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Original Article

Association of Neuregulin Levels and Neuregulin-1 Polymorphism with Short-term Morbidities in Preterm Neonates

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ABSTRACT

Background: Premature birth is linked to neonatal morbidity and mortality worldwide. Neuregulin (NRG) is a trophic factor from the growth factor (GF) of a transmembrane polypeptide, encoded by four different genes, including NRG-1 which acts as an endogenous protector in fetal development. Decreased levels of NRG-1 affect several organs. The relationship between NRG-1 polymorphism and the outcome of neonatal development has been widely studied. There are no studies that have assessed NRG-1 levels and NRG-1 rs35753505 C/T polymorphism in preterm neonates, as well as its association with short-term morbidities in Indonesia.

Methods: This cross-sectional study was conducted on preterm neonates with the gestational age of 32-36 weeks in Medan, North Sumatera, Indonesia, from December 2017 to December 2018. It aimed to evaluate the association of NRG-1 levels and NRG1 polymorphism with short-term morbidities. Samples were obtained from cord blood specimens. Enzyme-linked immunosorbent assay (ELISA) was used to determine NRG-1 levels, and NRG-1 polymorphism was sequenced by polymerase chain reaction (PCR). Observations in preterm neonates were made during the first 72 h to assess short-term morbidities.

Results: During the study period, 48 cord blood specimens from preterm neonates were found eligible for analysis. Preterm neonates with low NRG-1 levels had a 10-times higher risk of developing short-term morbidities. The presence of CC and CT genotypes increased the risk of developing short-term morbidities 13.33 times (*P=0.003*) and 6.19 times (*P=0.019*), respectively. The presence of the C allele in subjects' genotype increased the risk of short-term morbidities 4.04 times (*P=0.001*), compared to those with T allele.

Conclusion: As evidenced by the obtained results, preterm neonates with low NRG-1 levels had a higher risk of developing short-term morbidities. Furthermore, there was a significant association between NRG-1 rs35753505 C/T polymorphism and short-term morbidities.

Keywords: Neuregulinlevels, Neuregulin-1 polymorphism, Pretermneonates, Short-term morbidities

Introduction

Preterm neonates are defined as those delivered before the gestational age of 37 weeks (1). The incidence of premature labor in the world is quite high, approximately 15 million per year. In Indonesia, premature delivery occurs in 16%-18% of all live births, and its incidence is on a rise (2). Premature labor is still a major concern since it is closely related to perinatal and neonatal morbidity and mortality around the world up to 60%-80% (3).

The clinical conditions of preterm neonates differ significantly from those of term newborns and contribute to short-term morbidities. Clinical problems that occur in preterm neonates are associated with multiple organ dysfunction due to immaturity. These neonates are prone to

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neurological problems, such as intraventricular hemorrhage, hypoxic-ischemic encephalopathy, apnea of prematurity, and cerebral palsy (4). Impaired thermoregulation may cause temperature instability and lead to metabolic disturbances (5). Respiratory distress syndrome caused by surfactant deficiency mostly occurs in preterm neonates of < 32 weeks of gestational age (6).

Necrotizing enterocolitis, the most common gastrointestinal problem, and metabolic disturbances, such as hypoglycemia and hyperbilirubinemia, often occur in preterm neonates (7-10). The incidence of neonatal sepsis is higher in preterm neonates, compared to full-term ones, with a higher mortality rate in low birth weight neonates (11,12). Anemia may occur in preterm neonates characterized by the reduction of hemoglobin levels, low hematocrit values, low reticulocyte count, and low concentration of erythropoietin (13,14). Therefore, early detection of clinical problems is required (15).

Growth factor (GF) is useful for the stimulation of cell growth, cell proliferation, and cell differentiation. The ErbB receptor is a family of four transmembrane receptors which bind to multiple growth factors, including epidermal growth factor (EGF), transforming growth factor-a, and Neuregulin (NRG). The ErbB receptor plays a crucial role in the pathogenesis of development, inflammation, and angiogenesis throughout neonatal gestation (16). The NRG is a signaling protein, which is an intermediary in the process of interaction of the nervous system, heart, and other organs. The signaling neuregulin of NRG-producing cells becomes NRG-responsive cells that bind NRG to the extracellular region of the ERbB3 or ErbB4 tyrosine kinase receptors. Thereafter, a formation of homo or heterodimer ErbB begins the activation process of intracellular signaling pathways (17).

The NRG, coded by four different genes: NRG-1, NRG-2, NRG-3, and NRG-4, has NRG-1 as the most common identified characteristic among all. It functions as a mediator for inter-cell signaling in the growth and development of multiple organ systems (18). The NRG-1 affects the growth and differentiation of epithelial cells, glial cells, neuron cells, and myocytes. Moreover, it induces the expression of acetylcholine receptors in synapse vesicles during the formation of the neuromuscular junction, stimulates proliferation of Schwann cells, development of myocardial tissues, neuronal migration, and expression of neurotransmitter receptors in neuronal tissues (19,20). In the immune system, NRG-1 acts in the recruitment of T cells, B cells, and macrophages to produce the mediators of inflammation. It also performs a major role in glucose metabolism and systemic glucose homeostasis (21).

The NRG-1 gene has been identified with the length of 1.4 »1,4 megabases (»1/2000th dari genome)); less than 0.3% of the length of the protein-coding. Due to a large number of splicing alternatives and multiple promoters, there are at least 15 different NRG isoforms produced by a single NRG-1 gene (17). The NRG-1 gene is located on chromosome 8p12 (chromosome 8, short arm, position 12) (19).

Genetic and environment are the two factors which define human phenotype variation. The simplest genetic variation is the single nucleotide polymorphism (SNP). Most polymorphisms have no functional implication; therefore, no clinical symptoms appeared. Nonetheless, it is convinced that even SNP may affect people to develop certain diseases, compare to others (22, 23). There are more than 23.000 SNP in the NRG-1 gene, and several of them are identified. Researchers have recently focused on SNP rs35753505 on 5 untranslated regions (5'UTR) (20).

Transition in a single nucleotide, specifically on that region, will cause differentiation of composting amino acid; therefore, the result of mRNA translation will differ. This may contribute to the susceptibility of an individual to some morbidities (24, 25). The SNP is a manifestation of gene polymorphism. There are two alleles in NGR-1 gene, C allele, and T allele; therefore, there is a combination of CC allele, CT allele, and TT allele (20). A study conducted on 97 neonates demonstrated that 44%, 40%, and 12% of the neonates had CT, CC, and TT genotypes, respectively.

Neonates with CC genotype are at a lower risk of preterm birth, even though not statistically significant. The CC genotype has a significant association with decreased length of neonatal intensive care unit stay (OR 0.3; 95% CI 0.1-0.8) (26). The present study aimed to evaluate the association of NRG1 rs35753505 C/T polymorphism and NRG-1 levels with short-term morbidities in preterm neonates in Medan, North Sumatera, Indonesia. We aimed to identify one of the predictors of morbidity in premature neonates so that early treatment strategies can be planned to reduce complications and long-term effects on the development of premature neonates.

Methods

Study Design and Participants

This cross-sectional study was conducted on 62 preterm neonates with a gestational age of 32-36 weeks in several hospitals in Medan, Indonesia, from December 2017 to December 2018. The abstract was approved by the Medical Faculty of Universitas Sumatera Utara Health Research Ethical Committee, and written consent was obtained from the subject's parents. Eligible preterm neonates were included in the study with consecutive sampling techniques. The inclusion criteria were preterm neonates with a gestational age of 32-36 weeks delivered via normal spontaneous delivery or cesarean section. On the other hand, stillborn neonates and those with congenital malformation were excluded. All subjects' short-term morbidities (i.e., seizure, hypoglycemia, temperature instability, respiratory distress, anemia, and jaundice) were observed in the first 72 h.

Sample Collection and Measurement

Umbilical cord blood specimens were collected immediately after birth in an EDTA tube to determine NRG-1 levels with enzyme-linked immunosorbent assay (ELISA) and NRG-1 polymorphism with polymerase chain reaction (PCR). Every umbilical cord blood sample was centrifuged and frozen at a temperature of -70°C until the commencement of laboratory testing. The ELISA was performed using the DuoSetHuman ®NRG1-β1/HRG-β. Polymerase Chain Reaction was performed on 100 genomic DNA using primer 5'-ACC TAA GAT GTC CAA GAG ACA G-3' forward, 5'-GAC TGG AAG CCA TGT ATC TTT ATT GT-3' reverse (®Integrated DNA Technologies), and Go Taq® Green Master Mix (®Promega). There was no standardized cut-off value for the classification of NRG-1 levels from the previous study; consequently, we utilized this median value to classify as high and low values of NRG-1 levels. The NRG-1 genotype polymorphism is a variation of the NRG-1 gene at the 35753505 subunit receptor located at 5'UTR on chromosome 8p12. The result of real-time PCR differentiated among NRG-1 CC, CT, and TT polymorphisms and its allele.

Statistical Analysis

Continuous variables were presented as medians (minimum-maximum), while categorical variables were expressed as frequencies (%). Bivariate analysis was performed to analyze the association between NRG-1 levels or NRG-1 polymorphism with short-term morbidities using the chi-square test or Fisher's exact test. The data were analyzed in SPSS software (version 22.0) with a 95% confidence interval. A p-value of less than 0.05 was considered statistically significant.

Results

During the study period, 62 eligible preterm neonates were included in the study; nonetheless, 14 preterm neonates were ruled out due to lysis of the cord blood specimen. A total of 52.1% of subjects were male, and 56.3% of cases had birth weight <2,500 grams. The majority of neonates were born via cesarean section (95.8%) and late preterm at 35-36 weeks 7 days (66.7%). Most subjects had APGAR scores of 7 or more in the first min (79.2%) and the fifth min (97.9%) after birth. Maternal and preterm neonates' characteristics are described in Table 1. The most common short-term morbidities in this study were jaundice (42.5%), followed by hypoglycemia (32.5%), respiratory distress (22.5%), and anemia (2.5%). Any subject may have experienced more than one short-term morbidity (Table 2).

The median value of NRG-1 levels in the study subjects was 174.4 pg/ml. In the present study, the median value was used as a cut-off in categorizing high and low NRG-1 levels due to the lack of its standard levels and not normally distributed data in previous literature. Moreover, 50% of subjects had high NRG-1 levels (Table 3, Figure 1). The association between NRG-1 levels and short-term morbidities is displayed in Table 4. Preterm neonates with low NRG- 1 level had a 10-times higher risk of developing short-term morbidities.

The current study examined NRG-1 rs35753505 CC, CT, and TT polymorphisms. The frequency distribution of genotypes CC, CT, and TT from NRG-1 rs35753505 polymorphism of the subjects is displayed in Figure 2. Moreover, 42% of subjects had CT genotype, while alleles C and T were presented with the frequencies of 52% and 48%. There was a significant association between NRG-1 rs35753505 polymorphism and short-term morbidities. The presence of CC and CT genotypes increased the risk of developing short-term morbidities 13.33 times (P=0.003) and 6.19 times (P=0.019), respectively, compared to TT genotype. The presence of C alleles in subjects' genotype increased the risk of short-term morbidity 4.04 times (P=0.001), compared to those with T alleles (Table 5).

Table 1. Characteristics of maternal and preterm infants

Variable	n (%)
Maternal age (years), mean (SD)	31.0 (5.26)
Maternal body weight (kg), mean (SD)	69.3 (10.48)
Maternal gravida, n (%)	
Primigravida	36 (75.0%)
Multigravida	12 (25.0%)
Maternal parity, n (%)	
Multipara	8 (16.7%)
Primipara	40 (83.3%)
Mode of delivery, n (%)	
Normal spontaneous delivery	2 (8.0%)
Sectio caesarian	46 (92.0%)
Indication of preterm labor, n (%)	
Medical*	28 (58.3%)
Spontaneous	20 (41.7%)
Gestational age (weeks), n (%)	
32 – <35 weeks	16 (33.3%)
35 – <36 weeks 7 days	32 (66.7%)
Sex of preterm infants, n (%)	
Male	25 (52.1%)
Female	23 (47.9%)
Birth weight (g), n (%)	
<2.500	27 (56.3%)
>2.500	21 (43.8%)
1st min APGAR score, n (%)	
<3	1 (2.1%)
4-6	9 (18.8%)
>7	38 (79.2%)
5th min APGAR score, n (%)	
<3	0 (0%)
4-6	1 (2.1%)
>7	47 (97.9%)

*preeclampsia, premature rupture of membranes, fetal distress

Table 2. Short term morbidities of the patients

Short term morbidities*	n (%)
Seizure (n, %)	0 (0)
Temperature instability (n, %)	0 (0)
Respiratory distress (n, %)	9 (22.5%)
Hypoglycemia (n, %)	13 (32.5%)
Anemia (n, %)	1 (2.5%)
Jaundice (n, %)	17 (42.5%)

*) any patients may experience more than one short term morbidities

Table 3. NRG-1 levels in preterm infants

Variable	n (%)
NRG-1 levels, median (min-maks), pg/ml	174,4 (121,1 – 753,3)
High (≥174,4)	24 (50%)
Low (<174,4)	24 (50%)

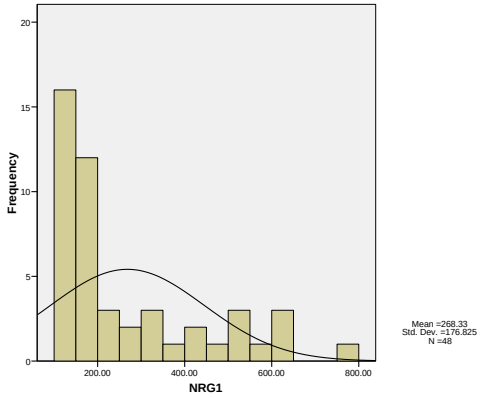

Figure 1. Distribution ofNRG-1 levels (pg/ml)

Table 4. Association between NRG-1 levels with short term morbidities in preterm infants

Variable	Short term morbidities		*P**	OR (95%CI)
	Yes-n (%)	No-n (%)		
NRG-1				
Low	20 (71.4)	4 (20.0)	<0.001	10.00 (2.54-39.29)
High	8 (28.6)	16 (80.0)		
Total	28 (100)	20 (100)		

*chi-square test

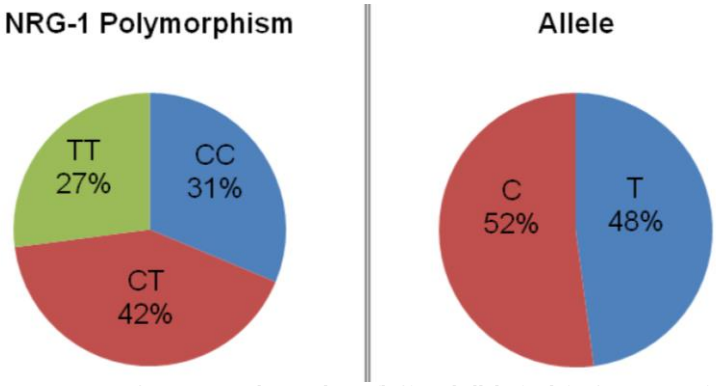

Figure 2. Frequency of genotype polymorphism (left) and allele (right) of NRG-1 rs35753505

Table 5. Association of NRG-1 rs35753505 polymorphism with short term morbidities

NRG-1 polymorphism	Short term morbidities		*P**	OR (95%CI)
	Yes-n (%)	No-n (%)		
CC genotype	12(42.8)	3 (15.0)	0.003	13.33(2.18-81.22)
CT genotype	13 (46.4)	7 (35.0)	0.019	6.19 (1.27 – 30.17)
TT genotype	3 (10.7)	10 (50.0)		
C allele	37 (66.1)	13 (32.5)	0.001	4.04 (1.70 – 9.58)
T allele	19 (33.9)	27 (67.5)		

**P*<0.05significant compared to TT genotype; chi-square test

Discussion

According to gestational age, preterm neonates are divided into extreme preterm (less than 28 weeks) which constitutes about 5% of preterm neonates, very preterm (28-31 weeks) which is about 15%, moderate preterm (32-33 weeks) around 20%, while the majority (60%-70%) of them are late preterm (34-36 weeks) (27). In the present study, the majority of preterm neonates were late preterm with a gestational age of 35 -<36 weeks 7 days (66.7%), while 33.3% of cases were moderate preterm. According to a previous study, preterm birth accounts for 11.1% of total births in the world (3). The majority of preterm neonates (71%) are born at 34-37 weeks of gestation (28, 29), and 55% of them are male (3). The proportions of male and female neonates in this study did not differ.

According to WHO, routine delivery with cesarean section is not recommended for premature neonates, unless it has medical indications (28). Cesarean section is indicated in labors with previous surgery, abnormal placental location, maternal heart disease, as well as maternal and fetal indications, such as cephalopelvic disproportion, failure of normal delivery, placenta previa, and fetal distress (30). In the present study, the majority of preterm neonates (n=48) were born via cesarean section with a medical indication, such as maternal complications and fetal distress.

Maternal characteristics were recorded, and mean maternal age was obtained at 31.0 years. The majority of mothers were primigravida. The findings differed from the previous study which reported that younger (adolescent) or older maternal age may contribute to increasing the risk of preterm delivery (3,29). Regarding gravida status, the risk of preterm delivery will decrease as the gravida status increases (31). The majority of preterm neonates were born with APGAR score ≥7, cried immediately as most of the neonates born on the gestational age of 35-<36 weeks and 7 days do since the transitional phase from intrauterine to extrauterine went well.

Since prematurity is a multifactorial problem, multiple studies have been conducted to shed light on the risk factors for preterm birth and effective measures to prevent short-term morbidity that will later have the potential to cause long-term

disorders. Preterm neonates are prone to clinical problems, such as hyaline membrane disease, bronchopulmonary dysplasia, apnea of prematurity, patent ductus arteriosus, hyperbilirubinemia, poor gastrointestinal function, hypothermia, hyperglycemia, hypocalcemia, and retinopathy (32,33). In the present study, 48% of preterm neonates developed short-term morbidities. This is almost the same as the percentage of neonatal morbidity and mortality reported in the world, about 60-80% (3). Those short-term morbidities will affect long-term growth and development if not addressed properly (29, 34).

The NRG-1 is a trophic factor that contains epidermal growth factor (EGF)-like domains that are signaled by stimulating ErbB receptor tyrosine kinases. The existence of NRG-1 has been widely studied and proven to be important in fetal development. Low NRG-1 levels will inhibit the formation of surfactants and affect the development of the heart, as well as nerves and immune system in preterm neonates(18,26,35-36). No literature has described cut-off normal levels of NRG-1 in neonates and children. The studies conducted on healthy populations obtained mean NRG-1 serum levels of 217 pg/mL with a range of 32-473 pg/mL (37). In the present study, there was no difference in the proportion of subjects with high and low NRG-1 levels(cut-off of 174.4 pg/ml). This is different from the previous study in which NRG-1 levels were lower since the neonate was premature [p1] (38).

The development of various organs is influenced by the presence of NRG-1, including the brain, lungs, intestines, kidneys, and heart (36). If these organs function properly, the short-term morbidities that threaten neonates can be reduced (17). In a similar vein, the present study pointed to the relationship between NRG-1 levels and short-term morbidities of preterm neonates. [p2] Low NRG-1 levels will lead to a 10-fold increase in the incidence of short-term morbidities, compared to those with high NRG-1 levels.

As mentioned earlier, genetic factors affect the incidence of preterm births. These manifestations are caused by an evolutionary process that causes genetic changes (39, 40). Genetic tests were carried out in this study to assess the coding gene of NRG-1 rs35753505 polymorphism. In the present study, most polymorphisms were CT genotype. This finding is in agreement with those obtained by Knickmeyer et al. who reported that out of 272 late preterm neonates, 46%, 41%, and 16% were heterozygote CT, TT, and CC, respectively (41).

In the current study, SNP was discovered on the NRG-1 rs35753505 gene, related to the incidence of short-term morbidities. The discovered CC, CT genotypes, and alleles C increase the risk of short-term morbidities in preterm neonates. Overall NRG-1 polymorphism did not affect the incidence of short-term morbidities. Only the CC genotype significantly increased the incidence of short-term morbidities, compared to the TT genotype. This is in line with the results of a study by Hoffmann et al. who indicated that neurologic morbidities, such as periventricular leukomalacia, cerebral palsy, and developmental delay, were seldom found in neonates with C alleles (38).

The results of the present study are expected to be useful as an alternative effort to prevent short-term morbidity by providing NRG-1 preparations to prevent short-term morbidity in preterm neonates. In previous studies, NRG-1 has begun to be administered to adult patients with heart and hemodynamic disorders. Among the notable limitations of the present study, we can refer to non-involvement of control term neonates; therefore, no evaluation regarding NRG-1 rs35753505 polymorphism, NTRG-1 levels, and their association with short-term morbidities can be established. Long-term evaluation to determine the association of NRG-1 rs35753505 polymorphism and NRG-1 levels with long-term morbidities is also warranted. It is hoped that the results of the present study will be used as a reference for future studies related to NRG-1 polymorphism rs35753505 C / T and NRG-1 protein levels as predictors of short-term complications in premature neonates.

Conclusion

Preterm neonates with low NRG-1 levels have a higher risk of developing short-term morbidities. The most common manifestation of NRG-1rs35753505 polymorphism is the heterozygote CT genotype with the C allele. The risk of short-term morbidities will be increased in preterm neonates with CC and CT genotypes, compared to the TT genotype. Preterm neonates with C allele also have a higher risk of short-term morbidities, compared to those with T allele.

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None.

Conflicts of interest

The authors declare that they have no conflict of interest.

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