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# Polymorphism of vascular endhothelial growth factor (VEGF) gene insertion/ deletion -2549 as risk factor of diabetic retinophathy in Javanese patients with type 2 diabetes

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## ABSTRACT

Diabetic retinopathy (DR) is a visual disorder caused by the diabetic microvascular complications. Genetic polymorphism in the vascular endothelial growth factor (VEGF) gene plays an importan role in the susceptibility of DR. The aim of this study was to evaluate the association of the polymorphism of VEGF gene insetion/deletion (I/D) -2549 with DR in Javanese type 2 diabetes mellitus (DM) patients. This was a case control study involving 40 Javanese type 2 DM patients with DR as case subjects and 40 Javanese type 2 DM patients without DR as control subjects. Type 2 DM patients with DR were recruited from Eye Polyclinic, whereas type 2 DM patients without DR were recruited from Endocrine Polyclinic of Dr. Sardjito General Hospital, Yogyakarta. Genotyping of VEGF gene I/D-2549 was conducted using PCR-RFLP method. Plasma VEGF levels were measured using Enzym-Linked Immunosorbent Assay (ELISA). The genotype distribution of DD (67.5%) and the allele frequency of D (82.5%) in type 2 DM patients with DR was higher than those without DR (27.5% for DD genotype and 56.3% for D allele). The OR of DD and ID genotypes versus II genotype between type 2 DM patients with DR and without DR was 6.882 (95%CI: 0.789-60.060; p = 0.048), whereas OR for the D allele versus I allele between type 2 DM patients with DR and without DR was 3.667 (95%CI: 1.773-3.667; p = 0.000). The plasma VEGF levels of DD genotype (92.16 ± 49.73 pg/mL) were significantly higher than ID genotype (42.70 ± 33.29 pg/mL) in type 2 DM patients (p = 0.000). In conclusion, the polymorphism of VEGF gene I/D -2549 is associated with DR in Javanese type 2 DM patients. The DD genotype and D allele of the VEGF gene polymorphism are the risk factor of DR in those patients. The association of the polymorphism of VEGF gene with DR may be explained with the high plasma VEGF level.

## ABSTRAK

Retinopati diabetes (RD) adalah gangguan visual akibat komplikasi mikrovaskular pada diabetes. Poliorfisme genetik di gen VEGF berperan penting terhadap terjadinya RD. Tujuan penelitian ini adalah untuk mengkaji hubungan polimorfisme insersi/delesi -2549 gena VEGF dengan kejadian RD pada penderita DM tipe 2 suku Jawa. Penelitian ini merupakan penelitian potong lintang yang melibatkan 40 penderita DM tipe 2 dengan RD sebagai kasus dan 40 penderita DM tipe 2 tanpa RD sebagai kontrol. Penderita DM tipe 2 dengan RD direkrut dari Poliklinik Mata, sedangkan penderita DM tipe 2 tanpa RD direkrut dari Poliklinik Endokrin, Rumash Sakit Umum Dr. Sardjito, Yogyakarta. Genotiping I/D -2549 gena VEGF dilakukan menggunakan metode PCR-RFLP. Kadar

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VEGF plasma ditetapkan menggunakan metode *Enzym-Linked Immunosorbent Assay* (ELISA). Distribusi genotip DD (67,5%) dan frekuensi alel D (82.5%) pada penderita DM tipe 2 dengan RD lebih tinggi dibandingankan dengan tanpa RD (27,5% untuk genotip DD dan 56,3% untuk alel D). Nilai OR genotip DD dan ID dibandingakan genotip II antara penderita DM tipe 2 dengan RD dan tanpa RD adalah 6,882 (95%CI: 0,789-60,060; p = 0,048). Sedangkan nilai OR untuk alel D dibandingkan alel lantara penderita DM tipe 2 dengan RD dan tanpa RD adalah 3,667 (95%CI: 1,773-3,667; p = 0,000). Kadar VEGF plasma genotip DD (92,16 ± 49,73 pg/mL) lebih tinggi secara bermakna dibandingkan genotip ID (42,70 ± 33,29 pg/mL) pada penderita DM tipe 2 (p = 0,000). Dari hasil penelitian ini dapat disimpulkan polimorfisme I/D -2549 gena VEGF berkaitan dengan kejadian RD pada penderita DM tipe 2. Genotip DD dan alel D polimorfisme gena VEGF merupakan faktor risiko RD pada pasien DM. Hubungan polimorfisme gena VEGF dengan RD kemungkinan berkaitan dengan tingginya kadar VEGF plasma.

***Keywords***: vascular endothelial growth factor (VEGF) - polymorphism - diabetes - retinopathy - Javanese patients

## INTRODUCTION

Diabetes Mellitus (DM) is a chronic disease, which occurs when the pancreas does not produce enough insulin or when the body can not effectively use the insulin. Diabetes mellitus leads to increased blood glucose level or hyperglycaemia.[1] Hyperglycaemia is a risk factor in diabetic microvascular complications which are the major cause of morbidity and early mortality in diabetes.[2-4] Diabetic retinopathy (DR) is a visual disorder caused by the diabetic microvascular complications. It is characterized by vascular permeability, tissue ischemia and neovascularisation. During hypoxia, hypoxia inducible factors bind to the hypoxia-response and induce the expression of VEGF which leads to the stimulation of angiogenesis and increases of microvascular permeability.[5-7]

Genetic factors have been proven to be able to influence in the susceptibility to diabetic nephropathy and retinopathy.[8] Vascular endothelial growth factor (VEGF) is disulfide-linked dimer glycoprotein or homodimeric glycoprotein, which plays an important role in the pathogenesis of DR. VEGF promotes angiogenesis, stimulates endhotelial disfunctions and increases microvascular permeability in many type of tissues including retinal blood vessel.[6-8]

Genetic polymorphism in the VEGF gene that influences its VEGF protein expression level has been reported.[4-8] The human VEGF gene that is located on chromosome 6 (6p21.3) and consists of 8 exons and 7 introns is highly polymorphic. Several different isoforms of VEGF gen have been reported.[9] One of the isoforms of VEGF gen that is associated with DR is insertion/deletion (I/D) polymorphism of the 18 bp fragment at -2549 posistion of the promoter region. Buraczynska *et al.*[12] suggested that the polymorphism of VEGF gene I/D -2549 is associated with DR in Caucasian type 2 DM patients. However, no significant association between the polymorphism of VEGF gene I/D -2549 and DR was observed in Egyptian type 2 DM patients.[7]

The study concerning genetic polymorphism of VEGF gene, especially I/D polymorphism at -2549 position in the association with DR in Indonesian DM patients has not been reported yet. The objective of this study was to investigate the association of the polymorphism of VEGF gene I/D -2549 with DR in a Javenese population with type 2 DM.

## MATERIALS AND METHODS

### Subjects

This was a case control study involving 40 Javanese type 2 DM patients with DR as case and 40 Javanese type 2 DM patients without DR as control subjects. Type 2 DM patients with DR were recruited from Eye Polyclinic, whereas type 2 DM patients without DR were recruited from Endocrine Polylinic of Dr. Sardjito General Hospital, Yogyakarta. Subjects aged 30-65 years, were diagnosed with type 2 DM 5 for at least 5 years, did not suffer from hypertension and were neither obese nor dyslipidemia. Informed consent was obtained from each participant, and the study was approved by the Medical and Health Research Ethics Committee, Universitas Gadjah Mada, Yogyakarta.

### Screening of DM and DR

The diagnosis and calassification of DM was based on clinical and laboratory examination and the guidelines in the recent Expert Committee Report of the American Diabetes Association. Subjects were diagnosed DM if the fasting plasma glucose (FPG) ≥ 126 mg/dL or random plasma glucose (RPG) ≥ 200 mg/dL or the oral glucose tolerance test (OGTT) ≥ 200 mg/dL.

The diagnosis DR was performed by independent opthalmologist based on fundus photography and the Early Treatment of Diabetic Retinopathy Study (ETDRS) protocol. Subject was considered DR if it was found at least one of retinophaty signs namely microaneurism, abnormality of intraretinal microvascular, venouse beading/looping, blot or dot bleeding, soft exudate, hard exudates, neovascularisation on pupil or other places, preretina bleeding and retina fibrosis with or without edema macula.[13]

### VEGF gene I/D -2549 polymorphisms genotyping

Genotyping of VEGF gene I/D-2549 was conducted using PCR-RFLP method as conducted by Fouad *et al.*[5] DNA sample was isolated from buffy coat. The VEGF gene I/D -2549 polymorphism was analyzed using the following primers i.e. forward primer 5'–GCTGAGAGTGGGGCTGACTAGGTA-3' and reverse primer 5'-GTTTCTGACCTGGC-TATTTCCAGG-3'. Genomic DNA was amplified using the following PCR conditions i.e early denaturation at 95 °C for 6 minutes followed by further denaturation of 35 cycle at 94 °C for 1 minute, annealing at 60 °C for 1.5 minutes, and extention at 72 °C for 2 minutes. A final extention was performed at 72 °C for 10 minutes. The amplification product was separated by electrophoresis on agarose gel 3.2 % and visualized with ethidium bromide. Elektrophoresis was carried out for 45 minutes, with 100 volt and the result was observed with UV ray. II genotype (homozygous mutation) had 1 band of 229 bp. DD genotype (wild type) had also 1 band of 211 bp, while ID genotype (heterozygous mutation) had 2 band of 229 bp and 211 bp.[12]

### Plasma VEGF concentrations assay

Plasma VEGF levels were measured using Enzym-Linked Immunosorbent Assay (ELISA) as performed by Awata *et al*.[14] A monoclonal antibody specific for human VEGF has been coated onto the 96-well microplate. One hundred µL of plasma sample and standard of known human VEGF concentration were pipetted into these wells and then incubated at room temperature for 2 hours. After washing with wash buffer for twice to three times (400 µL each), 200 µL a polyclonal antibody anti VEGF-HRP were added into these wells and incubated again at room temperature for 2 hours.

After washing again with wash buffer, 200 µL substrate solution were added and incubated at room temperature for 25 minutes. Following the incubation, 50 µL stop solution were added in these wells. The solution in the microplate was mixed by shaking it gently. Optic density of the microculture plates was then measured in an ELISA plate reader at l 450 nm. The standard curve was made and used to determine the VEGF plasma level in the unknown samples.

**Statistical analysiss**

Depending on the types of data, data were expressed as mean ± standard deviation (SD) for quantitative variables and as number and percentage for qualitative values. Variables including age, IMT, SBP, DBP, FPG, OGTT, triglyceride, total cholesterol and HDL cholesterol were analyzed using independent t-test or Mann Whitney test. Statistical differences between genotype distribution were tested using Chi-square (÷2) test and between plasma VEGF level were tested using independent t-test. Associations of genotypes and alleles were assessed as OR and 95% confidence intervals (95% CI). Differences by univariate methods (÷2 test, independent t-test) were analyzed together in a logistic regression analysis to test the significant risk factors for diabetic retinopathy. All statistical analysis were considered significant if p value < 0.05.

## RESULTS

The characteristics of subjects both type 2 DM patients with DR and without DR are presented in TABLE 1. No statistically significant difference in age between type 2 DM with and withour DR was observed (p = 0.272). Moreover, the distribution of gender was

TABLE 1. Characteristics of type 2 DM patients with and without DR

| Variables | Type 2 DM with DR (n=40) | Type 2 DM without DR (n=40) | p |
|---|---|---|---|
| Gender | | | |
| • Male (%) | 15 (37.5%) | 13 (32.5%) | |
| • Female (%) | 25 (62.5%) | 27 (67.5%) | |
| Age (years) | 54.90±7.55 | 56.75±7.69 | 0.272 |
| IMT (kg/m$^2$) | 22.02±3.06 | 23.27±1.87 | 0.052 |
| SBP (mmHg) | 123.25±10.95 | 123.00±12.24 | 0.905 |
| DBP (mmIIg) | 79.00±8.41 | 80.00±8.17 | 0.622 |
| FPG (mg/dL) | 144.73±52.60 | 138.11±67.66 | 0.142 |
| OGTT (mg/dL) | 239.99±82.40 | 202.30±93.25 | 0.041 |
| Triglyceride (mg/dL) | 120.49±47.61 | 140.70±122.35 | 0.722 |
| Total Cholesterol (mg/dL) | 174.27±32.56 | 168.31±25.31 | 0.634 |
| HDL cholesterol (mg/dL) | 77.25±16.81 | 71.06±12.97 | 0.050 |
| LDL cholesterol (mg/dL) | 107.48±22.74 | 102.46±16.65 | 0.264 |

*Data are n (%); Values are presented as mean ± standard deviation (SD); DM: diabetes mellitus; DR: diabetic retinopathy; statistical analysis uses independent t-test or Mann Whitney test depending on the type of data*

similar in type 2 DM with DR and without DR. With regard to clinical characteristics, patients of type 2 DM with DR and without DR were not different except for OGTT where type 2 DM patients with DR had a significantly higher plasma glucose level after OGTT than type 2 DM patients without DR (p = 0.041).

PCR amplification products of VEGF gene I/D -2549 polymorphism for type 2 DM patients with and without DR are presented in FIGURE 1. Wild type genotypes (DD), homozygous mutation (II) and heterozygous mutation (ID) were observed in both type 2 DM patients with DR and without DR (FIGURE 1).

The genotype distributions (DD, ID and II) and allele frequencies (D and I) of VEGF gene I/D -2549 polymorphism in type 2 DM patients with and without DR are presented in TABLE 1. The genotype distributions did not deviate significantly from the Hardy-Weinberg equilibrium (p=.986). In type 2 DM patients with DR, the genotype distribution of DD was highest followed by ID and II (p=0.001). Moreover, the allele frequency of D was higher than I (p=0.001), while in type 2 DM patients without DR, the highest genotype distribution was ID followed by DD and II (p=0.001). However, the allele frequency of D was also higher than I (p=0.001). Further analysis showed that the genotype distribution of DD and allele frequency of D in type 2 DM with DR were higher than in type 2 DM without DR (p=0.001). Conversely, the genotype distribution of ID and II and allele frequency of I were lower (p=0.001). The OR of DD and ID genotypes versus II genotype between type 2 DM patients with DR and without DR was 6.882 (95%CI: 0.789-60.060; p=0.048), whereas OR for the D allele versus I allele between type 2 DM patients with DR and without DR was 3.667 (95%CI: 1.773-3.667; p=0.000).

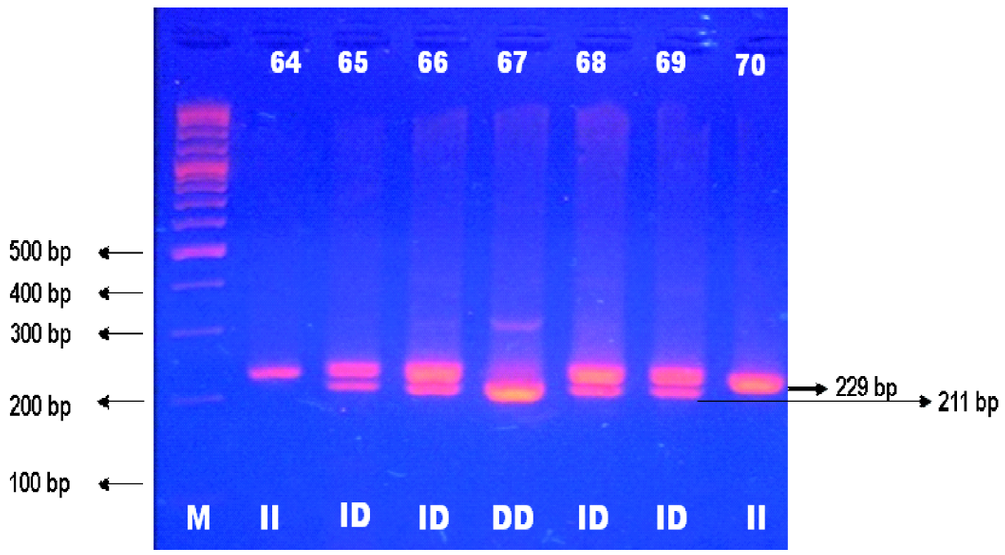


FIGURE 1. PCR amplification products of VEGF I/D -2549 gene polymorphism for type 2 DM patients. M: PCR marker of 100 bp; 64-70: patients number; DD: wild type; ID: heterozygous mutation; II: homozygous mutation

TABLE 2. Genotype distribution and allele frequencies of VEGF gene I/D-2549 polymorphism in type 2 DM patients with and without DR

| Variables | Type 2 DM with DR (n=40) | Type 2 DM without DR (n=40) | p | OR | 95% CI |
|---|---|---|---|---|---|
| Genotype | | | | | |
| • DD | 27 (67.5%) | 11 (27.5%) | | | |
| • ID | 12 (30.0%) | 23 (57.5%) | 0.001 | 6.882 | 0.789-60.060 |
| • II | 1 (2.5%) | 6 (15.0%) | | | |
| Allele | | | | | |
| • D | 66 (82.5%) | 45 (56.3%) | 0.001 | 3.667 | 1.773-7.582 |
| • I | 14 (17.5%) | 35 (43.8%) | | | |

*Data are n (%); : statistical analysis uses Pearson's Chi square ($\div^2$) test*

In order to evaluate the influence of the VEGF gene I/D -2549 polymorphism of type 2 DM patients on VEGF gene expression, the plasma VEGF levels were measured. Among 80 plasma samples obtained from all type 2 DM patients, only 27 samples from patients with the DD genotype and 29 samples from patients with the ID genotype in which the plasma VEGF level could be measured. Plasma VEGF level in patients with II genotype could not be determined due to damaged samples during storage. The plasma VEGF levels of DD genotype were significantly higher than ID genotype in all type 2 DM patients (p=0.000) as shown on TABLE 3. Moreover, a significantly higher plasma VEGF levels of DD genotype compare to ID genotype in type 2 DM with DR (p=0.000) was also observed in this study (TABLE 4).

TABLE 3. Plasma VEGF levels of DD and II genotypes of all type 2 DM patients

| Subject | VEGF level (pg/mL) | | p |
|---|---|---|---|
| | DD genotype (n=27) | ID enotype (n=29) | |
| Type 2 DM with and without DR | 92.16±49.73 | 42.70±33.29 | 0.000 |

*Values are presented as mean ± standard deviation (SD); Statistical analysis uses independent t-test*

TABLE 4. Plasma VEGF levels of DD and ID genotype of type 2 DM patients with DR

| Subject | VEGF level (pg/mL) | | p |
|---|---|---|---|
| | DD genotype (n=22) | ID genotype (n=7) | |
| Type 2 DM with DR | 90.27±52.17 | 7.50±3.36 | 0.000 |

*Value are presented as mean ± standard deviation (SD); Statistical analysis uses independent t-test*

The difference of the mean plasma VEGF level between the type 2 DM patients with and without DR was also evaluated (TABEL 5). The mean plasma VEGF level of type 2 DM patients with DR was higher than patients without DR. However, it was not significantly different (p=0.0611). Further analysis was conducted to evaluate the mean plasma VEGF levels in type 2 DM patients with poliferative diabetic retinopathy (PDR) and with nonproliferative diabetic retinopathy (NPDR). The results showed that the mean plasma VEGF level of type 2 DM patients with PDR was higher than with NPDR, although it was also not significantly different (p=0.611).

TABLE 5. Plasma VEGF levels of type 2 DM patients with and without DR

| Variable | Type 2 DM with DR (n=40) | Type 2 DM without DR (n=40) | p |
|---|---|---|---|
| Plasma VEGF level (pg/mL) | 70.29±57.82 | 58.36±38.69 | 0.611 |

*Values are presented as mean ± standard deviation (SD); Statistical analysis uses independent t-test*

TABLE 6. Plasma VEGF levels of type 2 DM patients wit PDR and NPDR

| Variable | Type 2 DM patients | | p |
|---|---|---|---|
| | PDR case (n=21) | NPDR case (n=8) | |
| Plasma VEGF level (pg/mL) | 73.54±62.49 | 61.75±45.84 | 0.696 |

*Values are presented as mean ± standard deviation (SD); Statistical analysis uses independent t-test*

## DISCUSSION

In this study, the variables such as age, BMI, blood pressure, FPG, OGTT, triglyceride, total cholesterol, HDL and LDL cholesterol between type 2 DM patients with and without DR were controlled with matching. These variables can be a risk factor of DR in type 2 DM patients. Patients who aged more than 60 years have higher the risk of retina disease, such as age-related macular degeneration (AMD). Hypertension is the risk factor of retinopathy hypertension and dyslipidemia is a risk factor of nonproliferative retinopathy. Statistical analysis showed that the type 2 DM patients with DR and without were not different except for OGTT where type 2 DM patients with DR had a significantly higher plasma glucose level after OGTT than type 2 DM patients without DR (p = 0.041).

Obesity was also controlled in this study because obesity is often related to dyslipidemia. However, duration of DM was a variable which was difficult to control in this study. Riyanto[15] reported that risk factor recall data have a potential for recall bias. Moreover, medical record as data resources was often incomplete and even inaccurate. These were the limitations of this study.

This study demonstrated the increase of genotype distribution of DD and allele frequency of D of the VEGF gene I/D polymorphism in type 2 DM patients with DR

compared with patients without DR suggesting that DD genotype and D allele were the risk factors for DR among Javenese type 2 DM patients. It was found that there were almost 7 fold increase risk of DR associated with DD and ID genotype between type 2 DM patients with DR and without DR (OR:6.882; 95%CI: 0.789-60.060; p=0.048) and almost 4 fold increase risk with D allele versus I allele between type 2 DM patients with DR and without DR (OR:3.667; 95%CI: 1.773-3.667; p=0.000).

Studies concerning the VEGF gene I/D polymorphism in association with DR in type 2 DM patients in different ethnic groups have been reported by some authors. Buraczynska *et al.*[12] reported that the polymorphism of VEGF gene I/D -2549 is associated with retinopathy but not nephropathy in Caucasian type 2 DM patients. The DD genotype was significantly higher in type 2 DM with DR compared with without DR (44 vs 21%; p<0.01). Moreover, the D allele of VEGF gene I/D polymorphism was an independent risk factor of retinopathy with OR of 2.27 (95%CI: 1.59-3.15). Otherwise, Fouad *et al.*[7] reported that the polymorphism of VEGF gene I/D -2549 is not significantly associated with retinopathy in Egyptian type 2 DM patients. Despite no significant results, the DD genotype was higher in type 2 DM with DR compared with without DR (40.9 vs 27.9%; p=0.162). Moreover, this study adjusted OR of 2.25 (95% CI: 0.672- 7.538; p=0.185) for D/D genotype versus I/I genotype between type 2 DM patients with DR and healthy subjects and the OR of 1.6 (95% CI: 0.873- 2.891; p=0.129) for the D allele versus I allele between type 2 DM with DR and healthy subjects, while the OR of 1.2 (95% CI: 0.64- 2.29; p=0.539) the D ellele versus I allele between type 2 DM without DR patients and healthy subjects.

Diabetic retinopathy may be the most common diabetic *microvascular complication.* Diabetic retinopathy is characterized with vascular permeability, tissue ischemia and neovascularisation. VEGF plays an important role in the pathogensis of diabetic microvascular complications. During hypoxia, hypoxia inducible factors bind to the hypoxia-response and induce the VEGF expression which leads to the stimulation of angiogenesis and increases of microvascular permeability.[6,7] Genetic polymorphism in the VEGF gene has been reported to influence the VEGF protein expression level which is implicated in the development of DR in DM patients.

In this study, the plasma VEGF level of all subjects was measured and its association with the VEGF gene polymorphism was also evaluated. The results showed that there was a strong association between DD genotype of VEGF gene with the high plasma VEGF level. This result is in agreement with the results reported by some authors.[16-18] *In vitro* and *in vivo* study in animal model showed that the presence of the D allele at -2549 in promotor region of the VEGF gene leads to enhanced expression of the gene.[16,17] Other study also showed that polymorphisms in the promotor region of the VEGF gene affect the VEGF production.[18,19] Moreover, Yang *et al.*[20] have reported that there was an increase of the serum VEGF levels in type 1 DM patients with DR with the DD genotype compared with those with the II genotype. Therefore, the association of the DD genotype or D allele of the VEGF gene with the susceptibility to DR among Javenese type 2 DM patients can be explained with the high plasma VEGF level.

## CONCLUSION

In conclusion, the polymorphism of VEGF gene I/D -2549 is associated with DR in Javanese type 2 DM patients. The DD genotype and D allele of the VEGF polymorphism are

the risk factor of DR in those patients. The association of the polymorphism of VEGF gene I/D -2549 may be explained with the high plasma VEGF level.

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