

# The Role of Angiopoietin-2 Gene Mutation on Clinical Severity of Dengue Disease in Children

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## Abstract

**BACKGROUND:** In general, angiopoietin-2 levels are increased concomitantly with dengue clinical severity.

**AIM:** This research aims to determine the role of mutation on angiopoietin-2 on dengue clinical severity.

**METHODS:** A cross-sectional study of 108 children with dengue disease grouped by severity. Angiopoietin-2 level was examined by enzyme-linked immunosorbent assay. Polymerase chain reaction and double nucleic acid sequencing are using 2 Exon 4-F primers.

**RESULTS:** Angiopoietin-2 levels on rs7834131 mutant are higher in dengue fever ( $p < 0.05$ ) and dengue hemorrhage fever group than non-mutant, while on dengue shock syndrome, it is lower than non-mutant.

**CONCLUSION:** Angiopoietin-2 mutation on rs7834131 might have a protective effect on dengue disease severity.

**Edited by:** Ksenija Bogoeva-Kostovska  
**Citation:** Mayetti M, Izzah AZ, Jamsari J, Darwin E, Somasetia DH. The Role of Angiopoietin-2 Gene Mutation on Clinical Severity of Dengue Disease in Children. Open Access Maced J Med Sci. 2020 Oct 30; 8(B):1110-1115. https://doi.org/10.3889/oamjms.2020.5288  
**Keywords:** Angiopoietin; Dengue; Mutation  
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**Received:** 20-Jul-2020  
**Revised:** 15-Oct-2020  
**Accepted:** 19-Oct-2020  
**Copyright:** © 2020 Mayetti Mayetti, Amirah Zatil Izzah, Jamsari Jamsari, Eriyati Darwin, Dadang Hudaya Somasetia  
**Funding:** This study was supported by Andalas University, Padang, Indonesia  
**Competing Interests:** The authors have declared that no competing interests exist.  
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## Introduction

Dengue virus infection is still one of the major health problems worldwide. An estimated 50 million people infected each year with a death rate of 22,000 per year [1]. Plasma leakage is a major factor that distinguishes the severity of the disease-causing dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). There are many theories stating that many molecules play a role in causing endothelial damage that triggers vascular leakage. Vascular integrity and plasma leakage on dengue virus infection is affected by some factors such as (1) pro-inflammatory cytokines (TNF- $\alpha$ , IL1, IL-6, and IL-12) release from monocyte infected by dengue virus that will activate the endothelial cell [2]. These cytokines will increase the expression of adhesion molecule such as vascular cell adhesion-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), selectin, and ligand integrin [3] and [2] activation of angiogenic protein that affects the integrity of junctional endothelium, which are vascular endothelium growth factor (VEGF) and angiopoietin [4], [5] Angiopoietin-2 level is reported to be the primary protein marker of

plasma leakage on DHF and DSS [6], [7], [8]. The research of Li *et al.* shows that the genetic variation of ANGPT-2 is associated with an increased risk of developing ARDS [9].

A study in Haiti showed that although dengue infection is caused by multiple serotypes, none of which have DHF and DSS [10]. Research in Cuba has found that the severity of dengue infection is milder in blacks than in whites. Both of these have led to theories on the existence of genetic influences, in the form of mutations and polymorphisms that affect the severity of the disease due to dengue virus infection [11].

The angiopoietin-2 protein-coding gene is located on chromosome 8 and is a highly polymorphic gene. The genetic variation of angiopoietin-2 causes different variations in angiopoietin protein expression and vascular angiogenesis [6, 12, 13].

Angiopoietin-2 gene polymorphism research on dengue disease has not been done, so the researcher is interested to see the genetic role of angiopoietin-2 gene polymorphism on the clinical severity of dengue disease on children.

## Materials and Methods

This was observational research using a cross-sectional design. The research population was patients diagnosed with dengue virus infection based on the World Health Organization (WHO) 2011 clinical criteria with positive immunoglobulin M (IgM) anti-dengue or IgM and immunoglobulin G (IgG) anti-dengue positive in the Pediatric Department of Dr. M. Djamil Padang/Faculty of Medicine Andalas University. The research conducted from August 2016 to June 2017. Subjects were part of the population that meets the inclusion criteria. Inclusion criteria are patients aged 1 month–14 years old with consent collected from the parents to participate in the research, exclusion criteria are patients with expanded dengue syndrome.

Routine blood, IgM, and IgG anti-dengue were examined at the Clinical Pathology Laboratory of Dr. M. Djamil Padang. Examination of angiopoietin-2 level was performed by Enzyme-Linked Immunosorbent Assay using ANGPT-2 Kit, Ray Biotech. An examination of angiopoietin-2 polymorphism was initially started with genomic DNA isolation using genomic DNA Mini Kit. The DNA was extracted from each subject's blood sample. For polymerase chain reaction and DNA sequencing, 2 Exon 4-F primers were used, including forward 5'-CACCCATATCCCACCTATCCT-3' and reverse 5'-TGCCCAGTCTCATCCTTCTA-3' primer. The primer was synthesized by Integrated DNA Technologies, Singapore, which was designed based on the ANGPT-2 Homo sapiens gene sequence (NCBI Accession No. NG\_029483.1) as a template.

The data obtained were analyzed by the computer system and presented in the form of tables and graphs. Characteristics of research subjects related to the severity of dengue disease were tested with Chi-square and analysis of variance (ANOVA). Different independent variables with disease severity were tested with ANOVA, with a significance of  $p < 0.05$ .

This study had passed ethical clearance No. 147/KEP/FK/2015 from the Committee of Ethics of Medical Faculty of Andalas University.

## Results

### Subjects characteristics

During the research periods, 121 subjects with dengue virus infection were selected by consecutive sampling. A total of 13 subjects were excluded due to insufficient blood sample volume in 6 subjects and lysis of the blood sample in 7 other subjects. There were 108 total subjects who participated in the research, including DF in 34 subjects (31.48%), DHF in 39 subjects (36.11%), and DSS in 35 subjects (32.40%). Demographic data and characteristics of subjects summarized in Table 1.

Table 1 showed the number of female subjects is 58 people (53.71%), with the most age is 5-10 years. There were no significant differences between sex and age in DF, DHF, and DSS. The median values of hemoglobin and hematocrit subjects increased significantly with the severity of the disease ( $p < 0.05$ ). Post hoc tests of hemoglobin and hematocrit values showed significant differences between DF and DHF groups ( $p < 0.05$ ), but no significant differences were found between DF and DSS group and between DHF and DSS group ( $p > 0.05$ ). Platelet count was significantly lower in the DHF and DSS group compared to DF ( $p < 0.05$ ). Post hoc test showed that there was a significant difference in platelet count significant DF and DHF group and between DF and DSS group ( $p < 0.05$ ), but there was no significant difference between DHF and DSS group ( $p > 0.05$ ).

### Association of angiopoietin-2 level with severity of dengue disease

Table 2 shows that the median level of angiopoietin-2 in the DSS group was higher than in the DHF group and the median level in the DHF group was higher than in the DF group. The differences in the levels of these three groups were statistically significant ( $p < 0.05$ ). In the Mann-Whitney *post hoc* test, there was also a significant difference of angiopoietin-2 levels among all groups (between DF and DHF, DHF and DSS, as well as DF and DSS).

**Table 1: Clinical characteristics and laboratory of research subjects based on dengue virus infection severity**

Characteristic	DF (n=34)	DHF (n=39)	DSS (n=35)	p value
Clinical characteristic				
Sex, n (%)				
Male	18 (52,9)	13 (33,3)	19 (54,3)	0,897 <sup>a</sup>
Female	16 (47,1)	26 (66,7)	16 (45,7)	
Age, years old, mean (SD)	7,87 (3,59)	7,07 (3,25)	6,19 (4,06)	0,165 <sup>b</sup>
≤1	2	2	5	
1–5	8	9	12	0,344 <sup>c</sup>
>5–10	15	22	10	
>10	9	6	8	
Laboratory characteristics				
Hemoglobin (g/dl), Median (min–max)	12,2 (10,5–14,8)	13,3 (9,2–17,3)	13,5 (5,6–17,4)	0,010 <sup>c</sup>
Hematocrit (%), median (min-max)	37,5 (33–49)	41 (28–53)	41 (16–53)	0,016 <sup>c</sup>
Platelet count, (/mm <sup>3</sup> ),median (min-max)	78 500 (13.000–247.000)	40 000 (13.000- 133.000)	40 000 (4.000-131.000)	0,001 <sup>c</sup>

<sup>a</sup>Chi-square, <sup>b</sup>One way ANOVA, <sup>c</sup>Kruskal-Wallis.

**Table 2: Level of angiotensin-converting enzyme 2 in different severity disease**

Dengue severity	n	Level of angiotensin-converting enzyme 2 (pg/ml)	p value
Dengue fever (DF)	34	397,45 (64,48–1895,07)	< 0.001
Dengue hemorrhage fever (DHF)	39	558,52 (79,36–2797,71)	
Dengue shock syndrome (DSS)	35	1393,82 (58,52–6310,49)	

**Angiotensin-converting enzyme 2 gene polymorphism**

Angiotensin-converting enzyme 2 gene sequencing results were analyzed using the Geneious bioinformatics software. Number of SNPs with global minor allele frequency (GMAF) ≥0.01 registered in single nucleotide polymorphism database (dbSNP) of the National Center for Biotechnology (NCBI) is 7 SNPs, and after sequencing all of the samples, 10 SNPs were obtained. The 2 additional SNPs are SNP with GMAF < 0.01 and 1 new SNP with unknown (Figure 1). Figure 1 showed the characteristics of SNP found in ANGPT-2 gene sequencing, and it was found that as many as 5 SNPs were in exon 4, namely: 4c.46981, rs149699486, rs55633437, rs3020221, rs374966371, and 4 SNPs were in intron 3, that is, rs7834131, rs963495, rs145450899, rs115694540, and 1 SNP was in intron 4, which was rs963496. Gene 4c.46981 is a new SNP that has not been registered in NCBI

that is located on the order of base no 232 (marked with purple box).

Amino acid changes were also found in two other SNPs undergoing a mutation, that is, SNP rs149699486 (T/C) where valine amino acid become alanine (Val (V) 235Ala (A)) and SNP rs374966371 (A/T) where asparagine become isoleucine (Asn (N) 257Ile (I)). To detect the SNP in the sequencing result, the entire sequence of samples was aligned with the angiotensin-converting enzyme 2 reference gene (NG\_029483.1) using multiple alignment with the help of Geneious software.

**Association of angiotensin-converting enzyme 2 gene polymorphism with angiotensin-converting enzyme 2 level**

The relationship between angiotensin-converting enzyme 2 gene polymorphism and angiotensin-converting enzyme 2 levels can be seen in Table 3. Only 5 SNPs can be statistically analyzed because in 2 other SNPs there are no mutations and 3 SNPs have very few mutations.

Table 3 shows that the levels of angiotensin-converting enzyme 2 in the rs7834131, rs115694540, 4c.46981, and rs3020221 mutant group were higher than the normal

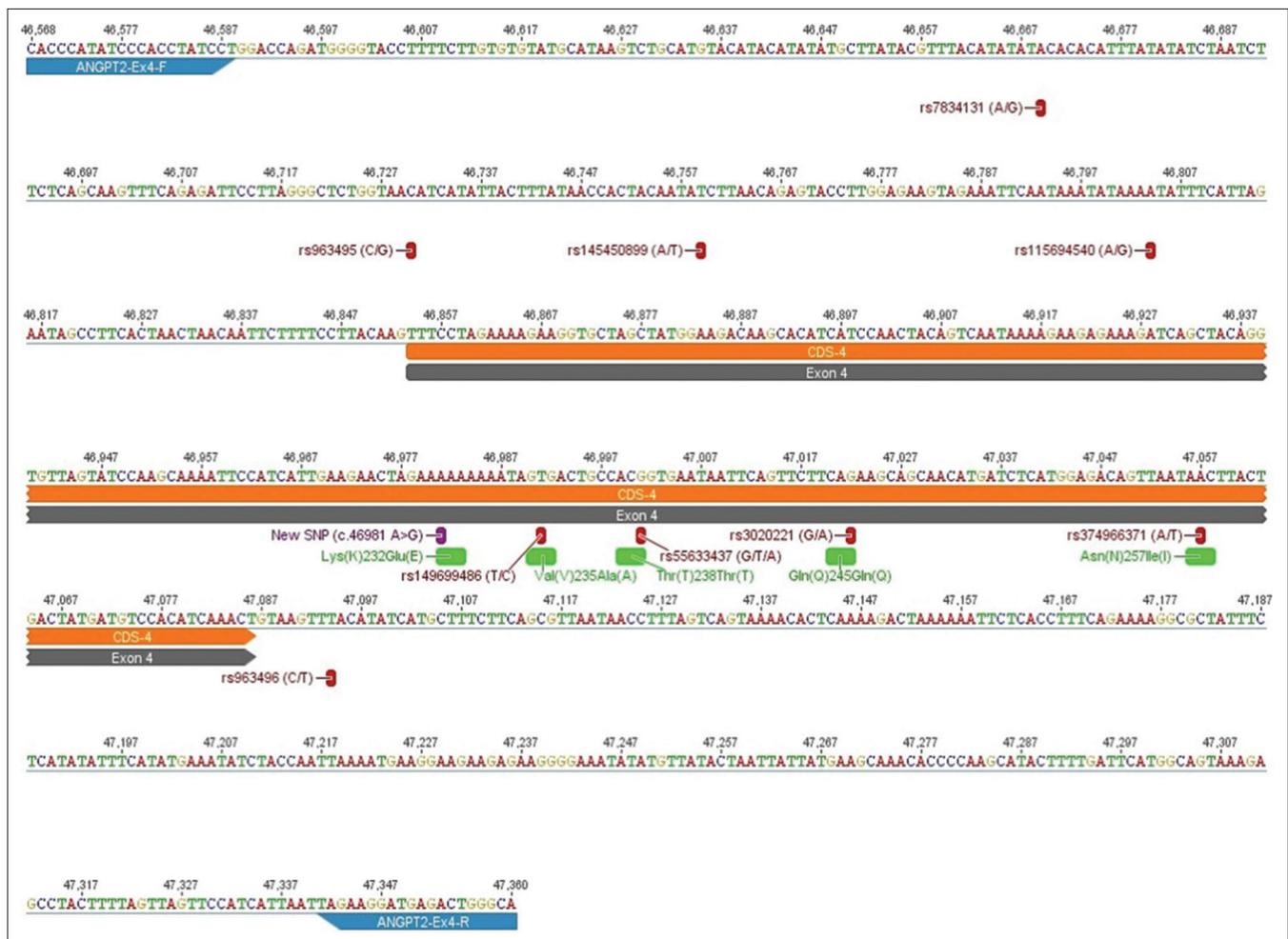


Figure 1: Results of genes sequencing angiotensin-converting enzyme 2 exon 4 using designed primer. There was one new SNP that has not been registered in NCBI that is 4c.46981 located on the order of base to 232 (marked with red box). In this SNP mutation, allele A changed into allele G, the allele changes cause the amino acid to change resulting the alteration from lysine to glutamate (Lys (K) 232Glu (E))

**Table 3: Relation of angiotensin-2 gene polymorphism with angiotensin-2 levels**

SNP	Allele	n = 108	Angiotensin-2 levels (pg/ml) Median (minimum-maximum)	p-value
rs7834131	Mutan	23	1075.962 (112.09–2567.49)	0,012
	Normal (Wild type)	85	550.26 (58.52–6310.49)	
rs115694540	Mutan	9	1020.95 (88.29–1561.49)	0,645
	Normal (Wild type)	99	597.13 (58.52–6310.49)	
4c.46981	Mutan	9	1020.95 (88.29–1561.49)	0,645
	Normal (Wild type)	99	597.13 (58.52–6310.49)	
rs55633437	Mutan	16	556.38 (79.36–2848.65)	0,318
	Normal (Wild type)	92	603.24 (58.52–6310.49)	
rs3020221	Mutan	38	887.48 (109.12–3845.02)	0,073
	Normal (Wild type)	70	554.39 (58.52–6310.49)	

group, but the difference in levels was only significant at SNP rs7834131 ( $p < 0.05$ ).

### Association of rs7834131 mutations with angiotensin-2 level and dengue disease severity

Table 4 showed there was an increasing level of angiotensin-2 on the DF group with mutations compare with non-mutations (wild type) and it is statistically significant ( $p < 0.05$ ). The median value of angiotensin-2 on the DHF group with mutations was higher than non-mutations (wild type), but it is not statistically significant. In the DSS group the median value of angiotensin-2 levels with mutations was lower than non-mutations but it is not statistically significant ( $p > 0.05$ ).

**Table 4: Association of rs7834131 mutation with angiotensin-2 levels on dengue disease severity**

Group		n = 108	Angiotensin-2 levels (pg/ml) Median (minimum-maximum)	p value
DF	Mutan	5	1477.36 (112.09–1895.07)	0.044
	Normal (Wild type)	29	263.88 (64.48–965.93)	
DHF	Mutan	8	798.43(209.9–1179.88)	0.543
	Normal (Wild type)	31	558.519 (79.36–2797.71)	
DSS	Mutan	10	1303.15(597.13–2567.47)	0.855
	Normal (Wild type)	25	1503.85 (58.52–6310.49)	

## Discussion

Clinical characteristics of subjects, including sex and age, did not differ significantly among the three groups. The group of the age of 5–10 years old was the most affected age group in this research. This is in accordance with the research by Sumarmo and Mayetti [14], [15].

This research showed no association between disease severity and gender. Research by Ditjen PP and PL of Indonesian Health Department in 2008 and Sumarmo also showed the same result where there was no gender difference in children with dengue virus infection [14], [16].

This research found that there were significant differences in hemoglobin, hematocrit, and platelet levels among the three groups. A significant difference in hematocrit levels indicated a plasma leakage in the

more severe disease group that leads to hypovolemic shock [17].

We also found that the platelet count was significantly lower in the more severe disease group (DHF and DSS). Thrombocytopenia leads to low levels of angiotensin-1 because angiotensin-1 was also present in large amounts in platelets. A low level of angiotensin-1 causes an imbalance between angiotensin-1 and angiotensin-2 levels that play a role in plasma leakage. The results from this research are similar to those obtained by Rampengan who reported that angiotensin-2 was instrumental in the pathogenesis of transient vascular leakage in DHF, an increase in angiotensin-2 caused the instability of the blood vessels leading to increased vascular permeability that eventually led to the occurrence vascular leak.<sup>6</sup> This research found there was a significantly higher level of angiotensin-2 in the DSS group than the DHF group and a significantly higher level of angiotensin-2 in the DHF group than the DF group.

The examination of angiotensin-2 gene polymorphism in this research focused on exon region 4. The reason for choosing exon 4 was a region that has been widely studied since it is a highly polymorphic area. Ward *et al.* reported that the angiotensin-2 gene had a high degree of polymorphism [18]. Another research used prime exon 4 to detect angiotensin-2 gene polymorphism on different diseases. In addition, the exon region 4 had more SNPs than any other region with a relatively large minor allele frequency [19], [20].

We performed the sequencing from intron 3, exon 4 to intron 4. The sequencing result showed polymorphism on 8 of 10 SNPs that persisted throughout the region. Nine SNPs examined were old SNPs that were already known, and we also found 1 new SNP that has not been registered in NCBI, namely, 4c.46981 located in exon 4 on the sequence of base no. 232.

Mutation on genotype A/G in rs7834131 related with increasing of angiotensin-2 levels significantly in children with dengue virus infection. Angiotensin-2 level in mutation group was higher than non-mutations group, and this difference is statistically significant. Effect of A/G mutation toward increasing of angiotensin-2 levels clearly visible on DF group, where 4 of 5 subjects with mutations in DF group have angiotensin-2 levels above group median value, in addition, 3 of them also has the highest extreme value in DF group on subject 3, 13, and 32.

Further analysis showed that A/G mutation on rs7834131 caused a significant increase of angiotensin-2 levels in the DF group; meanwhile, in the DHF group, this increment is not statistically significant. On the contrary, angiotensin-2 levels in DSS with the mutation group clearly lower than non-mutation even though the differences are statistically significant. It proved that increasing angiotensin-2 levels due to mutation of A/G in rs7834131 might have

a protective effect to prevent leakage. It means that the higher the level of angiopoietin-2 due to mutation on rs7834131, the more protection it has toward plasma leakage, thus decreasing the severity of the disease. This is probably due to the mutation causing functional changing of angiopoietin-2, where even though the amount released still high, but the ability of bonding with the Tie-2 receptor was interrupted.

Some previous studies had varied results about the role of genetic factors in dengue virus infection. Single nucleotide polymorphism examination in the MBL2, TNF- $\alpha$ , Fc $\gamma$  gene receptors, CTLA-4, TGF- $\beta$ 1, HPA, DC-SIGN, TAP, VDR, and JAK1 were associated with the level of protection, susceptibility, and severity of dengue infection, while SNP examination in IL-4, IL-1RA, IFN- $\gamma$ , IL-genes 6, TLR4, and IL-10 were not related to the severity of dengue virus infection [21].

To date, this research was probably the first to examine the genetic polymorphism/mutation of the angiopoietin-2 gene in pediatric patients infected with the dengue virus. However, in some studies, G/A mutations were not associated with the incidence of type 2 DM. Pietrowski *et al.* obtained G/A polymorphisms on exon-4 angiopoietin-2 genes were not associated with the incidence of idiopathic recurrent miscarriages in white female populations in Central Europe [19]. G/A polymorphisms were also not associated with the incidence of intrauterine fetal distress (IUGR) in Caucasian populations [22], and also not associated with an increased risk of gynecological cancer in Turkey [23].

The severity of the disease due to dengue virus infection was determined by the severity of plasma leakage that occurs. The process of plasma leakage begins from endothelial injury and involves the interaction of other factors that are very complex. The results of this research indicated that the presence of an A/G mutation at rs7834131 was associated with an increase in levels of angiopoietin-2, but this mutation was not related to the severity of the disease. This reinforces the notion that the A/G mutation at rs7834131 might be protective against plasma leakage. There were other factors that were not examined in this research that may cause an increase in the levels of angiopoietin-2 which play a more role in triggering plasma leakage in patients with dengue virus infection.

The effect of plasma leakage due to angiopoietin-2 will occur only if angiopoietin-2 binds to the Tie-2 receptor so that subsequent cascade activation occurs. Some studies also show plasma leakage can be prevented if the Tie2 receptor is occupied by ligands other than angiopoietin-2, or there is a deletion of angiopoietin-2 genes [24]. In this research, we found that patients with rs7834131 mutation also had an increment in angiopoietin-2 levels in the DF group, but this increase did not cause plasma leakage. It is suspected that mutations not only cause an increase in angiopoietin-2 level but also change the structure of

angiopoietin-2 so that it is not effective to bind to the Tie-2 receptor as a result of heparanase release and the structure of the glycocalyx remains intact. Kumpers *et al.* found that protection against plasma leakage was given by exogenous Tie-2 stimulation, angiopoietin-2, or genetic deletions of angiopoietin-2 in various models of sepsis partly due to the defense of glycocalyx [25].

In conclusion, gene polymorphism found in children with dengue virus infection. Angiopoietin-2 gene mutation at rs7834131 has been assumed to have a protective effect on the severity of the disease due to dengue virus infection in children.

## Acknowledgments

This research was supported by Andalas University, Padang. We thank our colleagues from the Department of Pediatrics, Andalas University, Padang, Indonesia, who provided insight and expertise that greatly assisted the research.

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