Gene polymorphism associated with FOXP3, CTLA-4 and susceptibility to pre-eclampsia: a meta-analysis and trial sequential analysis

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ABSTRACT

Previous studies have detected the correlation of polymorphisms in regulatory T cells associated genes FOXP3 and CTLA-4 with pre-eclampsia (PE) risk, but the results are inconsistent among studies. Eligible studies were retrieved in several database. Odds ratios (ORs) with 95% confidence intervals (CIs) were utilised to evaluate the relationship between FOXP3 rs3761548, FOXP3 rs2232365, CTLA-4 rs231775 polymorphisms, and PE susceptibility in the genetic models. The subgroup analysis and trial sequential analysis were performed. Twelve studies with a total of 4658 participants were included. There was a statistically significant association between FOXP3 rs3761548 polymorphism and PE within the recessive model in Asian (OR = 0.54, 95% CI = 0.34–0.86). Trial sequential analysis indicated sufficient proof of such association in the Asian population. This meta-analysis provides sufficient statistical evidence indicating an association between FOXP3 rs3761548 polymorphism and PE risk in Asian.

IMPACT STATEMENT

- What is already known on this subject? FOXP3 and CTLA4 are markers of regulatory T cells which play a crucial role during a preeclamptic pregnancy.
- What do the results of this study add? Eleven studies with a total of 4658 participants were included. There was a statistically significant association between FOXP3 rs3761548 polymorphism and pre-eclampsia (PE) within the recessive model in Asian (OR = 0.54, 95% CI = 0.34–0.86). Trial sequential analysis indicated sufficient proof of such association in the Asian population. However, there was no enough evidence could prove significant association between FOXP3 rs2232365 or CTLA-4 rs231775 polymorphism and PE.
- What are the implications of these findings for clinical practice and/or further research? This meta-analysis provides sufficient statistical evidence indicating an association between FOXP3 rs3761548 polymorphism and PE risk in Asian. The findings in this study may provide a basis for the further study on FOXP3 rs3761548 polymorphism in future research.

Introduction

Pre-eclampsia (PE) is a severe complication of pregnancy with a worldwide incidence of 5%–8% (Amaral et al. 2015). It is also one of the leading causes of maternal and perinatal morbidity and mortality. Despite extensive research, the etiology of the disease is still unknown. However, one of the likely hypotheses for the pathogenesis of the disease is the poor adaptation of the maternal immune system to the foetal alloantigen (Dekker et al. 1998; Dekker and Sibai 1999). It has been proposed that a Th1/Th2 cell disequilibrium occurs because of an abnormal activation of a Th1-type immune response during a preeclamptic pregnancy followed by the breakdown of peripheral tolerance to the foreign antigens of the foetus (Saito et al. 1999; Yoneyama et al. 2002).

Regulatory T (Treg) cells play a crucial role in preventing destructive immunity in all the tissues via various mechanisms such as regulation of TH1/TH2 balance (Aluvihare et al. 2004; Zenclussen et al. 2005). Tregs are characterised by expression of CD4, CD25, FOXP3 (Forkhead box P3) and their ability to produce inhibitory cytokines (TGF\(\beta\), IL-10, IL-35) and inhibitory receptors (CTLA4 (cytotoxic T-lymphocyte antigen), LAG-3) (Norouzian et al. 2016).

FOXP3, a member of the Forkhead transcription factor family encoded in the X chromosome, is required for the transformation of naive T cells into Tregs that play a role in protecting successful pregnancies and developments and functioning of Tregs (Sakaguchi et al. 2010; Norouzian et al. 2016). Various functional polymorphisms of the FOXP3 gene have been investigated in the pathogenesis of various human diseases (Park et al. 2005; Gao et al. 2010) and FOXP3 variants may play a role in the development of PE through quantitative and functional effects on TregCD4\(^+\)CD25\(^+\) (Gholami et al. 2018). Although FOXP3’s involvement in Treg biology is 

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Supplemental data for this article can be accessed online at https://doi.org/10.1080/01443615.2021.2002285.

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well established, the potential influence of FOXP3 variants on PE was not clear.

CTLA-4 gene is located in the chromosomal region 2q33 and codes for a protein that works as a negative regulator of T cell responses, leading to the preservation of T cell homeostasis (Daraiavach et al. 1988). The function of the CTLA-4 gene in the placenta is not widely studied, but it has been abundantly expressed in placental tissue at the maternal–foetal interface throughout gestation (Kaufman et al. 1999). Further, decidual T cells have been shown to express intracellular CTLA-4 (Heikkinen et al. 2004). Although the significance of CTLA-4 has not been thoroughly evaluated in pregnancy complications, the associations between polymorphisms of the CTLA-4 gene and pregnancy complications have been reported in women with recurrent pregnancy loss and severe PE (Samsami Dehaghani et al. 2005; Wang et al., 2006).

FOXP3 and CTLA-4 have many polymorphisms. The widely studied ones are FOXP3 rs3761548, FOXP3 rs2232365, FOXP3 rs2280883, and CTLA-4 rs231775 in the field of PE.

Studies have generally concluded that there was no association between FOXP3 rs2280883 polymorphism and the risk of PE (Metz et al. 2012; Chen et al. 2019). The association between FOXP3 rs3761548, FOXP3 rs2232365, and CTLA-4 rs231775 polymorphisms and risk of PE has been discussed in many studies with inconsistent results. Therefore, we conducted a meta-analysis of studies on the FOXP3 and CTLA-4 polymorphisms with the aim of providing a more comprehensive summary of currently available research to evaluate the relationship between the above polymorphisms and PE risk.

Material and methods

Search strategy

Two examiners (JL and GS) independently searched for the significant studies in databases, including PubMed, Web of Science, EMBASE, and Scopus on September 8, 2021. Searching words are the ‘pre-eclampsia’, ‘(polymorphism) or ‘variant’ or ‘mutation’, and ‘(FOXP3’ or ‘CTLA-4’). The details of the search strategy are shown in Table S1.

Inclusion and exclusion criteria

Original studies were eligible if they met the following criteria: (I) case–control studies; (II) full text in English or Chinese available; (III) literature on associations between FOXP3 and CTLA-4 genetic polymorphisms (rs3761548, rs2232365, and rs231775) and PE.

Original studies were ineligible if they: (I) were reviews, letters, or case reports; (II) did not contain polymorphisms data which could be used to calculate odds ratio (OR) and 95% confidence interval (CI); (III) laboratory animal literature. If there were several publications from the same study, the study with the most cases and relevant information was included.

Data extraction

Reviewers (JL and GS) was assigned to extract data independently. The third reviewer (GZ) will solve the disagreement if it is existing. The extract information included the polymorphism types, first author, published year, country/ethnicity, control source, genotyping method, and genotype distribution in case and control groups.

Assessment of literature quality

The Newcastle–Ottawa Scale (NOS) was used to assess the quality of all studies included, similar as our previous research (Liu et al. 2020). The NOS quality score ranges from 0 to 9 stars. Two authors independently assessed the quality of the studies included. Disagreements were resolved by discussion.

Statistical analysis

We calculated the OR by genotype and allele model comparisons of FOXP3 rs3761548, FOXP3 rs2232365, and CTLA-4 rs231775 polymorphisms between cases and controls. The P value for Hardy–Weinberg equilibrium was calculated in the control group using the chi-square test. Heterogeneity was assessed by using the I² statistics. If there was no heterogeneity (P >.1 or I² < 50%), a fixed-effects model was used to estimate the pooled OR; otherwise, a random-effects model was utilised. Subgroup stratified analysis was performed using the genotyping method, ethnicity, or Hardy–Weinberg equilibrium. Publication bias was evaluated with Egger’s test. Sensitivity analyses were directed to assess the influence of individual study on the overall estimate. Statistical analyses were performed with Stata (version 16.0; StataCorp, College Station, TX, USA).

Trial sequential analysis (TSA)

The TSA (version 0.9.5.10, http://www.ctu.dk/tsa/) was conducted to maintain a 95% confidence interval, a 20% relative risk reduction, overall 5% risk of a type-I error and 20% of the type-II error (a power of 80%) in this study to reduce the risk of type-I error. When the cumulative Z-curve crossed the trial sequential monitoring boundary or exceeded the required information size line, it was considered to be an indicator of sufficient and firm evidence, with no further studies required. Otherwise, additional studies were needed.

Results

Literature search

112 potentially eligible studies were retrieved. After the application of the criteria, eleven studies were kept (Figure 1). Characteristics of the 12 studies are shown in Table 1 (Samsami Dehaghani et al. 2005; Jaaskelainen et al. 2008; Pendeloski et al. 2011; Chen et al. 2013, 2015, 2019; Jahan et al. 2013; Norouzian et al. 2016; Zhou et al. 2016; Gholami et al. 2018; Cekin et al. 2020; Pan et al. 2020). The published
year ranged from 2005 to 2020. The subjects in nine were Asians, Caucasians in two studies, and Mulatto in one study. All were hospital-based design. Four types of genotyping methods in these studies. NOS scores were between five and seven (Supplementary Table S2). In a total of 4658 subjects were involved, including 2079 PE patients and 2579 healthy women (Supplementary Table S3).

Quantitative synthesis

There was statistically significant association between FOXP3 rs3761548 polymorphism and PE within the recessive model (OR = 0.62, 95% CI = 0.39–0.98, Table 2, Supplementary Figure S1) and the homozygous model (OR = 0.57, 95% CI = 0.33–0.86, Table 2, Supplementary Figure S2). However, there was no statistically significant association between FOXP3 rs3761548 polymorphism and PE within other three genetic models: allele (OR = 0.81, 95% CI = 0.62–1.07), dominant (OR = 0.82, 95% CI = 0.60–1.11), and heterozygous models (OR = 0.91, 95% CI = 0.77–1.08).

There was statistically significant association between FOXP3 rs2232365 polymorphism and PE within the recessive model (OR = 0.77, 95% CI = 0.64–0.94, Table 2, Supplementary Figure S3). However, there was no statistically significant association between FOXP3 rs2232365 polymorphism and PE within other four genetic models: allele (OR = 1.00, 95% CI = 0.82–1.22), dominant (OR = 1.16, 95% CI = 0.77–1.75), heterozygous (OR = 1.32, 95% CI = 0.75–2.32), and homozygous models (OR = 1.00, 95% CI = 0.80–1.24).

Subgroup analysis

Subgroup analysis was conducted to reveal some details regarding potential associations between FOXP3 rs3761548, FOXP3 rs2232365, and CTLA-4 rs231775 polymorphisms and PE risk (Supplementary Tables S4–S6). There was a statistically significant association between FOXP3 rs3761548 polymorphism and PE within the recessive model in Asian (OR = 0.54, 95% CI = 0.34–0.86, Figure 2).

Sensitivity analysis and publication bias

The results of sensitivity analysis and publication bias were shown in Table 2. Most results were stable after the deletion of any of the studies involved in the investigation. No obviously publication bias was detected.

TSA

The cumulative Z-curve passed the trial sequential monitoring boundary, indicating sufficient proof of such association.
### Table 1. Characteristics of published studies included in the meta-analysis.

| Polymorphism | First author (year) | Country/ethnicity | Control source | Genotyping method | Newcastle–Ottawa Scale | Case (n)* | Control (n)* | Hardy–Weinberg equilibrium (P) |
|--------------|---------------------|------------------|----------------|-------------------|------------------------|-----------|--------------|---------------------------------|
| FOX3 rs3761548, 3279 C > A | Chen (2013) | China (Asian) | Hospital-based | PCR-SSP | 7 | 156 | 114 | 40 | 2 | 252 | 184 | 62 | 6 | .775 |
| | Jahan (2013) | India (Asian) | Hospital-based | PCR-SSP | 7 | 282 | 70 | 134 | 78 | 215 | 19 | 79 | 117 | .288 |
| | Norouzian (2016) | Iran (Asian) | Hospital-based | PCR-SSP | 6 | 81 | 34 | 42 | 32 | 90 | 42 | 41 | 7 | .486 |
| | Gholami (2018) | Iran (Asian) | Hospital-based | PCR-RFLP | 6 | 133 | 47 | 69 | 17 | 143 | 47 | 63 | 33 | .187 |
| | Chen (2019) | China (Asian) | Hospital-based | PCR-RFLP | 7 | 203 | 112 | 86 | 5 | 243 | 143 | 95 | .016 |
| | Cekin (2020) | Turkey (Caucasian) | Hospital-based | PCR-RFLP | 6 | 500 | 85 | 270 | 145 | 500 | 70 | 265 | 165 | .026 |
| | Pan (2020) | China (Asian) | Hospital-based | PCR-RFLP | 7 | 158 | 88 | 63 | 7 | 157 | 87 | 82 | 8 | .037 |
| FOX3 rs2232365, 924 A > G | Chen (2015) | China (Asian) | Hospital-based | PCR-SSP | 6 | 156 | 63 | 72 | 21 | 252 | 111 | 103 | 38 | .087 |
| | Jahan (2013) | India (Asian) | Hospital-based | PCR-SSP | 6 | 81 | 13 | 38 | 30 | 89 | 19 | 42 | 28 | .661 |
| | Norouzian (2016) | Iran (Asian) | Hospital-based | PCR-SSP | 6 | 81 | 13 | 38 | 30 | 89 | 19 | 42 | 28 | .661 |
| | Gholami (2018) | Iran (Asian) | Hospital-based | PCR-RFLP | 6 | 133 | 47 | 69 | 17 | 143 | 47 | 63 | 33 | .187 |
| | Chen (2019) | China (Asian) | Hospital-based | PCR-RFLP | 7 | 203 | 112 | 86 | 5 | 243 | 143 | 95 | .016 |
| | Gholami (2018) | Iran (Asian) | Hospital-based | Tetra-ARMS PCR | 6 | 133 | 25 | 80 | 28 | 143 | 30 | 66 | 47 | .446 |
| | Chen (2019) | China (Asian) | Hospital-based | PCR-RFLP | 7 | 158 | 88 | 63 | 7 | 157 | 87 | 82 | 8 | .037 |
| CTLA-4 rs231775, 49 A > G | Jääskeläinen (2005) | Finland (Caucasian) | Hospital-based | PCR-SSCP | 6 | 133 | 25 | 80 | 28 | 143 | 30 | 66 | 47 | .446 |
| | Samsami Dehaghani (2005) | Iran (Asian) | Hospital-based | PCR-SSCP | 6 | 133 | 25 | 80 | 28 | 143 | 30 | 66 | 47 | .446 |
| | Pendeloski (2011) | Brazil (Mulatto) | Hospital-based | PCR-RFLP | 6 | 158 | 88 | 63 | 7 | 157 | 87 | 82 | 8 | .037 |
| | Zhou (2016) | China (Asian) | Hospital-based | PCR-RFLP | 7 | 158 | 88 | 63 | 7 | 157 | 87 | 82 | 8 | .037 |

PCR-RFLP: polymerase chain reaction-restriction fragment length polymorphism; PCR-SSCP: polymerase chain reaction—single-strand conformation polymorphism; PCR-SSP: polymerase chain reaction-sequence-specific primers; Tetra-ARMS PCR: tetra-primer amplification refractory mutation system–polymerase chain reaction.

**Note:** AA, AB and BB represented wild homozygous genotype, heterozygous genotype and mutation homozygous genotype, respectively.

### Table 2. Meta-analysis of association between FOX3, CTLA-4 polymorphisms and risk of pre-eclampsia.

| Comparison | Type of Model | Odds Ratio (95% CI) | P | i² (%) | P | Statistical Model | Test of Publication Bias | Sensitivity Analysis | Odds Ratio (95% CI)_{min} | Odds Ratio (95% CI)_{max} |
|------------|---------------|---------------------|---|--------|---|-------------------|--------------------------|-----------------------|------------------------|------------------------|
| FOX3 rs3761548 | Allele | 0.81 (0.62–1.07) |.140 | 82.3 |<.001 | Random | .449 | .77 (0.57–1.04) | .90 (0.80–1.02) |
| | Dominant | 0.82 (0.60–1.11) |.197 | 69.3 | .003 | Random | .719 | .76 (0.54–1.07) | .93 (0.78–1.11) |
| | Recessive | 0.62 (0.39–0.98) |.042 | 68.4 | .004 | Random | .194 | .54 (0.34–0.86) | .78 (0.62–0.98) |
| | Homozygous | 0.91 (0.77–1.08) |.278 | 37.1 | .145 | Fixed | .890 | .86 (0.71–1.04) | .97 (0.82–1.17) |
| | Heterozygous | 0.57 (0.33–0.99) |.048 | 68.3 | .004 | Random | .228 | .53 (0.29–1.01) | .71 (0.53–0.96) |
| FOX3 rs2232365 | Allele | 1.00 (0.82–1.22) |.981 | 65.1 | .014 | Random | .206 | .97 (0.77–1.21) | 1.06 (0.88–1.28) |
| | Dominant | 1.16 (0.77–1.75) |.487 | 81.5 |<.001 | Random | .130 | .96 (0.76–1.22) | 1.30 (0.86–1.95) |
| | Recessive | 0.77 (0.64–0.94) |.008 | 8.4 | .362 | Fixed | .291 | .75 (0.61–0.92) | 0.81 (0.66–0.998) |
| | Heterozygous | 1.32 (0.75–2.32) |.336 | 88.6 |<.001 | Random | .167 | 1.02 (0.79–1.31) | 1.49 (0.81–2.37) |
| | Homozygous | 1.00 (0.80–1.26) |.964 | 33.4 | .186 | Fixed | .360 | .83 (0.62–1.12) | 1.08 (0.86–1.36) |
| CTLA-4 rs231775 | Allele | 1.19 (0.99–1.43) |.062 | 25.3 | .260 | Fixed | .366 | 1.11 (0.90–1.38) | 1.33 (1.06–1.66) |
| | Dominant | 1.42 (0.88–2.29) |.150 | 64.2 | .039 | Random | .014 | 1.14 (0.75–2.94) | 1.73 (0.86–3.49) |
| | Recessive | 1.17 (0.82–1.67) |.393 | 10.0 | .170 | Fixed | .181 | .93 (0.59–1.47) | 1.36 (0.89–2.06) |
| | Heterozygous | 1.42 (0.83–2.43) |.203 | 68.9 | .022 | Random | .048 | 1.09 (0.81–1.46) | 1.75 (0.79–3.87) |
| | Homozygous | 1.34 (0.90–1.98) |.150 | 0 | .392 | Fixed | .756 | 1.09 (0.67–1.79) | 1.65 (1.03–2.67) |

*CI: confidence interval; OR: odds ratio. Significant results (P < .05) are marked in bold.*
between FOX3 rs3761548 polymorphism and PE in Asian (Figure 3). The cumulative Z-curve did not pass trial sequential monitoring boundary or the required information size line, indicating that more studies are needed for further analysis for FOX3 rs2232365 (Supplementary Figure S5) and CTLA-4 rs231775 (Supplementary Figure S6).

| Study          | %     | OR (95% CI) | Weight |
|----------------|-------|-------------|--------|
| Asian          |       |             |        |
| Chen (2013)    |       | 0.53 (0.11, 2.67) | 6.17   |
| Jahan (2013)   |       | 0.32 (0.22, 0.47) | 22.52  |
| Norouzian (2016)|     | 0.78 (0.24, 2.56) | 9.49   |
| Gholami (2019) |       | 0.49 (0.26, 0.93) | 17.44  |
| Chen (2019)    |       | 1.20 (0.34, 4.21) | 8.86   |
| Pan (2020)     |       | 0.98 (0.35, 2.76) | 11.21  |
| Subtotal (I-squared = 41.6%, p = 0.128) |       | 0.54 (0.34, 0.86) | 75.69  |
| Caucasian      |       |             |        |
| Cekin (2020)   |       | 0.83 (0.63, 1.08) | 24.31  |
| Subtotal (I-squared = .%, p = .) |       | 0.83 (0.63, 1.08) | 24.31  |
| Overall (I-squared = 68.4%, p = 0.004) |       | 0.62 (0.39, 0.98) | 100.00 |

NOTE: Weights are from random effects analysis

Figure 2. Forest plot for the association between FOX3 rs3761548 polymorphism and pre-eclampsia risk using ethnicity subgroup analysis in the recessive model (AA vs. CC + CA).

Figure 3. Trial sequential analysis of pre-eclampsia risk associated with FOX3 rs3761548 polymorphism in the recessive model in Asian.
Discussion

Increasing number of researches revealed the single-nucleotide polymorphisms of these T-cell regulatory genes have been associated with PE. However, the results have limited statistical power because most studies focus on partial polymorphisms and only have relatively small sample sizes. Therefore, the conclusions of those studies are inconsistent and incomplete. Therefore, we performed a meta-analysis and TSA analysis to obtain more conclusive and credible results.

This is the first meta-analysis to summarise evidence of associations between FOXP3 and CTLA-4 polymorphisms and PE. The results showed a significant association between the FOXP3 rs3761548 and PE risk in Asian. TSA analysis confirmed the above result. The association between FOXP3 rs2232365 and PE, and the null association between CTLA-4 rs231775 and PE, both need more evidence.

FOXP3 gene is known to be a major control gene for the development and function of Tregs that play an important role in maintaining self-tolerance and mediated maternal tolerance to foetal development (Jahan et al. 2013). Inadequate expression of FOXP3 may lead to weaker suppression of Treg cells, which ultimately plays a role in the development of PE (Bacchetta et al. 2006; Rahimzadeh et al. 2016). Downregulation of FOXP3 is also associated with other pregnancy abnormalities such as recurrent spontaneous abortion, unexplained infertility, and failure to implant (Jasper et al. 2006; Saito et al. 2007; Shima et al. 2010; Wu et al. 2012).

Down-regulation of FOXP3 in PE has been reported in previous studies and thus results in the decreased number of FOXP3 regulatory T cells in PE (Gholami et al. 2018). CTLA-4 appears to block more efficiently naive and type 1 than type-2 T-cell responses, preventing allograft rejection and inducing tolerance (Donner et al. 1997; Maurer et al. 2002). The rs231775 (−49 A/G) polymorphism in the CTLA-4 gene has previously been associated with autoimmune disorders and collectively, a priori genetic information links the pathogenesis of autoimmune diseases to the G allele of the CTLA-4 gene (Heward et al. 1999; Kouki et al. 2000). Similarly, susceptibility to severe PE and recurrent pregnancy loss have been associated with the G allele of the CTLA-4 gene in two case–control studies (Samasmi Dehaghami et al. 2005; Wang et al. 2005), suggesting that the immunosuppressive nature of the CTLA-4 gene is modified and maternal immunologic tolerance thus becomes impaired.

Small sample studies limited statistical power and reduced the feasibility of conclusions. They usually focussed on a specific ethnicity. For many of these studies, the meta-analysis was usually performed in terms of genetic association studies of complicated diseases. We found FOXP3 rs3761548 polymorphism reduced the risk of PE by 41% in the recessive genetic model with high heterogeneity ($I^2 = 72.5\%$). To prevent interference from heterogeneity, we did the subgroup analysis by ethnicity. After stratification, we found a low heterogeneity ($I^2 = 33.5\%$) and an association between FOXP3 rs3761548 polymorphism and PE in the Asian subgroup. However, in the Caucasian, this association didn’t exist supported by existing data.

Meta-analysis has its potential limitation, resulting in spuriously overestimated (type I errors) or spuriously underestimated (type II errors) effects (Wetterslev et al. 2017). For example, failure to include grey literature in this meta-analysis can lead to an increase in type-I errors. As a complement of meta-analysis, TSA was designed. TSA could reduce the risk of type-I error by estimation of required information size with an adjusted threshold for statistical significance, and estimate whether further additional trials are needed (Wetterslev et al. 2008). If the cumulative Z-curve touches the trial sequential monitoring boundary or the required information size line, it shows firm evidence for such study. If not, additional studies are necessary to reach a consistent conclusion (Wetterslev et al. 2008). In our TSA results, the cumulative Z-curve of rs3761548 crosses the trial sequential monitoring boundary. There is sufficient evidence to support the association between FOXP3 rs3761548 polymorphism and PE risk in Asian. However, there is insufficient evidence to support the association between FOXP3 rs2232365 and CTLA-4 rs231775 polymorphisms and PE risk.

There were limitations in this study. First, the sample size in each subgroup was small. Second, different subgroups may have not consistent exclusion criteria. Third, there was no study that includes the Blacks or Hispanics.

Conclusion

This meta-analysis provides sufficient statistical evidence indicating an association between FOXP3 rs3761548 polymorphism and PE risk in Asian.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was financially supported by the National Natural Science Foundation of China [Grant No. 81871173].

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