Malay premature infants with retinopathy of prematurity: risk factors and screening of NDP gene mutation

Abstract — The aim of this study is to determine the risk factors for retinopathy of prematurity (ROP), and also to screen Norrie Disease Pseudoglioma (NDP) gene mutation in order to determine if mutation in the NDP gene may play a role in the development of ROP among Malay premature infants. This was a case control study among Malay premature infants from Hospital Universiti Sains Malaysia (USM) conducted from August 2011 to May 2013. Written consent were taken from their parents before conducting the study. The stage of ROP, systemic risk factors (gestational age and birth weight) and enviromental risk factors (oxygen exposure and duration of ventilation) were reviewed from patients’ medical records. DNA was extracted from venous blood and subjected to polymerase chain reaction (PCR) before direct sequencing of NDP gene. A total of 56 Malay premature infants (Case group = 28 ROP premature infants, Control group = 28 non-ROP premature infants) from Hospital USM were enrolled in this study. Out of 28 premature infants with ROP, 11 (39.3%) premature infants were in stage 3. Only 1 (3.6%) premature infant in stage 4 and 2 (7.2%) premature infants in stage 5. The gestational age (p = 0.010) and birth weight (p = 0.010) were the significant risk factors for ROP. There was no significant difference of environmental risk factors between the two groups. The NDP gene mutation was not detected in Malay premature infants with ROP and also in control group. The gestational age and birth weight were important risk factors of ROP. Although NDP gene mutations were being linked to ROP but NDP gene mutation was not detected in premature infants with ROP as well as premature infants with non-ROP among Malay ethnic background.

Keywords — Low birth weight, Malay, Mutations, NDP gene, Prematurity, Retinopathy of prematurity

1 INTRODUCTION

Retinopathy of prematurity (ROP) is the leading cause of preventable childhood visual loss even in the developed world and accounts for 3 to 11% of blindness in children [1]. Major systemic risk factors that are associated with ROP include prematurity and also low birth weight (LBW), with prematurity as the primary risk factor for ROP development. Severity of ROP and prematurity are closely related to each other; as there is an increase in the incidence and severity of ROP when there is a decrease in gestational age.

LBW was found to be another risk factor and an important predictor for ROP other than prematurity. The improving survival rate of extreme low birth weight (ELBW) (< 1000 g) infants in recent years has increased the chances of developing ROP [2]. Overall incidence rate was estimated to be as high as 68% among infants who were born with birth weight 1251 g and 93% for birth weight of 750 g [3].

The link between supplemental oxygen and ROP was first recognized in 1951 [4]. High oxygen exposure is one of the environmental risk factors that refer to a condition where oxygen saturation (SpO2) in neonates’ blood is 95% or more for higher than 40% of daily percentage time [5]. The incidence of ROP decreased when infants with a birth weight 1250 g or less had a reduced SpO2 level (SpO2 < 95%) in the first week of life [6].

Mechanical ventilation is another factor, which has adversely contributed to several devastating complication including ROP and chronic lung diseases [7]. Many studies have found that mechanical ventilation acts as an independent risk factor for ROP, which is proportionate to the duration of ventilation [5,7].

Genetic influences on the pathogenesis of ROP have been implicated on human as well as experimental animal study [8]. Genetic factor is associated with ROP in recent years since infants
from similar risk factor group have developed different phenotypes of ROP. Investigators discovered that about 70% of the variance in susceptibility to ROP was the result of genetic factor alone which consequently encouraged the study of sequence variants in genetic components that control Wnt signalling factors. These factors play a key role in the growth and establishment of vascular development in retina [9]. Common Wnt signalling factors are norrin protein, frizzled-4 (FZD4) protein and lipoprotein receptor-related protein 5 (LRP5) [10]. Norrie Disease Pseudoglioma (NDP), FZD4 and LRP5 genes are related to this Wnt signalling factors respectively [10].

NDP gene is located in long arm (q) of chromosome X at position 11.4 (Xq11.4) and is expressed in the retina, choroid and brain. It is approximately 24 kb in length from base pairs 43,808,021 to 43,832,920 and has three exons with 1.85kb transcript area.

NDP gene provides instruction for making norrin protein, which is 133 amino acids in length and is involved in blood vessel formation. Norrin protein play a role in Wnt signaling pathways which is important for cell proliferation, attachment, adhesion migration and many other cellular activities. Norrin protein binds with the receptor FZD4 protein, which is produced by FZD4 gene and initiates a serial process that regulates the activity of certain genes. Besides, norrin protein and FZD4 protein also play critical roles in the specialization of cells in the retina and the establishment of blood vessel formations.

More than 75 mutations have been identified in the NDP gene and these mutations affect the ability of norrin protein to bind with FZD4 protein, interfering with the specialization of retinal cells and development of blood vessels. Deficiency of the protein causes retarded retinal vascularization leading to retinal ischaemia and the accumulation of hypoxia induced angiogenic factors.

In addition, NDP gene mutations may influence the course or progression of ROP in premature infants. Infants with ROP usually may experience improvement of the condition over time, but some NDP gene mutations have been associated with a worsening of the condition. NDP gene mutations also may cause other retinal disorder, such as Coats disease and familial exudative vitreoretinopathy (FEVR) [11].

NDP gene mutations were seriously being linked to ROP based on previous reports [3,12] in which the mutations in ROP patients had been identified. Screening of NDP gene mutation will provide early identification of infants at very high risk in the development of advanced ROP, thereby allowing early aggressive intervention.

The aim of this study is to determine the risk factors for ROP, and also to screen NDP gene mutation in order to determine if mutation in the NDP gene may play a role in the development of ROP among Malay premature infants.

2 METHODS
2.1 Patients
This was a case control study among Malay premature infants with ROP as a case group while non-ROP premature infants as a control group. It was conducted in Hospital Universiti Sains Malaysia (USM) from August 2011 to May 2013. The patients were enrolled in this study after written consents were taken from the parents. Premature infants with gestational age more than 34 weeks, and birth weight of more than 1500 g were excluded from the study.

Paediatric Ophthalmology Team did the screening for the presence of ROP and the staging of ROP was based on the International Classification of Retinopathy of Prematurity (ICROP) [10]. The systemic risk factors; gestational age ≤ 34 weeks and birth weight ≤ 1500 g, and environmental risk factors; oxygen exposure (SpO2 level ≥ 95% daily percentage time) and duration of ventilation (day) were reviewed from patients' medical records. This study had received ethical approval from USM Ethical Committee for Human Research (USMKK/PPP/JEPeM[242.3.(9)]).

2.2 Genetic Analysis
Venous blood (0.5 ml to 1.0 ml) was collected in EDTA tube (BD, New Jersey, USA) and stored in -20°C prior to extraction. Genomic deoxyribonucleic acid (DNA) from each infant was extracted from venous blood using QiaAmp DNA blood mini kit (Qiagen, Hilden, Germany) according to manufacturer's recommended protocols and subjected to polymerase chain reaction (PCR) for amplification of target sequences.

The primer for PCR for this study was designed based on a complementary DNA (cDNA) sequences of NDP gene (NM 000266.3) obtained from a GenBank (National Centre for Biotechnology Information) and encode from the previous study [10]. These designated primers
then underwent blasting process using the software OLIGO version 7. A total of three pairs (forward/reverse) of primer were designed to amplify three exons of NDP gene. Exon 1 is 201 bp length and does not have coding sequence region (CDS) and only contained untranslated region (UTR). Exon 2 is 381 bp and contained UTR as well as CDS regions. Exon 3 is the longest (1257 bp) and contained the main CDS region. Table I showed the selected 3 pairs of primer sequences and Figure 1 showed the position of the primers target area in their respective exons.

PCR was done using Phusion High-Fidelity DNA Polymerase reagent kit (Thermo Fisher Scientific, Massachusetts, USA) by following manufacturer’s exact protocols and 5x HF buffer was chosen just to decrease the error rate. Amplification was conducted for 30 cycles for every step of denaturation (98°C for 30 sec), annealing (Primer 1 = 61°C for 10 sec, primer 2 = 60°C for 10 sec and primer 3 = 66°C for 15 sec) and finally extension (72°C for 10 sec for primer 1 and 2, 15 sec for primer 3). Purification of PCR product was then done using QIAquick PCR Purification kit (Qiagen, Hilden, Germany) before samples were sent to First BASE Laboratories Sdn. Bhd. Malaysia (604944-X) for direct sequencing of NDP gene.

Electropherogram of the nucleotide sequences received were analyzed using ‘Bio Edit Sequence Alignment Editor Software’ version 7.1.3.0 in order to identify the presence of any mutations.

Table I: Primer sequences of NDP gene [10].

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequences</th>
<th>Size (bp)</th>
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<tbody>
<tr>
<td>Primer 1 Forward 1</td>
<td>CGC CTG ATT</td>
<td>322</td>
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<tr>
<td></td>
<td>GAT ATA TGA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CTG CAA TGG C</td>
<td></td>
</tr>
<tr>
<td>Reverse 1</td>
<td>GCT CGG TTT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GGA AAG AAG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CGA TTT CCT</td>
<td></td>
</tr>
<tr>
<td>Primer 2 Forward 2</td>
<td>TTC TGG GTA</td>
<td>471</td>
</tr>
<tr>
<td></td>
<td>AAT AAT TCT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GGG G</td>
<td></td>
</tr>
<tr>
<td>Reverse 2</td>
<td>GTT TCT GAG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GGA AAT GCT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CTC CTC ACA</td>
<td></td>
</tr>
<tr>
<td>Primer 3 Forward 3</td>
<td>TAA GGT TGT</td>
<td>690</td>
</tr>
<tr>
<td></td>
<td>GGC ATG CCC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ACA GAG TAA</td>
<td></td>
</tr>
<tr>
<td>Reverse 3</td>
<td>CAG AAG ATG</td>
<td></td>
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<tr>
<td></td>
<td>TCC CAG GAA</td>
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<td></td>
<td>AAG CTG GCC</td>
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Figure 1: Position of the primers target area in their respective exons.
2.3 Data Analysis
Data entry and analyzes were performed using Statistical Package for Social Sciences version 20. Independent t-test was used for comparison of gestational age, birth weight and duration of ventilation. Significance of difference in values was determined by the p value < 0.05.

3 RESULTS
3.1 Patient Characteristic and Stages of ROP
A total of 56 Malay premature infants (ROP premature infants = 28, non-ROP premature infants = 28) were recruited. Out of 56 premature infants in our study, 32 were boys (57.1%) and 24 were girls (42.9%). Out of 28 premature infants with ROP, 11 (39.3%) premature infants were in stage 3. Only 1 (3.6%) premature infant in stage 4 and 2 (7.2%) premature infants in stage 5 (Table II).

3.2 Risk Factors
The mean gestational age of the premature infants with ROP was 27.8 weeks (SD 2.4) and was significantly less in gestational age than those with non-ROP (30.2 SD 2.5 weeks, p = 0.010). Birth weight was also significantly lower in the ROP premature infants group as compared to the non-ROP group (986 g vs 1495 g, p = 0.010). All premature infants in both groups were ventilated and had oxygen exposure. The mean duration of ventilation was 12.3 days (SD 22.5) in ROP group and 4.3 days (SD 16.9) in non-ROP. There was no significant difference of mean duration of ventilation between the two groups (p = 0.207) (Table II).

3.3 Screening of NDP Gene Mutation
A total of 56 blood samples from both ROP and non-ROP premature infants were successfully analysed in order to detect NDP gene mutation. The electropherogram sequences were satisfactory for almost all samples. Figure 2, Figure 3 and Figure 4 showed electropherogram graphs of one sample ROP premature infant (sample 48) for all three exons (exon 1, exon 2 and exon 3 respectively). Clustered sequences reading for all of the samples revealed no sequence NDP gene variants or NDP gene mutations detected in Malay premature infants with ROP as well as in control group (premature infants with non-ROP).

<table>
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<th>Table II: Gender distribution, stages of ROP and risk factors among Malay premature infants.</th>
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<tr>
<td>Gender</td>
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<tr>
<td>(n, %)</td>
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<tr>
<td>Boy</td>
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<tr>
<td>Girl</td>
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<tr>
<td>Stages of ROP</td>
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<td>(n, %)</td>
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<tr>
<td>Stage 1</td>
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<td>Stage 2</td>
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<td>Stage 3</td>
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<td>Stage 4</td>
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<td>Stage 5</td>
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Systemic risk factors
Gestational age < 34 weeks
Mean (SD) 27.8 (2.4) 30.2 (2.5) 0.010
Birth weight < 1500 g
Mean (SD) 986 (235) 1495 (406) 0.010

Environmental risk factors
Oxygen exposure (SpO2 ≥ 95%)
Mean (SD) 28 (100) 28 (100)
Duration of ventilation (day)
Mean (SD) 12.3 (22.5) 4.3 (16.9) 0.207

Independent t-test, P < 0.05 significant
ROP: retinopathy of prematurity, NA: not applicable, SpO2: oxygen saturation

Figure 2: Electropherogram of exon 1 for ROP sample 48.
4 DISCUSSION

In our study, the mean gestational age was 27.8 (SD 2.4) week and 30.2 (SD 2.5) weeks in ROP and non-ROP premature infants respectively. The mean gestational age in our study was lower compared to Iran infants as the reported data were 30.2 weeks and 32.4 weeks in both ROP and non-ROP group respectively [13]. The mean gestational age of our study was similar to a study conducted in Singapore where it had similar geographical strata and comparable socioeconomic status [14].

In countries with low socio-economic status, the incidence of ROP is much lower due to less survivability of premature infants. In developed countries with advanced neonatal care, there is an increased survival rate of premature infants which indirectly increases the incidence of ROP. This may explain why we have observed the differences in gestational mean age of ROP in the above studies, where health services and socioeconomic status of the different countries are not equal.

The incidence of ROP is inversely related to the degree of LBW. Lower birth weight, coincide with higher incidence of ROP. In our study, ROP were detected among premature infants born with mean birth weight of 986 (SD 235) g, compared to 1495 (SD 406) g in non-ROP premature infants.

The link between intensive oxygen supplement in premature infants and ROP was first reported by Campbell et al [4]. In a recent prospective study, Vanderveen DK et al [6] reported that there was lower incidence of ROP in premature infants (with equal gestational age strata) in lower SpO₂ group (85%-93%) with incidence of 5.5% and 17.5% in higher SpO₂ (>95%) group. It is supported by Tin W [15] who reported that the incidence of ROP was 27.2% in infants that required high SpO₂ (88-98%) compared to 6.2% in infants with low SpO₂ (70-90%). In our study, both groups of premature infants (ROP and non-ROP) were exposed to SpO₂ >95%.

Several other studies [7,16] attempted to link ROP and duration of ventilation. It was found that premature infants who were having mechanical ventilation longer than 7 days were at a higher risk to get ROP [16]. In our study, duration of ventilation was prolonged in premature infant with ROP.

Molecular pathogenesis in ROP is still inconclusive although many studies [17,18] have been conducted to link of the genetic factor as aetiology or as part of the risk factors for certain infants to develop ROP. Almost all of the previous studies [17,18] attempted to relate Wnt signaling factors gene as a potential cause for advanced ROP since these factors are highly expressed in the retina and play a vital role for normal completion of retinal vascular development. The main three genes that control Wnt signaling factors are NDP gene, FZD4 gene and LRP5 gene [10]. In our study, we performed NDP gene screening among Malay Kelantan subgroup that is known to have uniform ethnic background based on established Malay genomics data [19].

In our study, three exons of NDP gene were screened. There was no sequence variations seen in ROP Malay premature infants. There was also absence of sequence variations in control group. We could not rule out that there was no mutation at all in NDP gene due to limited number of exons tested and thus not representing the whole region of the NDP gene.

In one pilot study done by Shastry BS et al [12], they found that four out of sixteen children had severe ROP with missense mutations detected. However, research done by Kim JH et al [20] had failed to find any NDP gene mutations among Korean ROP infants. In general, NDP gene mutations were detected in a small percentage of ROP cases except in the study reported by Shastry BS et al [12]. These might be due to the low frequency of the NDP gene in ROP population. Besides, this wide diversity in the studies’ outcome that is related to NDP gene and ROP suggests that ROP is a complex disease
and population stratification may contribute to its genotype differences.

In our study, only a small number of severe ROP (11 premature infants) and advanced ROP (3 premature infants) were recruited. This may have compromised the effectiveness of the study in the screening of NDP gene mutations.

Although we could not find any NDP gene variants in our study population, our hypothesis was based on previously reported epidemiological studies [3,21] where genetic variances were influenced by ethnicity and geographical area of study. Furthermore, the absence of NDP gene sequence variants in our study could be due to low frequency nature of the NDP gene in ROP population and polygenetic nature of the disease. Multiple genes such as FZD4 gene and LRPS5 gene rather than a single gene (NDP gene alone) are postulated to be contributing to the accumulative effect of the disease process.

5 CONCLUSION
The gestational age and birth weight were important risk factors of ROP. Although NDP gene mutations were being linked to ROP, NDP gene mutation was not detected in premature infants with ROP as well as premature infants with non-ROP among Malay ethnic background from our study's population.

CONFLICTS OF INTEREST
The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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