IPV and survival rate

Kaplan–Meier curves were constructed for patients with low and high Tac-IPV (Figure 3), age <41 and age ≥41 (Figure 4), gender (Figure 5), and acute rejection in the first post-transplant year (Yes/No; Figure 6). There is no statistically significant founded between survival rate of transplanted kidneys in low (<41) and high (≥41) age, and in male and female patients (x² = 0.375, 0.891; P > 0.05). The survival rate of transplanted kidneys in low Tac-IPV patients was higher than high Tac-IPV patients. The survival rate of patients who did not develop acute rejection in the first post-transplant year was higher than those who developed acute rejection. The difference was statistically significant (x² = 5.22, 44.21; P < 0.05).

4. Discussion

Nowadays, increased efforts have been made to improve and optimize the sample preparation and throughput of the LC-MS/MS method. Hiruyuki Ono et al [16] developed a high-sensitive and high-throughput ultra-performance liquid chromatography with tandem mass spectrometry method (UPLC–MS/MS) for simultaneous quantification of tacrolimus and its metabolites. Sample preparation involved protein precipitation followed by solid phase extraction (SPE). A high-throughput LC-MS/MS method with one-step protein precipitation was introduced by Zf-Shan Gong et al [14]. ZnSO₄ followed by acetonitrile were used to
precipitate the proteins for sample preparation. The method used protein precipitation only, but had three steps for sample preparation. In this study, sample preparation was modified in one step which 50% methanol/water and 50 mmol/L ZnSO4 were used to promote both erythrocyte lysis and protein precipitation. A rapid, simple and economical method for sample preparation was developed. Another outstanding advantage of our method is its high throughput. Many studies improve the throughput by shortening the LC run time while retaining the resolution using smaller-diameter separation media [11, 14]. Hiroyuki Ono et al. [16] and Soma Bahmany et al. [17] used a UPLC BEH C18 column (1.7 μm, 50 × 2.1 mm) for chromatographic separation and the total run time was 3 min [10]. A rapid and accurate LC-MS/MS method for quantifying tacrolimus in capillary blood collected by volumetric absorptive microsampling was developed by Camille Tron et al. [18]. A C18 Hypersil Gold column (30 × 2.1 mm, 3 μm) fitted with a guard column (10 × 2.1 mm, 3 μm) was used and the total run time was 2.5 min. The linearity of the assay was 2.25 ng/mL to 42.9 ng/mL. In this study, separation was achieved using a Phenomenex Kinetex 2.6 μm XB-C18 2.1 × 50 mm column. The mass spectrometer was coupled with parallel HPLC systems, through which, not only a shorter analyzing time for each sample of 1.75 min was achieved, rejection of impurities that do not need to be tested could also avoid contamination of the mass spectrum. Although the total run time of each HPLC system was 3.5 min, it carried out a shorter analyzing time 1.75 min for each sample, which was more rapid than previous LC-MS/MS method [16, 17, 18, 19]. It is indicated that the two simultaneously operating LC systems may be a feasible route to improve the throughput. The method we construct may be further contributable for the labs, which have two HPLC systems. Since the development of the method so far, we have also found that the blood concentration of tacrolimus is particularly high in some patients, some of which can reach more than 50 ng/mL or even more than 100 ng/mL. We do not know whether this is due to the peak concentration samples submitted, but a wider quantitative range will be more conducive to clinical practice. In this method, it could obtain quantitative results of concentration in 0.37–343.20 ng/mL through dilution, with a linearity range of 0.37–42.9 ng/mL. Altogether, a simple, superior and high throughput method is developed for tacrolimus detection, which will contribute to the clinical decision making and precision medicine.

At the same time, clinical data were applied for assessing the value of Tac-IPV. It was found to have reliable prognostic value in organ transplants in recent years. But much less research has investigated whether Tac-IPV is valuable for Chinese. Our study shows that patients with a high Tac-IPV between 6 and 12 months after surgery had a 4.7 times higher risk of reaching the composite endpoint. The study of Borra demonstrated that patients with a high IPV (>16.2%) had a 1.4 times higher risk. Shuker later confirmed the findings through a larger cohort of 808 kidney recipients [22, 24]. One of the differences between our study and the two studies is the detection method. Borra and Shuker used several kinds of immunoassays. Therefore, our method may perform better in predicting long-term outcomes for kidney transplanted patients.

There are several inadequacies in this study. Some studies have demonstrated that, there are multiple factors cause Tac-IPV, such as the

Table 6. Results of the multivariable Cox regression analysis. Impact of tacrolimus intrapatient variability on the composite end point censored for death.

| Index                                      | Hazard ratio (95% CI) | P-value |
|--------------------------------------------|-----------------------|---------|
| Tac C0 (ng/mL) between 6 and 12 months     | 22.75 (5.73–90.34)    | <0.001  |
| Serum creatinine (mol/L) between 6 and 12 months | 1.01 (0.99–1.02)     | 0.526   |
| eGFR (mL/min/1.73 m2) between 6 and 12 months | 0.97 (0.92–1.03)     | 0.347   |
| Tac-IPV (high)                             | 4.75 (1.23–18.36)    | 0.024   |
timing of Tac dosing in relation to food ingestion, food, and drug-drug interactions [25, 26, 27, 28]. In our study, Chinese herbal formulas may also cause Tac-IPV. We will explore this in further study [29, 30]. For the same batch of samples, cross-validation was not done between our method and immunoassays. The number of patients in our study was small, and we did not promote Tac-IPV with our method for other organ transplants. In further studies, we will increase our sample size, compare the Tac-IPV prognostic value between our method and other LC/MS-MS methods, and assess the Tac-IPV prognostic value in other organ transplants based on our method.

Figure 3. Kaplan-Meier survival curves for patients with low (≤23.71%) and high (>23.71%) Tac-IPV. These groups were compared using the long-rank test ($x^2 = 5.22; P = 0.022$).

Figure 4. Kaplan-Meier survival curves for patients with low (<41) and high (≥41) age. These groups were compared using the long-rank test ($x^2 = 0.38; P = 0.54$).
Figure 5. Kaplan-Meier survival curves for male and female patients. These groups were compared using the long-rank test ($\chi^2 = 0.89; P = 0.345$).

Figure 6. Kaplan-Meier survival curves for patients with developing acute rejection and not developing acute rejection in the first post-transplant year. These groups were compared using the long-rank test ($\chi^2 = 44.21; P < 0.001$).

**Declarations**

**Author contribution statement**

Ke-Wei Yu: Performed the experiments; Wrote the paper.

Bing-Ling Li: Performed the experiments.

Ying-Shi Yuan; Jia-Min Liao: Analyzed and interpreted the data.

Wei-Kang Li; Heng Dong; Pei-Feng Ke; Xing Jin; Lu Chen; Jing-Jing Zhao; Heng Wang; Shun-Wang Cao; Wei-Ye Chen: Contributed reagents, materials, analysis tools or data.
Xian-Zhang Huang; Bei-Bei Zhao; Chun-Min Kang: Conceived and designed the experiments.

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Data availability statement
Data will be made available on request.

Declaration of interests statement
The authors declare no conflict of interest.

Additional information
No additional information is available for this paper.

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