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Impact of the *Neuregulin* rs35753505 C/T Polymorphisms on Neuregulin 1 Levels in Preterm Infants

Bugis Mardina Lubis[1*], Sjarif Hidajat Effendi[2], Ratna Akbari Ganie[3], Oke Rina Ramayani[1]

[1]Department of Child Health, Faculty of Medicine, Universitas Sumatera Utara, Medan, Indonesia; [2]Department of Child Health, Faculty of Medicine, Universitas Padjajaran, Bandung, Indonesia; [3]Department of Clinical Pathology, Faculty of Medicine, Universitas Sumatera Utara, Medan, Indonesia

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***Correspondence:** Bugis Mardina Lubis. Department of ChildHealth, Faculty of Medicine, Universitas Sumatera Utara, Medan, Indonesia; 2Department of ChildHealth, Faculty of Medicine, Universitas Padjajaran, Bandung, Indonesia. E-mail: bugismardina@yahoo.com

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Abstract

BACKGROUND: Neuregulin (NRG) 1 plays an important role in the development of various organ systems in human. Single nucleotide polymorphisms rs35753505 C/Tof the gene encoding NRG1 evident as allele C and T with genotypes of CT, CC, and TT are believed to have an impact on NRG1 levels.

AIM: To determine the impact of the NRGrs35753505 C/T polymorphisms on NRG1 levels in preterm infants.

METHODS: A cross-sectional study was conducted from February to December 2018, whereas 48 eligible preterm infants with a gestational age of 32- < 37 weeks were enrolled. An umbilical cord blood specimen was collected for determination of NRG1 levels with enzyme-linked immunosorbent assay (ELISA) and NRG1 polymorphisms with polymerase chain reaction (PCR). Statistical analysis was performed with 95%CI and P value of < 0.05 was considered statistically significant.

RESULTS: Median value of NRG1 levels (174.4 pg/ml) served as a cut off value. NRG 1 polymorphisms composed distribution of CC (31%), CT (42%), TT (27%) genotypes and distribution of C and T alleles were 52% and 48%. The median NRG1 levels in CC and CT genotypes were significantly lower compared to TT genotype (151.1 pg/ml vs 407.2 pg/ml, P = 0.005 and 159.1 pg/ml vs 407.2 pg/ml, P = 0.009). Subjects with C allele had significantly lower median NRG1 levels than T allele (151.1 pg/ml vs 407.2 pg/ml, P = 0.002). Subjects with CC and CT genotypes had higher risk to develop lower NRG1 levels compared to TT genotype (OR = 8.25, P = 0.016 and OR = 10.74, P = 0.005, respectively).

CONCLUSION: Allele C is associated with lower NRG1 levels. Preterm infants with CC and CT genotypes pose a higher risk to have lower NRG1 levels.

Introduction

The neuregulin (NRG) protein, encoded by the neuregulin gene, is a membrane glycoprotein that mediates inter-cell signals and plays an important role in the growth and development of multiple organ systems. It has ligand to thyroxine kinase receptors ERBB3 and ERBB4. It concomitantly recruits co-receptors ERBB1 and ERBB3, which stimulate thyroxine phosphorylation and activation of ERBB receptors. These isoforms affect the growth and differentiation of epithelial cells, glial cells, neurons, and muscle cells; induces expression of acetylcholine receptors in synaptic vesicles during neuromuscular junction generation; stimulates lobuloalveolar budding and milk production; stimulates Schwann cell proliferation; myocardial development such as trabeculation during heart development; neuronal migration and regulation of neurotransmitter receptors on neurons; play a role in the development of motor and sensory neurons [1], [2].

Neuregulin 1 is a trophic factor which is a subclass of transmembrane GF polypeptide owned by family epidermal growth factor (EGF) signalled by stimulating ERBB receptor tyrosine kinases. It is a family of GF encoded from four different genes of NRG-1, NRG-2, NRG-3, and NRG-4, where NRG1 is the best characteristic. EGF-like domains are located within the membrane-proximal region of the

extracellular region, which allows the activation of the ErbB receptor tyrosine kinase. NRG1 plays a role in stimulating a family of single-transmembrane receptor tyrosine kinases called ErbB proteins [3].

The last important finding is sequencing and the identification of all NRG 1 genes in human that have been successfully identified. It has a length of ≈ 1.4 megabases (≈ 1/2000th of the genome); less than 0.3% of its length encodes the protein. Due to the many alternative splicing and multiple promoters, there are at least 15 different NRG isoforms produced by a single NRG1 gene. The three important structural characteristics that distinguish isoforms in in-vivo functions and biological cells are types of EGF-like domains (αorβ), N-terminal sequences (type I, II, or III) or whether the isoform is directly synthesised as a transmembrane or non-membrane protein [4]. Single nucleotide polymorphisms rs35753505 C/Tof the gene encoding NRG1evident as allele C and T with genotypes of CT, CC, and TT are believed to have an impact on the NRG1 levels.

Methods

A cross-sectional study was conducted from February to December 2018, whereas 48 preterm infants from 5 hospitals in Medan with a gestational age of 32- < 37 weeks were enrolled. Infants with severe congenital malformation were excluded. Umbilical cord blood specimens were collected immediately after birth in an EDTA tube for determination of NRG1 levels with enzyme-linked immunosorbent assay (ELISA). A blood sample was centrifuged and frozen at a temperature of -700°C until laboratory testing commenced. ELISA was performed using the ®DuoSetHuman NRG1-β1/HRG-β. As much as 100 µl sample was added in each well-containing reagent diluents and incubated for 2 hours. Detection antibody (100 µl) was added, and the solution was incubated for 2 more hours. Working dilution (100 µl) was then added into the solution and incubated for 20 minutes. The solution was incubated for 20 more minutes after the addition of 100 µl substrate solution. At the last step, 50 µl of stop solution was added to each well. After being centrifuged, the solution's optical density was determined using a microplate reader set to 450 nm.

Polymerase Chain Reaction was performed on 100 of genomic DNA using primer 5'-ACC TAA GAT GTC CAA GAG ACA G-3' forward and 5'-GAC TGG AAG CCA TGT ATC TTT ATT GT-3' reverse (®Integrated DNA Technologies) and Master mix Go Taq® Green Master Mix (®Promega). Thirty-six cycles were performed with 15 minutes denaturation at 95°C, 1-minute annealing at 68°C and 1-minute extension at 72°C. The difference in NRG1 levels was analysed using the Mann-Whitney test. The association between polymorphisms and NRG1 levels was analysed using the chi-square test. Statistical analysis was performed using statistical software at 95% CI, and a P value of < 0.05 was considered significant. This study was approved by the Health Research Ethical Committee, Medical School, University of Sumatera Utara.

Results

The median value of NRG1 levels was 174.4 pg/ml and served as a cut off value. Neuregulin 1 polymorphisms rs35753505 C/Tcomposed distribution of CC, CT, and TT as many as 31%, 42%, and 27%, respectively. Distribution of C and T alleles were 52% and 48%. Baseline characteristics of subjects are shown in Table 1.

Table 1: Baseline characteristics of subjects

Characteristics	n (%)
Mode of delivery, n (%)	
Normal	2 (4.2%)
Caesarean section	48 (95.8%)
Indication for preterm labor, n (%)	
Medical indication	28 (58.3%)
Spontaneous	20 (41.7%)
Gestational age, week, n (%)	
32 – < 35 weeks	16 (33.3%)
35 – < 37 weeks	32 (66.7%)
Gender, n (%)	
Male	25 (52.1%)
Female	23 (47.9%)
Birth weight, gram, n (%)	
< 2.500	27 (56.3%)
≥ 2.500	21 (43.8%)

Mann-Whitney test was used to determine the differences in serum NRG1 levels between genotypes and alleles of neuregulin rs35753505 C/T polymorphisms. The median NRG1 levels in CC and CT genotypes were significantly lower compared to TT genotype (151.1 pg/ml vs 407.2 pg/ml, P = 0.005 and 159.1 pg/ml vs 407.2 pg/ml, P = 0.009). Subjects with C allele had significantly lower median NRG1 levels than their counterparts (151.1 pg/ml vs 407.2 pg/ml, P = 0.002) (Figure 1).

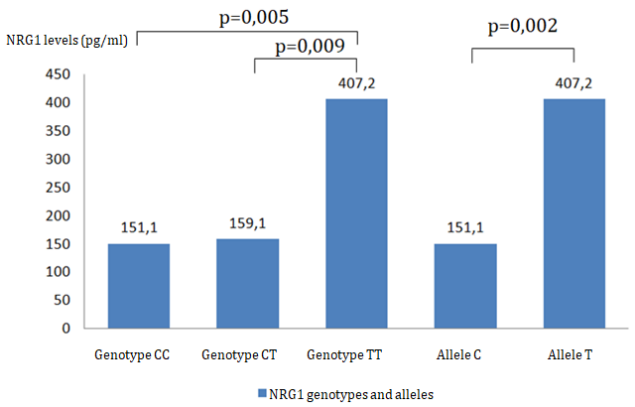

Figure 1: The differences in serum NRG1 levels between genotypes and alleles of NRG rs35753505 C/T polymorphisms

Chi-square test was conducted to determine the association between NRG1 rs35753505 C/T polymorphisms and NRG1 levels in this study. We found a significant association between NRG rs35753505 C/T polymorphism with NRG1 levels. Subjects with CC and CT genotypes had higher risk to have lower NRG1 levels compared to TT genotype (OR = 8.25, 95%CI = 1.32-51.26, P = 0.016 and OR = 10.74, 95% CI = 1.74-59.65, P = 0.005, respectively). Subjects with C allele had higher risk to have lower NRG1 levels compared to T allele (OR = 2.78, 95% CI = 1.21-6.36, P = 0.014) (Table 2).

Table 2: The association between NRG rs35753505 C/T polymorphism with NRG1 levels

NRG rs35753505 C/T polymorphism	NRG1 levels		*P*	OR (95%CI)
	Low n (%)	High n (%)		
CC genotype	9 (37.5)	6 (23.0)	0.016#	8.25 (1.32 – 51.26)
CT genotype	13 (54.1)	7 (26.9)	0.005#	10.74 (1.74 – 59.65)
TT genotype	2 (8.3)	13 (50.0)		
C allele	31 (64.6)	19 (39.6)	0.014*	2.78 (1.21 – 6.36)
T allele	17 (35,4)	29 (60,4)		

*$P < 0.05$; #significant compared to TT genotype.

Discussion

The presence of NRG1 has been widely studied and has been shown to play a role in fetal development. Low NRG1 levels will inhibit surfactant formation and affect heart development, nerves, and the immune system in premature infants [3], [5], [6], [7], [8], [9], [10]. There is no literature that publishes the normal cut-off levels of NRG1 in children. Studies conducted on the adult population with coronary heart disease shows the median NRG1β level in plasma and serum in individuals with ischemia of 4100 pg/mL and 2400 pg/mL, respectively, whereas for individuals without ischemia at 3300 pg/mL and 1600 pg/mL. The study also states that NRG1β levels in plasma are more accurate than those in serum [11]. Studies conducted in clinically healthy populations obtained a mean serum NRG1 levels of 217 ng/mL with a range of 32 ng/mL to 473 ng/mL [12]. Neuregulin 1 is also found in the human cornea; prior studies using the PCR showed no difference in the replication of the NRG1 coding gene in the cornea and epithelium. This demonstrates that NRG1 is produced by relatively equal numbers by epithelial and stromal cells [13]. The median NRG1 level in this study was 174.4 pg/mL. Using the median value as a cut off to determine high and lower NGR1 levels, an equal proportion of subjects with high and lower NRG1 levels was obtained. This is different from the previous study. The previous study has suggested that NRG1 levels will be lower as more premature the baby is [14]. The NRG1 gene, together with the NRG2, NRG3, and NRG4 genes, functions to encode the NRG1 protein [7]. In these genes, DNA sequences variations can occur in the form of SNPs that can affect NRG1 production [10]. A study conducted by Hoffmann, et al., showed the presence of NRG 221533 coding gene polymorphism, which resulted in 77% of infants having at least one C allele [14]. By Hardy-Weinberg law, the polymorphism will be passed to the next generation in the same proportion if no mutation occurred. The impact of these polymorphisms will also persist in the next generation until other mutations or genetic changes occurred [15]. In this study, a genetic examination was conducted to determine the NRG rs35753505 C/T polymorphism. Based on the results of the examination, it was found that 42% of subjects had CT genotypes, while CC and TT genotypes were detected in 31% and 27% of subjects, respectively.

There was an association between genotypes and alleles of the NRG rs35753505 C/T polymorphism with NRG1 levels in this study. Subjects with CC and CT genotypes had lower NRG1 levels than subjects with TT genotype (P = 0.005 and P = 0.009). The presence of C allele was associated with a decrease in NRG1 levels in the study subjects (P = 0.02). The NRG1 rs35753505 polymorphism was associated with a decrease in NRG1 levels in this study. Subjects with CC and CT genotypes showed a decrease in NRG1 levels of 8.25 times (P = 0.016) and 10.74 times (P = 0.005) compared to subjects with TT genotypes. The presence of C allele will increase the risk of decreasing NRG1 levels by 2.78 times (P = 0.014). The previous study reported different results. This is different from the results of previous studies where neonates, who had C alleles were less often born prematurely and had higher NRG levels than neonates who had T alleles [14].

In conclusion, the median value of NRG1 levels in the study subjects was 174.4 pg/ml. The most frequent NRG rs35753505 C/T polymorphism in this study was heterozygous CT genotype (42%). The c allele was present in 52% of subjects. The serum neuregulin level was lower in subjects with CC and CT genotype than subjects with TT genotypes. Subjects with C allele showed lower serum NRG1 levels compared to subjects with T allele. There was a significant relationship between NRG rs35753505 C/T polymorphism and NRG1 levels. Subjects with CC genotype had a risk of 8.25 times higher having lower NRG1 levels than subjects with TT genotype while subjects with CT genotype had 10.74 times higher risk. The presence of C allele had a risk of 2.78 times higher for low NRG1 levels.

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