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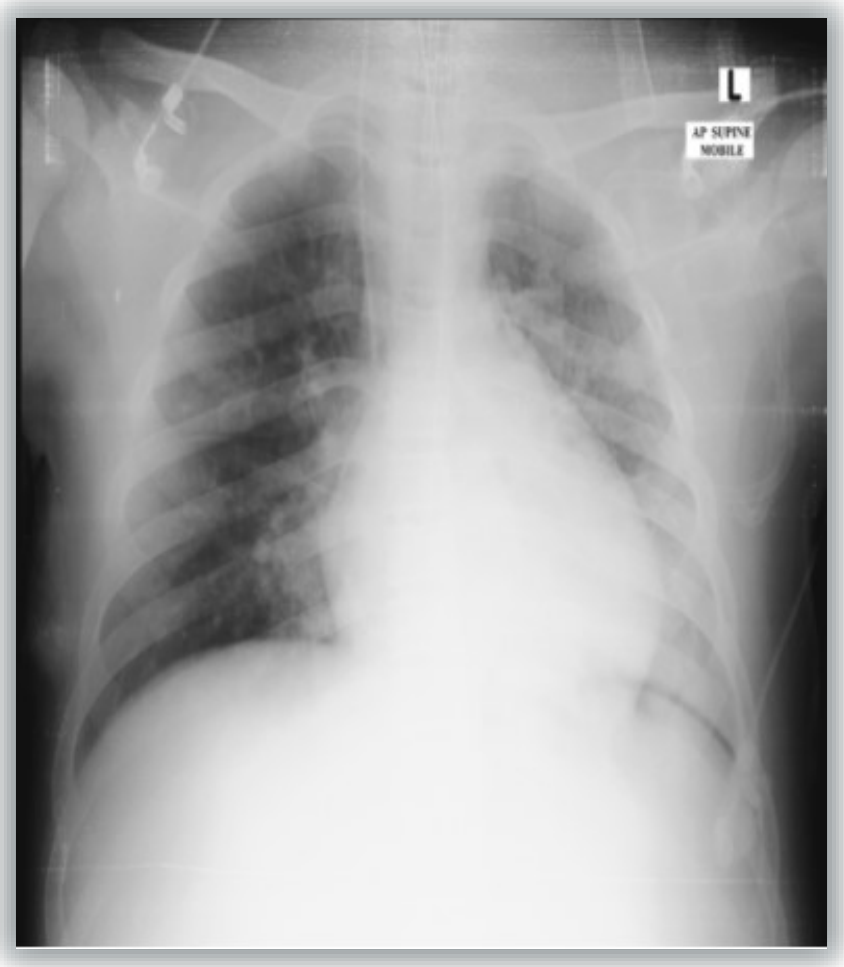

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Foreword from the Editor

Dear JUMMEC readers,

It is the time of the year again, where we each reflect on the achievement and failure for 2016, and making resolution and plans for 2017. In the field of health and research, 2016 has been a challenging year. In February 2016, the World Health Organization (WHO) declared Zika a Public Health Emergency of International Concern (PHEIC) due to the virus association with microcephaly and other neurological disorders, which has also affected the Rio 2016 Olympic Games. The PHEIC was then lifted by the WHO on 18 November 2016. In June, we saw the end of the largest-ever Ebola outbreak, which started in March 2014 in West Africa that has claimed more than 11 thousand life in the affected countries. Then in December, the experimental Ebola vaccine, rVSV-ZEBOV, was reported to be highly protective against the virus in a major trial in Guinea.

The economic and political situation around the world, with the slowing economy, Brexit in June and the unexpected results from the United States of America presidential election, have put pressure on the field of health and research. One such issue is the agreement by the Unites States in September 2016 to ratify the Paris climate change agreement may fall trough. Now, coming back to JUMMEC, for the second issue un 2016 (Issue 2, volume 19), it is my pleasure to introduce to you four interesting articled from researchers in the field of Health and Translation Research in the regions. Breast cancer is one of the major cancer among women both in high-income, and low- and middle-income countries (LMIC). Research focus to in identifying biomarker that can be used to predict the prognosis of the breast cancer is valuable. In the research by Siregar KB from University of Sumatera Utara in Indonesia, the vascular endothelial growth factor receptors (VEGFR) was investigated as a novel predictive and prognostic biomarker for breast cancer. The researcher found that overexpression of VEGFR to be potentially valuable biomarker for targeted therapy. Moving on from breast cancer, low-birth weight infants is another important health issue, especially in resources poor regions of LMIC. Finding a low-resource and low-cost methods to manage them in pertinent. Lumbanraja SN also from University of Sumatera Utara conducted a trial comparing kangaroo mother care (KMC) and conventional care with the use of incubators among 40 low-birth weight newborns. The researcher found no difference in the outcome for the two groups, which means that KMC could be promoted among resource poor regions.

Besides original research, it is also important to review and summaries the current research findings. Al-Khateeb A and Al-Talib H conducted a review on the genetic research among familial hypercholesterolemic population in Malaysia. The researchers found 11 articles, which reported on different aspect; i.e., form gene variants and mutations, and phenotype-genotype associations. They concluded that the research in Malaysia is encouraging, however, there is still room for more multicentre research. The final article was a case report on a patient with haemophagocytic lymphohistiocytosis associated with acute parvovirus B19 infection in a young healthy patient with underlying hereditary spherocytosis. Although a case report is not high on the evidence list, it is always important for clinician to share unique and interesting cases.

JUMMEC welcome articles on health and translational research, including case-report that will interest our local and regional readers. With the continued support from contributors, readers and the Faculty of Medicine, University of Malaya, JUMMEC will continue to strive to bring you good quality publication that contributes to the body of evidence in health and translational research. Till next year, JUMMEC editorial board would like to wish you happy holidays and Happy New Year 2017!

Victor Hoe
Managing Editor (Volume 19 Number 2)

GENETIC RESEARCHES AMONG MALAYSIAN FAMILIAL HYPERCHOLESTEROLAEMIC POPULATION

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ABSTRACT

Background:

Familial hypercholesterolaemia (FH) is one of the most frequent inherited metabolic disorders that can lead to a risk of premature cardiovascular disease. Publications on FH are mainly from western patients as there is little research on Asians, including Malaysians. The aim of this review is to provide an up-to- date information on Malaysian studies on FH genotyping and its relation to the phenotype of the affected patients.

Method:

A search was conducted for data from online databases on FH in Malaysia.

Results:

The mutation spectrum for FH among Malaysian patients was extremely broad. The gene variants were located mainly in the low-density lipoprotein receptor (LDLR) and apolipoprotein B-100 (APOB-100) genes rather than in the proprotein convertase subtilisin kexin type 9 (PCSK9) gene. The exon 9 and 14 were the hotspots in the LDLR gene. The most frequent mutation was p.Cys255Ser, at 12.5%, followed by p.Arg471Gly, at 11%, and the most common single nucleotide polymorphism (SNP) was c.1060+7 T>C at 11.7%. The LDLR gene variants were more common compared to the APOB-100 gene variants, while variants in the PCSK9 gene were very few.

Phenotype-genotype associations were identified. Subjects with LDLR and APOB-100 genes mutations had a higher frequency of cardiovascular disease, a family history of hyperlipidaemia and tendon xanthoma and a higher low-density lipoprotein cholesterol (LDL-C) level than non-carriers.

Conclusion:

Research on Malaysian familial hypercholesterolaemic patients by individual groups is encouraging. However, more extensive molecular studies on FH on a national scale, with a screening of the disease-causing mutations together with a comprehensive genotype-phenotype association study, can lead to a better outcome for patients with the disease.

Keywords: *apolipoprotein B-100, familial hypercholesterolaemia, low-density lipoprotein receptor, Malaysians, and proprotein convertase subtilisin kexin type 9.*

Introduction

Familial hypercholesterolaemia (FH) is an autosomal dominant inherited disorder in man (1), characterised by an increase in the level of low-density lipoprotein cholesterol (LDL-C), tendon xanthoma (TX) together with an increase in the risk of premature cardiovascular disease (PCVD)

(2, 3). It is the first genetic disorder of lipid metabolism that was characterised both clinically and molecularly (1). Heterozygous FH is the commonest monogenic disorder, affecting 1 in 200–250 people, which is double what had been reported previously (4). Its penetrance rate is more than 90% (5). The frequency was reported to be even higher in the Afrikaners, French-Canadians and Christian Lebanese (6), due probably to a founder effect (1). Data from a large community study in Denmark reported that the prevalence of FH reached up to 1:137 (7). It is believed that there are about 34 million FH patients globally (8) with about 3.6 million in the Asia-Pacific region (9).

Despite the high prevalence of FH and the considerable advantage of its early detection and treatment, only about 1% of FH cases have been diagnosed worldwide (8). There are a few exceptions; in the Netherlands and in Norway, where 71% and 43% of patients respectively, with FH, were diagnosed (8). The prevalence of FH in Malaysia is unknown, and there are no published reports on the systematic analyses of mutations underlying FH.

The most important genes in familial hypercholesterolaemia

Monogenic FH is mainly attributed to defects in three genes: low-density lipoprotein receptor (LDLR) gene, apolipoprotein B-100 (APOB -100) gene, and proprotein convertase subtilisin kexin type 9 (PCSK9) gene.

The LDLR is a transmembrane protein which is encoded by the LDLR gene. It was first described by Goldstein et al. (10). This gene is located on the short arm of the chromosome 19 at p13.1-p13.3 (11), comprising of 18 exons and 17 introns and spanning 45 kilobases (kb) (12). The LDLR protein is a cell surface receptor and is responsible for the removal of the LDL cholesterol particles from the plasma (13). The uptake of LDL into cells follows a receptor-mediated endocytosis (RME) pathway that was originally described by Brown and Goldstein in 1986 (14). When the LDLR protein function is diminished as a result of defects in the LDLR gene, the mechanism of uptake is inhibited, so that cholesterol is increased in plasma, and atherosclerosis will develop eventually (15).

Five classes of LDLR gene mutations have been identified: cLass 1 mutations where the LDLR protein is not synthesized, resulting in a receptor-negative mutation (14); class 2 mutations where the synthesized LDLRs are not transported to the Golgi apparatus; class 3 mutations where the LDLRs do not bind to the LDL particles; class 4 mutations where the LDLRs are not internalized from the surface of the cells; and finally, class 5 mutations where the LDL particles are internalized but are not released into the endosome. Mutations of classes 2-5 are classified as defective receptor mutations. The majority of mutations that have been identified to date are in class 2 and 3 mutations (15). More than 1100 different mutations have been identified in the LDLR gene (16).

The APOB-100 protein is responsible for the removal of LDL particles from the circulation through its role as a ligand for the LDLR (17). Studies that were done in the preceding decade demonstrated that similar clinical presentation of FH patients might be due to mutations in APOB-100 gene (18). APOB-100 gene spans 43 kb and is located on the chromosome 2p23-24. It encodes a 4,536-amino acid protein and is the only protein component of LDL particles acting as a ligand to the LDLR. A defect in the APOB-100 gene may disrupt the binding of LDL to its receptor, resulting in an improper clearance of the LDL from the plasma and subsequent hypercholesterolaemia (19). A clinical phenotype that is caused by a mutation in APOB-100 gene is the “familial defective apolipoprotein B-100” mutation defect or “FDB”. Mutations that can cause FDB are located at the LDL binding domain of the APOB -100 gene, at exons 26 and 29 (20, 21).

Several types of APOB-100 gene mutations have been reported, and the most characterised mutations reported are p.Arg3500Glu (22), p.Arg3500Tryp (23) and p.Arg3531Cys (24).

Autosomal dominant hypercholesterolaemia (ADH) can also be a result of a mutation in the PCSK9 gene (25) that spans around 25 kb, and resides on the chromosome 1p32 (26). The PCSK9 protein consists of 694 amino acids and is a part of the proprotein convertase subtilase family. It is secreted by hepatocytes and it produces its action by the down-regulation and the degradation of the LDLRs, instead of their recycling to the cell surface. PCSK9 gene defects that cause hypercholesterolaemia are probably gain-of-function mutations, as over-expression of the PCSK9 protein in the liver of mice produces hypercholesterolaemia by reducing the number of functional LDLRs (27). The loss-of-function mutations have an opposite effect with a reduction in both LDL-C and coronary heart disease (CHD) risks (28).

Apolipoprotein E (APOE) has an essential role in the metabolism of the highly atherogenic APOB containing lipoproteins (LDL) (29). Coronary heart disease risk are reported to be associated with the APOE gene variations (30). It was estimated that the APOE polymorphisms contribute to 2-16% of the changes in the LDL-C levels. The E4 allele and E2 allele are associated with a higher and a lower plasma LDL-C levels, respectively (31).

Hypercholesterolaemia can also be inherited in an autosomal recessive manner by a mutation in the low-density lipoprotein receptor adaptor protein (LDLRAP1) gene, known as the autosomal recessive hypercholesterolaemia gene (ARH). The gene is located at the chromosome 1p36.11, spanning about 47 kb and coding for a 308 amino acid putative adaptor protein that is used in the LDLR endocytosis process (32). This protein cooperates with the cytoplasmic tail of the LDLR (33). It acts during RME through the internalisation process by linking the LDLRs to their endocytic element of the coated pits (34). An ARH gene mutation can cause a defect in the adaptor protein function and restrict the uptake of LDL from the cell surface by LDLRs (33). Patients with ARH have a plasma LDL-C level that is intermediate between the FH heterozygous and homozygous patients (34).

Importance of the diagnosis of familial hypercholesterolaemia

It was reported that untreated FH patients had a 3–4 times higher risk for coronary heart disease (CHD) compared to unaffected subjects, with an occurrence of CHD at a decade earlier (35). It is for this reason that it is very important to diagnose FH not only for a better prognosis but also for the benefit of other family members. In the past, patients with FH typically presented with premature CHD, but presently for patients who may have a higher level of education, a greater awareness and the easier availability of biochemical investigations, an earlier diagnosis should be possible. Those with heterozygous phenotypes have tendon manifestations alone while those with a homozygous phenotype may have both tendon and cutaneous manifestations. In patients with a heterozygous phenotype, early signs of atherosclerosis may be identified during the second decade of life whereas, in patients with a homozygous phenotype, they can be clearly seen in the first decade of life.

Three groups have been established with the diagnostic tools for FH: the United States Med Ped Program (36), the Simon Broome Register Group in the United Kingdom (37) and the Dutch Lipid Clinic Network (38). Genetic counselling can improve the outcome for FH patients. Counsellors, together with the correct genetic testing results, are essential for proper patient support and the clarification and direction for family screening (39). Despite an extensive screening program that was established over twenty years ago, a substantial proportion of patients with FH are still undiagnosed and remain undertreated. A genetically based family cascade screening was performed in the Netherlands and over 64,000 persons were tested, of whom 40.3% were found to carry FH causing mutation. It is a challenge to arrange a country-wide program for the diagnosis and screening of FH (40), and genetic cascade testing approach has proved to be cost-effective (41).

The International FH Foundation has recently presented guidelines for FH (42) in Asia, and Japanese guidelines have been recently published (43). Most of the data of FH are from western investigators with few studies from Asia (44, 45). One such study is a review was published by Alex Livy and Say Hean Lye on genetic research among Asian familial hypercholestralaemic patients (46). Although FH is being identified and clearly diagnosed clinically in Malaysia, only a few genetic studies have been reported from the population. Here, we provide an overview of the molecular studies on SNPs and gene mutations of FH among Malaysians.

Studies in Malaysian familial hypercholestrolaemic subjects

An extensive search was done for data on FH among Malaysians based on online libraries: PUBMED, Scopus, Google Scholar, and Medline from 1990-2015. 11 articles were identified and reviewed.

In 1997, Khoo KL et al. screened for APOB -100 gene mutations in 163 clinically hyperlipidaemic patients with a high cholesterol level in Kuala Lumpur. Mutations in the APOB-100 gene were determined by using polymerase chain reaction (PCR) and Denaturating Gradient Gel Electrophoresis (DGGE), followed by a DNA sequencing to confirm the presence of an APOB -100 gene defect. Only three point mutations were identified among four patients. Two women, a Chinese and a Malay, had the same mutation, p.Arg3500 Trp. Two silent mutations, p.Ala3527 Ala and p.Leu3517 Leu, were identified. They concluded that the common p.Arg3500 Trp mutation could be identified among Asians supported by haplotype analysis of the APOB-100 gene. This gene might, therefore, have Asian ancestry as it appeared among Asians. The team recommended that further studies for the screening of APOB-100 gene mutations among a larger Asian population with ADH were needed (47).

In the year 2000, Khoo KL et al hypothesized that in Asia, heterozygous FH subjects had less severe clinical phenotype than subjects from other parts of the world. With the role of LDLR and APOB-100 gene mutations yet to be ascertained, Khoo KL et al. screened for both LDLR and APOB-100 genes among eighty-six Malaysian FH patients, each patient with a total cholesterol level >7.0 mmol/L, and with an ADH pattern of inheritance. PCR – DGGE analyses of the promoter and the 18 exons of the LDLR gene were done. Mutagenic PCR and restriction digestion with the endonuclease restriction enzyme, MspI, followed by electrophoresis was carried out for exon 26 of the APOB-100 gene. The sequence variants were confirmed by DNA sequencing. Eighteen mutations of the LDLR gene were reported with a frequency of 26%. No mutation was detected in the APOB-100 gene. More than 70% of FH patients had neither LDLR nor APOB-100 genes mutation, suggesting the existence of a third gene with a milder form of FH among Southeast Asians. The team recommended genetic screening for families with a clinical diagnosis of FH without an identified LDLR gene defect, for the detection of other genes causing a similar but a milder clinical presentation of FH (48).

Khoo KL and his team further reported the location of the LDLR gene defects and showed that 4% of the familial hypercholestrolaemic patients studied, had a mutation in the promoter region, whereas 35% carried the mutation in the ligand binding domain, of which 9% of the patients had the mutation in exon 4 (49). The LDLR gene mutation database has summarized by the British Heart Foundation with about 40.7% of the LDLR gene variants within the ligand binding domain in exon 2-4 whilst exon 4 carried the highest frequency (16). Such a high frequency was could either be explained by the big size of this exon (1), or to a bias in the selection of the patients, where subjects with a functional mutation in exon 4 presented with a more severe clinical phenotype (50). Exon 4 encodes for the 5th repeat of the LDLR protein, and this repeat is important for both the low-density lipids (LDL) binding through APOB and the very low-density lipids (VLDL) through the apoprotein-E

(APOE) (13), and might explain the severe phenotype presentation of the patient with the exon 4 genetic defect.

In 2006, Azian et al (51) investigated 72 FH subjects. Mutational screening analysis was performed by DGGE for all exons of the LDLR except for the promoter region, exon 4-3' and exon 18 that were screened by direct sequencing , due to difficulties in optimising DGGE, and for the APOB-100 genes. Positive mutations were confirmed by DNA sequencing. Four mutations were discovered in the LDLR gene among 19.4% of patients. No mutation in the APOB-100 gene could be reported. This was the first report for p.Cys234Ser mutation in exon 5. The p.Asp69Asn mutation in exon 3 had been reported among Malaysians, while p.Arg385Trp mutation in the exon 9 and p.Arg716Gly mutation of the exon 15 had not been reported locally. Additionally, four SNPs were identified: p.Arg450Arg, p.Asn510Asn, p.Asn570Asn, and p.Val632Val. As the LDLR mutation was only reported among 19% of the study cohort, about 80% of the subjects had no mutation, again suggesting that other genes might contribute to the FH phenotype. The DGGE method had a low sensitivity rate of 80% (48) and the use of a faster and more sensitive mutational screening method together with the search for additional genes that can cause a clinical disorder equivalent to FH was recommended (51).

Al-Khateeb et al in 2011 (52) studied further a group of 154 unrelated familial hypercholaestrolaemic patients from Hospital University Sains Malaysia (HUSM). The promoter region and exons 2-15of the LDLR gene were screened using denaturing high-performance liquid chromatography (DHPLC) to detect point mutations, small deletions and duplications. Multiplex ligation-dependent probe amplification (MLPA) was performed to detect large rearrangements. This study reported on 29 genes sequence variants with an overall mutation detection rate of 42.2% which was almost double that reported (51,48). Eight mutations and 21 variants were described, with eight novel gene sequence variants in the FH patients, but not in the controls: p.Asp100Asp, p.Asp139His, p.Arg471Gly, c.1705+117T>G, c.1186+41T>A, 1705+112C>G, Dup exon 12 and p.Trp666ProfsX45. The novel large rearrangement mutation was reported at a frequency of 1.3%. It was the first report of duplication in the LDLR gene among Malaysian. A higher frequency of 8% in the large LDLR rearrangement, had been reported in Taiwanese FH patients (53). The use of DHPLC had a higher sensitivity, and together with the applications of MLPA in the detection of LDLR mutation, contributed to the greater percentage of positive molecular diagnosis of FH.

A study showed that patients with a large rearrangement mutation in the LDLR gene had a higher LDL-C levels and that statin treatment was less able to lower LDL-C level. It was identified that the rearrangement in the LDLR gene would result in a "null-receptor" or a truncated protein that lacked essential domains for proper LDLR function (53). Eight mutations were reported to be pathogenic by an in-silico analysis. However, family and protein study would be needed to confirm the pathogenicity of those mutations.

The University Malaya Medical Centre (UMMC) reported on 144 clinically diagnosed FH patients. Screening for the 18 exons of the LDLR gene was completed by PCR and direct DNA sequencing. A novel missense mutation, p.Cys711Tyr, in exon 14 of the LDLR gene was reported for the first time in Asia. This mutation was identified as a probable damaging mutation based on PolyPhen prediction (54). The epidermal growth factor (EGF) -precursor homology domain of exons 7-14 is essential for the receptor recycling process and for lipoprotein release in low pH conditions (55). Mutation in this domain would disrupt the uptake of cholesterol-carrying particles into the cells, resulting in hypercholesterolaemia. An extensive determination of the pathogenicity for each reported mutation would help in the confirmation of the disease-causing mutation in the index cases. Screening of the family members would contribute to an earlier diagnosis and treatment, improving the outcome of FH patients.

A number of genetic markers, including SNPs and/or mutations in LDLR, APOB- 100 and PCSK9 genes have been reported worldwide. Those data are important for the purpose of a universal screening and management of ADH. However, such data from Malaysians are very few. This stimulated research to identify ADH-causing mutations and associated SNPs among the multiethnic Malaysian population (56), in LDLR, APOB-100 and PCSK9 genes in 140 ADH patients, diagnosed using the Dutch Lipid Criteria and 111 controls. Genotyping assay for 310 previously recognised point mutations and/ or SNPs was performed. Selection of SNPs was initiated from the online databases for the three genes through the British Heart Foundation (BHF) (http://www.ucl.ac.uk/ldlr/), dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/) and the SNPedia (www.snpedia.com). Microarray and genotyping assays were performed with the Illumina Golden Gate Genotyping (GGGT). The confirmation of the allele detected by the microarray was performed by sequencing of randomly selected samples. The team reported on 137 mono-allelic markers, 44.1%, and 173 polymorphic markers, 55.8%, among the autosomal dominant hyperchlesterolaemic studied population. Failure of the study to report on a minor allele among the subjects indicated that these genetic markers could not be used as biomarkers for FH among Malaysians ADH patients (56).

By comparing their findings to the public database (http://www.ncbi.nlm.nih.gov/projects/ SNP), only 23 markers showed significant differences in the allele frequency among Malaysians, European Whites, Chinese, Yoruba and Indians. There were five SNPs related to ADH in Malaysians that were non-conservative amino-acid changes in the public database. They concluded that the variants that contributed to ADH susceptibility among other populations might not be of concern among Malaysians. Genetic markers must be a population specific and further genetic studies to characterise the full range of alterations among Malaysians are indicated, including the association of the reported SNPs and /or mutations with the phenotype of ADH, and a segregation or haplotype study for those variants (56).

Hean Lye et al further hypothesised that SNPs could exert their effects separately and that multiple SNPs could act synergistically to alter the susceptibility to the disease. They investigated the association of previously reported genetic variants that were involved in regulation of lipid among Malaysian FH patients. The samples were collected from 141 patients with clinical diagnosis of FH and in 111 unrelated controls at the UMMC. Genetic variations were derived from three publically databases: British Heart Foundation (BHF), dbSNP, and SNPedia. Polymorphisms previously implicated in FH were sent to Illumina for designing the probes. Only 1536 variations were designed to the Illumina criteria. Then high throughput microarray genotyping analysis was performed. Fourteen SNPs were found to be significantly associated with FH; eleven were associated with an increasing FH risk, of which only one SNP was reported in LDLR gene, seven in the APOB -100 gene, and three in the PCSK9 gene. This report is the first to highlight the PCSK9 gene variants among Malaysian FH patients. Three SNPs were associated with decreasing FH risk.

The authors reasoned that the lack of an association with ADH could be explained by the mono-allelic nature (56). The lack of an association could be also be explained by the non-significant difference between the cases and the controls that were recruited in the study. Many of the published SNPs could be non-causative polymorphisms among Malaysian ADH patients and further validation in a larger cohort of Asian descent was recommended together with family and in vitro studies to confirm the pathogenicity of the variants (57).

There are few investigations in the Malaysian FH population to determine a gene variant in the APOB -100 gene. AL – Khateeb et al studied 164 patients attending HUSM. The subjects were selected according to the Simon Broome Register diagnostic criteria for FH (37). All patients were screened for APOB -100 gene variants. DHPLC and DNA sequencing were used to identify the mutations in exons 26 and 29 of this gene. The team was able to identify 10 variants. The five novel mutations discovered by this study were p.Gln2485Arg, p.Thr3526Ala, p.Glu3666Lys, p.Tyr4343CysfsX221, and p.Arg4297His. The focus for future study would be on both the APOB-100 and the LDLR genes as causes for ADH rather than on the LDLR gene alone (58).

Patients with LDLR gene variants have a different phenotype from those patients with APOB-100 gene variants, and the type of mutation is a well-known contributing factor to the clinical presentation of FH patients. In 2013. Al-Khateeb et al presented the association of different gene variants in LDLR and APOB-100 genes with the clinical phenotype among Malaysian FH subjects. A group of 164 patients with the clinical diagnosis of FH were recruited and analysed from HUSM. This study reported that carriers of APOB-100 gene mutation have a significantly higher frequency of CVD, 83.3 % vs. 64.9%, a higher LDL-C level, 5.2 mmol/l vs.4.7 mmol/l, and a higher TC:HDL-C ratio, 7.2 mmol/l vs 6.1 mmol/l, than the non-carrier patients with p of 0.045, p of 0.03 and p of 0.02, respectively (59). Mutations in this gene may result in a defective APOB protein function, so that the uptake of LDL-C from the circulation by LDLR is impaired, resulting in hyperlipidaemia, high LDL-C and the development of CVD. For patients with the LDLR gene defects, those with frame shift mutation showed the worst clinical presentation in terms of LDL-C level and cardiovascular disease frequency. They concluded that there was an association between mutations in LDLR and APOB -100 genes with a history of CVD, a younger age of clinical presentation, a family history of hyperlipidemia, TX and a higher LDL-C level (59).

Only one Malaysian case report could be identified (60). A twenty-two-year-old Malay woman presented with soft tissue injuries after a car accident. A positive family history of PCVD was elucidated, and a clinical examination revealed the presence of xanthelasma. Biochemical investigations demonstrated a very high total cholesterol (TC) of 15.3 mmol/L, and low-density lipid (LDL) -C of 3.9 mmol/L. A screening of the LDLR and the APOB-100 genes using DHPLC revealed a homozygous mutation of p.Cys255Ser at the exon 5 of the LDLR gene. This mutation had been previously described in the affected Malaysian population (51, 52).

This case report highlights the importance of a genetic screening in the clinically diagnosed patient in order to ensure an early confirmation and early treatment, to lessen the risk of the development of CHD.

Table 1, 2 and 3 summarised the genetic findings in the LDLR, APOB-100 and PCSK9 genes, respectively among Malaysian FH patients.

Discussion

Familial hypercholesterolaemia is a common monogenic disorder of lipid metabolism that is associated with an increasing the risk for premature CVDs. It is important to ensure the correct diagnosis so that an early and appropriate therapy can be assured. Diagnosis is based on the clinical presentation of the patient with high LDL-C levels and is confirmed by genetic testing. Most of the information about FH is based on the data from western countries, with few reports from in Asia including Malaysia. A variety of LDLR gene mutations have been reported among the Malaysians including silent, missense, splice site mutations and even large rearrangements; this may reflect the genetic heterogenicity of the Malaysian familial hypercholesterolemic population.

The spectrum of mutations among Malaysian FH patients is unlike that of other countries. The in-frame deletion of a single amino acid and the large deletion detected among European familial hypercholesterolaemic subjects (61), could not be identified among the Malaysians. Europe and South East Asia have received migrants from all parts of the world and the spectrum of LDLR gene mutations will reflect the greater diversity in the genetic background of

Table 1: Summary of the LDLR gene variants

No	Sample size	Method	Region	Mutation /variant	%	Reference
1-	86	DGGE, mutagenic PCR and restriction digestion	Promoter	-152 G<T	1.2	Khoo *et al.*2000
			Exon 2	77del GA	1.2	
			Exon 3	p.Asp69Asn	1.2	
			Intron 3	c. 313+1 G<A	1.2	
			Exon 4	p.Arg94His	2.4	
			Exon 5	p.Arg232Trp	2.4	
			Exon 6	p.Glu256 Lys	1.7	
			Exon 7	p.Cys308Tyr	2.4	
			Exon 8	p.Gln357ter	1.2	
				p.Lys372Asn	1.2	
			Exon 9	p.Leu393Arg	2.4	
				p.Ile402Thr	1.2	
				p.Asn407Lys	1.2	
			Exon 10	p.Gly457Arg	1.2	
				p.Asp471Asn	1.2	
			Exon 14	2108ins7bp	1.2	
				p.Ala663Thr	1.2	
				p.Cys675Thy	1.2	
2-	72	DGGE, mutagenic PCR	Exon 3	p.Asp69Asn	1.4	Azian *et al.* 2006
			Exon 5	p.Cys255Ser	12.5	
			Exon 9	p.Arg716Gly	2.8	
			Exon 15	p.Arg385Trp	1.4	
			Exon 10	p.Arg450Arg	Not reported	
			Exon 11	p.Asn510Asn	Not reported	
			Exon 12	p.Asn570Asn	Not reported	
			Exon 13	p.Val632Val	Not reported	
3-	154	DHPLC and Sequencing	Exon 2	p.Cys27Cys	0.6	Alyaa *et al.*2013
			Intron 2	c.190+58C>T	4.5	
				c.190+56G>A	1.9	
				c.190+4A>T	0.6	
			Exon 3	p.Asp100Asp	0.6	
				p.Glu101Lys	7.1	
			Exon 4	p.Asp139His	0.6	
				p.Glu201Lys	5.8	
			Exon 5	p.Cys255Ser	6.5	
			Exon 6	p.Asp304Asn	4.5	
			Intron 6	c.940+36G>A	1.8	
			Intron 7	c.1060+7 T>C	11.7	
				c.1060+10 G>C	3.2	
			Intron 8	c.1186+41T>A	0.6	
			Exon 9	p.Ile398 Ile	3.2	
			Intron 9	c.1359-30C>T	1.3	
			Exon 10	p.Arg471Gly	11	
			Exon 11	p.Pro539Pro	3.9	
			Intron 11	c.1705+56C>T	7.8	
				c.1705+117T>G	1.3	
				c.1706-69G>T	0.6	
				c.1705+112C>G	0.6	
				c.1706-55A>C	3.9	
			Exon 12	p.Asn591Asn	4.5	
				Dup exon 12	1.3	
			Exon13	p.Val653Val	0.6	
			Exon 14	p.Asp700Glu	3.2	
				p.Trp666ProfsX45	2.6	
			Exon 15	p.Arg744Arg	4.3	

No	Sample size	Method	Region	Mutation /variant	%	Reference
4-	141	Microarray	Intron 15 Intron 6	rs10422244 [T/C] rs61318752 [G/T]	Not reported	Alex *et al*. 2012
5-	141	Microarray	Not reported	rs2569556 [G/A]	Not reported	Lye *et al*. 2013
6-	144	Sequencing	Exon 14	p.Cys711Tyr	1.4	Chahil *et al*. 2012
7-	Case report	DHPLC and Sequencing	Exon 5	p.Cys255Ser	Not reported	Alicezah *et al*. 2014

Table 2: Summary of the *APOB-100* gene variants

No	Sample	Method	Region	Mutation /variant	%	Reference
1-	163	DGGE	Exon 26	p.Leu3517Leu p.Arg3500Trp p.Ala3527Ala	0.6 1.2 0.6	Choong et al. 1997
2-	141	Microarray	Exon 26 Exon 29	p.Ile2716Ile p.Leu2680Gln p.His3182Asn p.Val4128Met	Not reported Not reported	Alex *et al*. 2012
3-	141	Microarray	4291 1722 11742 28640 5287 22106 24881 7009	rs13306187[G/A] rs13306194[G/A] rs12714238[G/A] rs12720772[G/A] rs12720762[G/C] rs41291161[T/A] rs57825321[A/T] rs12714254[T/G]	Not reported	Lye *et al*. 2013
4-	164	DHPLC and Sequencing	Exon 26 Exon 29	p.Thr2515Thr p.Gln2485Arg p.Thr3526Ala p.Thr3567Met p.Glu3666Lys Thr3567Thr p.Arg4297His p.Ser4338Asn p.Arg4270Thr P.Tyr4343CysfsX2	49.4 7.3 3.7 1.8 0.6 2.4 1.8 2.4 19.5 2.7	Alyaa et al. 2013

Table 3: Summary of the *PCSK9* gene variants

No	Sample size	Method	Region	Mutation /variant	%	Reference
1-	141	Microarray	Intron 5	rs28385711 [C/G] rs17111555 [A/G] rs17111557 [T/C]	Not reported	Alex *et al*. 2012
2-	141	Microarray	15015 24382 24924	rs12084215[C/A] rs565436 [A/G] rs28362269[G/A]	Not reported	Lye *et al*. 2013

both the migrant and native population. For the LDLR gene, p.Cys255Ser mutation has been reported in more than one Malaysian study (51, 52, 60), with a higher frequency of 12.5% by Azian et al followed by 6.5% by Alyaa et al, and led to the conclusion that this mutation is common among the affected Malaysians. Other point mutations are common among the Christian Lebanese (62) and specific LDLR gene mutations in French Canadian subpopulation (63). From previous studies, the exon 14 and exon 9 were the hot spots as they carried the highest number of gene variants, the 6 and the 5 variant, respectively (48,52) yet the FH public database from http://www.ucl.ac.uk/fh. showed that exon 4 carried the highest variant number, and was not applicable to every sub-population group in the UK as Taylor et al. reported the highest variant number were in both exon 10 and 13 (64).

Among on the Malaysians, the mutation detection rate was variable ranging from 42.2% by Alyaa et al to 19% by Azian et al and to 26% by Khoo et al. The higher detection rate by Alyaa et al might be attributed to the use of DHPLC and sequencing plus MLPA that could detect the large rearrangement of the LDLR gene in comparison to the DGGE that was used by the other groups. A study in the UK using DHPLC/sequencing reported a mutation detection rate of about 51% (64).

An important challenge of the LDLR genetic testing is gene coverage. The promoter region cannot be sequenced by many clinical laboratories because the interpretation of gene variants in this area is difficult. Only a few variants have been identified within the promoter and the 5' UTR gene region among Malaysians (49, 65). To confirm the pathogenicity of mutations is not easy as the confirmation of the pathogenicity requires functional studies of segregation analysis and protein study.

Apart from Chong et al, who was able to confirm the p.Arg3500Trp mutation, the previously reported common global mutations of the APOB -100 gene could not be confirmed among Malaysian FDB. APOB-100 gene mutation was detected at a frequency of 12% among our population (58), this frequency is high in comparison to another study that also used DHPLC as a method of detection of 2.3 % (66). This can be explained by the wider region (exons 26 and 29) in comparison to only exon 26 that was screened by the other study.

The data about the PCSK9 is very limited among Malaysians with only two studies and a few variants reported. PCSK9 mutations are relatively common in Japan with a frequency of 5.9%, and cause a milder phenotype compared with the LDLR mutations (67).

The phenotypic expression of FH is dependent on the functional consequence of the mutation, the interactions with other genes that regulate the circulating lipid levels, and the effects of the environment (68). Despite the monogenic nature of the disorder, FH shows a large variability in phenotypic expression in terms of the lipid profile, the frequency of xanthomas, the age of onset and the severity of CVD (69).

Among the studies that have been carried out, those with LDLR gene mutations have a higher prevalence of CVD and a higher LDL-C in comparison to those without apparent genetic mutations (48, 52) and the LDLR gene mutation has been identified as a predictor for CVD among Malaysian FH patients (59). These two findings are in agreement with an observation among Taiwanese FH cases (53). The worst phenotype was reported among those with a frameshift mutation, in terms of CVD frequency and lipid profile parameters compared to those with the large rearrangement mutations, although both mutations are classified as a null mutation type (70). This finding is opposite to another study in which FH patients with large rearrangements are associated with a more severe biochemical phenotype compared to other mutation types (71). The latter result may be explained by the small sample size of the large rearrangement group. As a general rule, null mutations and LDLR-negative familial hypercholesterolaemic patients are associated with higher levels of LDL-C, more imaging markers of cardiovascular disease (CVD), and more adverse CVD outcomes (72). However, this clinical presentation was not seen clearly among the Malaysians.

Conclusion

The variety of mutations that have been reported in the LDLR gene suggests that the genetic background of Malaysian FH is diverse. Many FH patients do not express any sequence variant, suggesting that FH in Malaysia may be caused by mutations in genes other than LDLR or APOB-100 gene. Many of the reports were not able to identify the APOB -100 gene variant as a causative defect. Hypercholesterolaemia is a silent disease and the underlying aetiology of FH is still not well defined.

Recommendations

There is a strong need to engage in systematic sequencing studies of index patients to discover novel mutations and simultaneously to establish a cascade screening for the relatives of index cases so that the affected people can be traced early and managed properly. A population-based study is recommended to look for other gene defects that may predispose to FH-like phenotypes. Research on mutations in the genes controlling lipid metabolism is needed. In-vivo study to look for the pathogenicity of the gene variants in man is crucial. The research that was done by individual groups is promising. Appropriate genetic tests are needed to be adopted by the government at a national level for the establishment of a screening program to ensure an earlier diagnosis and management for a better outcome for the patient and family.

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PARVOVIRUS B19 ASSOCIATED HAEMOPHAGOCYTIC LYMPHOHISTIOCYTISIS IN HEREDITARY SPHEROCYTOSIS PATIENT: A CASE REPORT

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ABSTRACT

Haemophagocytic lymphohistiocytosis (HLH) is a clinico-pathologic entity caused by increased proliferation and activation of benign macrophages with haemophagocytosis throughout the reticulo-endothelial system. Virus-associated HLH is a well-recognised entity. Although majority of parvovirus B19 associated HLH does not require any specific treatment and carries good prognosis, outcome of children is worse than adults. We report here a case of HLH associated with acute parvovirus B19 infection in a young healthy patient with underlying hereditary spherocytosis, with bone marrow findings typical of parvovirus infection. Although this patient had spontaneous recovery of cell counts, he succumbed due to complication from prolonged ventilation. Unexpectedly, his immunoglobulin levels were inappropriately normal despite on-going ventilator associated pneumonia, which reflects inadequate humoral immune response towards infection.

Keywords: *Hemophagocytic lymphohistiocytosis; hereditary spherocytosis, Parvovirus.*

Introduction

Haemophagocytic lymphohistiocytosis (HLH) is a clinico-pathologic entity caused by a defect in inflammatory signals that results in increased proliferation and activation of benign macrophages with haemophagocytosis throughout the reticulo-endothelial system (1). The disease is seen in all ages and has no predilection for race and sex (2).

HLH is a reactive process resulting from an uncontrolled immune response triggered by different stimuli with an underlying inherited or acquired inability to regulate this trigger. Persistent stimulation of lymphocytes and histiocytes results in hypercytokinemia, leading to characteristic symptoms of HLH (3).

Characteristic symptoms of HLH are prolonged fever, hepatosplenomegaly and cytopenias. Other features of HLH is the presence of haemophagocytosis typically seen in involved organs such as liver (4) as well as bone marrow (5). However, haemophagocytosis is initially absent in majority of patients. In patients with pre-existing chronic haemolysis, parvovirus induced transient aplastic crisis may lead to severe anaemia (6). The typical changes in the bone marrow of patients with HLH secondary to parvovirus B19 infection are haemophagocytosis of mature and immature hematopoietic cells, in addition to myeloid and erythroid hypoplasia, as well as variable megakaryocyte hyperplasia (7). Intranuclear parvovirus B19 inclusions in red cell precursors have rarely been described in patients with transient aplastic crisis. Other laboratory values that suggest HLH include high ferritin, raised triglycerides, transamininitis, increased bilirubin and sCD25 (alpha-chain of the soluble interleukin-2 receptor) levels, and decreased fibrinogen. Although all these symptoms are characteristic, none is specific (2).

HLH can be categorised into genetic and acquired form, based on aetiology. The genetic form of HLH includes familial HLH, and those associated with other primary immunodeficiency syndromes. Familial HLH is an autosomal recessive disease caused by several mutations in the NK/T-cell cytotoxic pathway. Immune-deficiency syndromes known to be associated with HLH include Chediak-Higashi

syndrome, Griscelli syndrome type 2, Hermansky-Pudlak syndrome type 2 and X-linked proliferative syndrome (2).

Acquired HLH is often subcategorised into infection-associated HLH, malignancy-associated HLH and HLH in association with autoimmune disease (5). It has also been reported in patients receiving immunosuppressive therapy after transplant (2). The true incidence of acquired HLH is unknown and may be under-recognised in adult critical care units (8).

HLH associated with infection was initially described in immune-suppressed patients (9). Various infectious organisms have been associated with HLH. Viral infections associated with the syndrome include Epstein-Barr virus (EBV), cytomegalovirus (CMV), hepatitis viruses, human herpesvirus 8 (HHV8), influenza virus, parvovirus B19, and enterovirus (10). HLH may be the first manifestation of a human immunodeficiency virus (HIV) infection (11). However, EBV is the most common triggering agent.

Parvovirus B19 infection typically cause erythema infectiosum in children (12), acute polyarthritis in adults (13), and transient aplastic crisis in patients with chronic haemolytic anaemia such as sickle cell anaemia (14) or hereditary spherocytosis (15). Parvovirus B19 associated with HLH is less common than that of EBV. In previous case reports, parvovirus B19 associated HLH was more prevalent among adults patients (16). More than half of the patients have an underlying disease, the most common being hereditary spherocytosis (16). Other underlying conditions that were present in previous case reports include sickle cell anaemia and iron deficiency anaemia. Most of these patients had spontaneous recovery without specific therapy, while some of them were treated with corticosteroids or intravenous immunoglobulin (16-23). This condition has showed a benign clinical course in more than 80% of the cases. The low mortality suggests that parvovirus B19 associated HLH carries a better prognosis compared to other viral associated HLH (10).

HLH is a difficult diagnosis to make as it can be easily confused with any infective process and not all of the above clinical signs are present. It is the magnitude of clinical symptoms and laboratory abnormalities and especially their progression, which suggest the diagnosis when these patients do not respond appropriately to the treatment (2).

We report here a case of HLH associated with acute parvovirus B19 infection in a previously young and healthy patient with underlying hereditary spherocytosis who succumbed to complication related to HLH.

Case Presentation

A 15-year-old boy with underlying hereditary spherocytosis (HS), presented with 3-day-history of fever and chills. It was associated with nausea, vomiting and abdominal pain, as well as diarrhea. He also complained of lethargy, myalgia and loss of appetite. There was no history of headache or symptoms of respiratory tract infection. There was no significant travel history. The patient was conscious but disoriented upon arrival at the emergency department. He was febrile at 39.2˚C. He was also tachycardic. Abdominal examination revealed mild splenomegaly. There was neck stiffness and macular rash at the posterior aspect of his neck. Physical examination of other systems was unremarkable. Blood investigations showed that he was mildly anaemic, with a raised white cell count, and normal platelet level. The lactate dehydrogenase and bilirubin were elevated at 2030 U/L and 36 umol/L respectively, consistent with haemolysis likely secondary to underlying hereditary spherocytosis. Chest X-ray revealed mild cardiomegaly while CT scan of brain was normal. An empirical diagnosis of meningo-encephalitis was entertained and he was treated with intravenous ceftriaxone 2 grams twice daily and intravenous acyclovir 500 milligrams three times a day.

However, his condition rapidly deteriorated over the next 12 hours, with low blood pressure requiring inotropic support and mechanical intubation for respiratory failure. This was further complicated with disseminated intravascular coagulopathy the following day with persistent spiking fever. Antibiotic was upgraded to intravenous meropenem 500 milligrams four times a day. In view of the dropping haemoglobin level, he had two units of packed cells transfusion. On day 3 of admission, the maculopapular rash had spread from his neck to trunk and abdomen. There was now hepatomegaly. The full blood counts had worsened with hemoglobin 7.3 g/dL, white cell count $2.3x10^9$/L and platelet $18x10^9$/L. Serum ferritin was elevated at 103,700 µg/L, while his fasting triglyceride was mildly raised at 1.8 mmol/L. The HIV screening test was negative. A preliminary diagnosis of HLH was made in view of the results. Bone marrow examination was performed on day 4 of admission. The bone marrow was hypercellular but there was a lack of erythroid precursors together with giant proerythroblasts; several histiocytes were seen scattered among the haematopoietic cells with a number of them showing haemophagocytosis. There were also several large cells, which show large round or ovoid nuclei with peripheral chromatin condensation and intranuclear inclusion resembling 'lantern cells' seen in the marrow (Figure 1A and 1B). In view of this, parvovirus infection was suspected and this was later confirmed with a raised parvovirus B19 IgM.

Although his blood counts recovered spontaneously on day 11 of admission, he unfortunately developed multidrug-resistant Acinetobacter baumanii ventilator-associated pneumonia and acute respiratory distress syndrome (ARDS) (Figure 2). Serum immunoglobulin levels were inappropriately normal (IgG 1130 mg/dL, IgA 301 mg/dL, IgM 80 mg/dL) despite on-going infection. His condition deteriorated despite treatment with intravenous colistin 150 milligrams twice a day and succumbed to the complication on day 22 of admission.

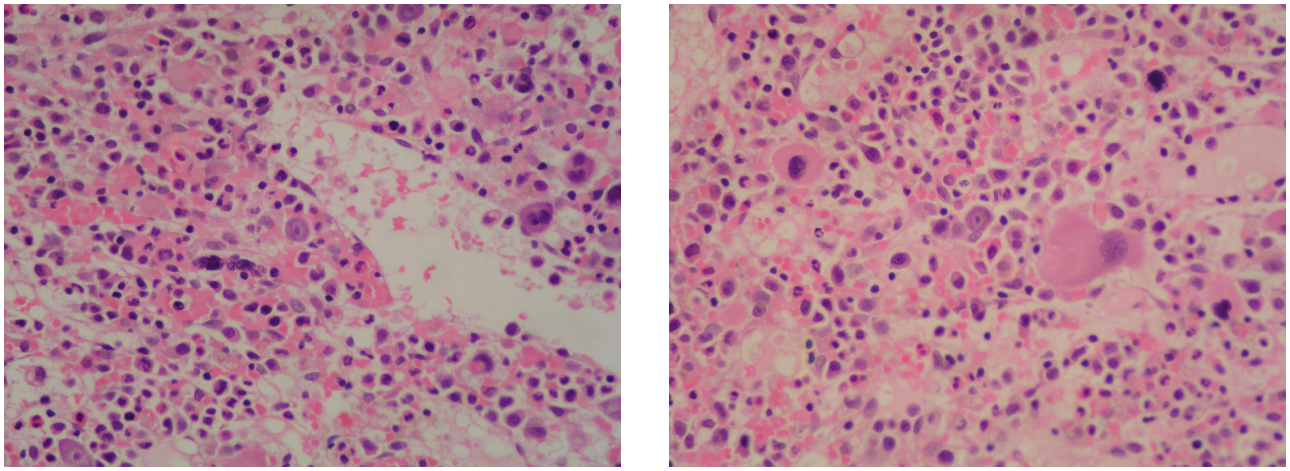

Figure 1A and 1B: Bone marrow trephine at 20x magnification. Erythroblast with intranuclear eosinophilic inclusions, suggestive of parvovirus B19 infection.

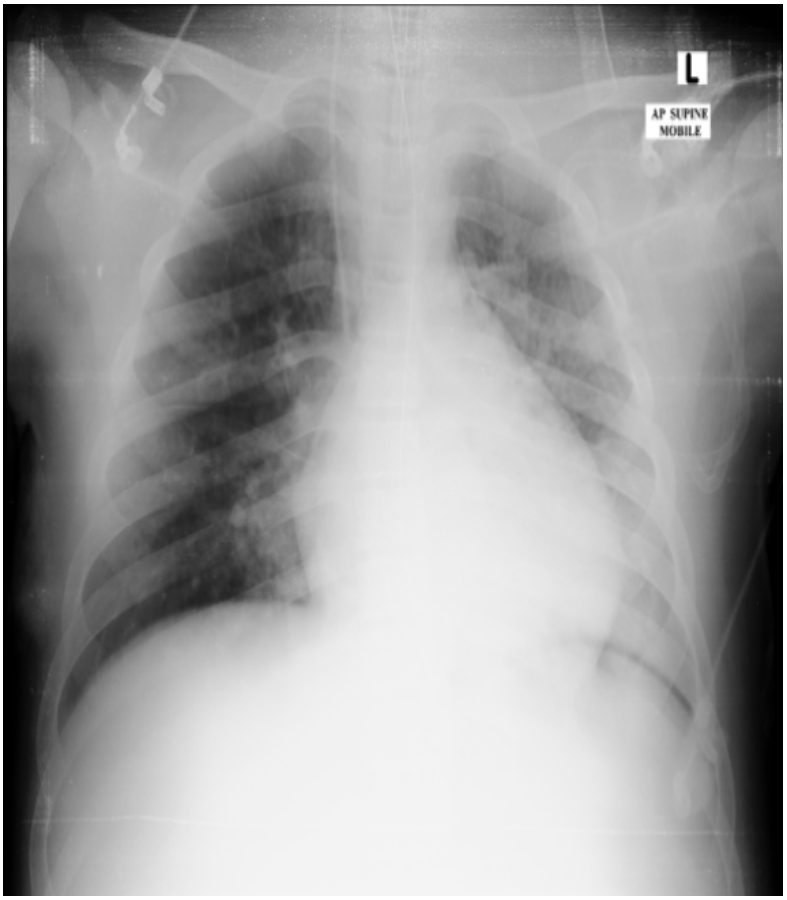

Figure 2: Chest X-ray showing acute respiratory distress syndrome picture.

Discussion

HLH is a rare and complex syndrome that can present with pancytopaenia. The diagnosis of this syndrome requires the presence of 5 or more of the following: fever, splenomegaly, cytopaenia affecting at least 2 lineages in the peripheral blood, hypertriglyceridemia or hypofibrinogenemia, low or absent natural killer cell activity, a serum ferritin level higher than 500 ug/L, soluble CD25 higher than 2,400 U/ml, or hemophagocytosis as demonstrated in bone marrow, spleen, or lymph node (24). This patient has fulfilled six out of eight criteria, except for measurement of natural killer cell activity and soluble CD25 levels, which were not available in our institution. However, it was adequate for the diagnosis of HLH. The aetiology of HLH was confirmed with positive IgM parvovirus B19 serology, as well as the

typical bone marrow findings of erythroid hypoplasia and giant proerythroblast, together with large cells with intranuclear inclusions resembling 'lantern cells' seen in acute parvovirus B19 infection.

There are 32 cases of parvovirus B19 associated HLH reported so far in the literature, including our case (16-23). Sixteen of the patients were adults while another sixteen patients, were children. Most of the patients with parvovirus B19 associated HLH patients in other case reports recovered spontaneously without receiving any specific treatment, suggesting better outcome compared with other virus-associated HLH (10). There is no specific antiviral drug against parvovirus B19 and the infection does not normally need treatment in immune-competent host (25). However, patients in more recent case reports have received intravenous immunoglobulin as part of the treatment. There was only one death (6.3%) reported among adult patients, compared to five mortalities (31.3%) among children. The low mortality among adult patients, despite the fact that majority of them not receiving any specific therapy, suggests better outcome in this cohort, compared to children who have higher mortality despite treatment.

In this previously health and young boy, the development of HLH secondary to parvovirus B19 infection has significantly affected his immune system, rendering ineffective NK and T cell activation to kill parvovirus infected cells and antigen presenting cells. The fact that this patient did poorer compared to other parvovirus B19 infected patients could be due to his inability to mount an immune response against infection as suggested by an inappropriately normal serum immunoglobulin levels for someone with overwhelming infection.

In previous study looking at characteristic immune abnormalities in HLH, most patients with HLH have normal quantitative serum immunoglobulin levels with no consistent pattern of immunoglobulin deficiency (26). While the humoral immunity is essentially intact, cellular function is abnormal in most. The authors concluded that HLH have consistent and significant abnormalities in cytotoxic immune function (26). This boy in our case has demonstrated signs of recovery based on laboratory parameters. However, secondary infection from prolonged intensive care unit stay and ventilation has led to his death, predisposed by immunocompromised state from HLH.

The HLH-2004 protocol recommends an 8-week induction therapy with corticosteriods, etoposide, and cyclosporin A upfront, for the treatment of HLH (24). In cases of infection-associated HLH, malignancy HLH or macrophage activation syndrome in idiopathic juvenile arthropathy, immediate treatment of the underlying disease is indicated (2). Among all virus associated HLH, EBV carries the worst prognosis (27). The addition of etoposide in the therapy, especially if initiated within the first 4 weeks from diagnosis, improves survival (28).

Early intervention and more aggressive therapy as recommended by HLH 2004 protocol may have been indicated in this patient, rather than supportive care to shorten the course of disease. Complications of prolonged ventilation may then be avoidable. This may alter the outcome of this patient.

Conclusion

HLH remains a rare condition that can lead to fatal complications without early recognition. Although parvovirus B19 infection associated HLH carries good prognosis without specific treatment in previous reports, there was still significant mortality among children. Early intervention and more aggressive treatment is indicated in patients who have evidence of immunocompromised state to shorten the course of disease and to improve outcome.

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INFLUENCE OF MATERNAL FACTORS ON GROWTH PARAMETERS IN LOW-BIRTH-WEIGHT BABIES WITH KANGAROO MOTHER CARE

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ABSTRACT

Background: Kangaroo mother care (KMC) in low birth weight newborns has been found to be beneficial, but studies have shown that maternal factors might be of concern in the successful application of KMC.

Aim: To study the influence of maternal factors on growth parameters in low-birth-weight babies with KMC.

Methods: This is a prospective cohort study of 40 low birth weight newborns in our institutions. We randomly assigned the newborns to the group which received KMC and to the group which received conventional care. Maternal factors were recorded. We measured weight, length, and head circumferences of newborns daily for thirty days. Data was processed by SPSS x22.0.

Results: A total of 40 newborns were recruited into the study. Weight parameters were significantly higher in the KMC group than in the conventional group except for the Z scores. Regarding maternal characteristics, only gestational age was found to influence the initial and the last head circumference (p=0.035). There were no differences in maternal age, parity, maternal education, mode of delivery, fetal sex, and initial Apgar score with any of the growth parameters.

Conclusion: There were no maternal and fetal differences in the growth parameters of the groups, except in the delayed growth of head circumferences in preterm infants.

Keywords: *Growth parameters*, *KMC method*, *low birth weight*

Introduction

Low birth weights (LBW) in newborns is one of the leading causes of neonatal morbidity and mortality. As many as 16% of newborns worldwide have low birth weights with an annual incidence of 18 million. In developing countries, the incidence of LBW was two times higher than in developed countries (1). LBW is associated with many complications such as growth and cognitive disorders, and the development of chronic diseases of the elderly later in life (2).

Maternal factors such as age, multigravidity, narrow pregnancy interval, socio-economic factors, a low level of education, and weight gain during pregnancy increased the incidence of LBW (3-5). Other studies showed a 1.2 times increased risk of low birth weight in women with low socioeconomic status, and 1.7 times increased risk in women with low levels of education (6). LBW is also closely linked with maternal height (7) Mumbare et al. (8) by logistic regression showed that maternal weight <55 kg (OR 4.81, 95%CI 2.53-9.15), maternal height <145 cm (OR 4.13, 95%CI 2.04-8.37), and inadequate antenatal care (OR 4.98, 95%CI 2.64-9.39), were the most significant factors associated with LBW. Taha et al. (9) by logistic regression showed that only maternal age and parity had significant effects on birth weight (p=0.014 and p=0.008). However, Negi et al. (10) on the other hand showed that only the primigravida had a higher risk of having an LBW baby (OR 3.21; p <0.01) and found no relationship between maternal age, maternal height and weight with LBW. In Indonesia, Trihardani et al. (11) showed that the risk factors associated with LBW were maternal body mass index (RP 5.4; 95CI 1.07-27.29), weight gain during pregnancy (RP 6.6; 95CI 1.30-33.01), and parity (RP=5.30; 1.24-22.56) while there was no significance between in maternal age, maternal height, frequency of antenatal care, and pregnancy interval, with LBW.

Pregnancy in women who were under 20 years of age or who were over 35 years old had an unmet need of adequate nutrition for fetal growth (12). A too narrow pregnancy interval also indicated a shared nutritional condition at risk; and an inadequate time for the recovery of the reproductive system so that the following fetal birth weight in utero would be affected (13).

LBW infants should be kept warm because of their vulnerability to hypothermia which could lead to life-threatening infection, apnea or massive bleeding (14). An incubator is a way to provide warmth for the LBW babies but it may prevent early maternal contact and

breastfeeding (15). Invasive procedures in the incubator also induced stress in babies as proven by some studies that showed increasing heart rates and breaths in infants treated in an incubator (16). Inspired by how marsupials keep their newborns warm, KMC was developed by Martinez and Rey in Bogota (1978)(17).

KMC is effective in the treatment of low birth weight newborns. It is also easy to apply and it is inexpensive. It is very important in many remote areas in Indonesia due to the lack of modern health technology for resuscitation facilities (18). The majority of studies showed better results in infants treated with KMC than with the conventional method, although some of these results are still controversial (19-21). Pratiwi et al. (22) showed that the BBL ≥ 1500 g (RR 0.4; 95%CI 0.23-0.73; p=0.001) and a neonatal age at the commencement of KMC > 10 days (RR 2.69; 95% CI 1:14 to 6:32; P = 0.003) were related to the successful use of KMC. Gestational age ≥ 34 weeks (RR 0.94; 95%CI 0.46-1.89; p=1.00), KMC duration ≥ 65 minutes (p=0.215), and a high maternal education level (p=0.408) did not influence the incidence of LBW. There is no study that specifically assessed the maternal factors that contribute to the success of KMC in LBW. Neonatalogists should be able predict the benefits at the beginning of a therapy because the neonatal period is the most vulnerable period in life and clinicians should apply a therapy that is of benefit.

The aim of the study was to determine the influence of maternal factors on growth parameters in low-birth-weight babies on KMC.

Methods

Study design

This was an analytical prospective cohort study that was conducted at the Adam Malik General Hospital and the Pirngadi Hospital, in Medan, Indonesia, from June to November 2015. Written informed consents were obtained from all parents. This study was approved by the Ethical Committee of University of Sumatera Utara.

Study population

In order to have a representative sample, we calculated the minimal sample size, and 20 babies were required in each study group. Forty consecutive low birth weight newborns, who were delivered in the two institutions, were enrolled into the study . A low birthweight is defined as a weight below 2500 g.The newborns were randomly assigned to the two groups. Randomization was done by computer generated-random numbers. In the first group, the newborn was given KMC and in the second group, they were cared in an incubator as the conventional method. This study only included babies with birth weight of 1000-2500 g, who were hemodynamically stable, who did not require oxygen therapy for most of the day, who had no need of continuous intravenous fluids, and with a mother who was healthy and willing to practice KMC. This study excluded mothers who were exclusively breastfeeding because, from prior studies, breastfeeding could significantly affect KMC outcome. Exclusion criteria were patients who withdrew from the study or who were lost to follow-up, babies with congenital anomalies, severe perinatal complications that required NICU care, malignancies, metabolic and cardiovascular disorders and death.

Study protocols

The baseline information of maternal and fetal demographics was recorded. Maternal factors included maternal age, gestational age, the number of parity, maternal education, and mode of delivery. This study also included fetal demographics such as fetal sex and the initial Apgar Score.

In the conventional group, the babies were placed in an incubator, according to the standard guidelines of the hospitals. In the KMC group, KMC therapy was initiated as soon as the babies were stable. At enrolment, the mothers were taught how to practice KMC. Mothers were seated in a comfortable chair close to the babies' cradle. They were shown how to hold their babies vertically, strapped to the middle of their (mothers') chests, with skin to skin contact so that the babies' skins were touching their mothers' skin. At other times, when not on KMC, the babies were placed in the cradle with their bodies covered. KMC was applied for 4-6 hours each day. Babies in both the groups were provided with vitamin and mineral supplementation as per the protocol.

The subjects were followed up for 30 days, with daily anthropometric measurements. Babies were weighed naked on an electronic weighing scale (GEA). The weighing machine was calibrated daily. The lengths were measured with an infantometer (GEA) at birth, day 10, day 20, and day 30. The head circumferences were measured with a standard tape (Butterfly) at birth, day 10, day 20, and day 30. All measurements were carried out by two blinded investigators. The mean of both measurements was taken. During the follow-up, if there were sucking disorders, breathing disorders, or loss of consciousness, the babies were treated appropriately and were excluded from the study.

The babies' weights were plotted using Fenton's growth charts. We calculated the accurate weight gain velocity with the following formula GV = [1000 x (Wn-W1)] : [(Dn-D1) x (Wn+W1)/2] and the estimated weight gain velocity with the formula GV [1000 x ln (Wn/W1)] : (Dn-D1). The magnitude of errors was reflected in the percentage of absolute difference with the formula 100 x (Estimated GV – Accurate GV) : Accurate GV. We also calculated a z-score for weight in www.peditools.org/fenton/2013 at birth and day 30.

Data analysis

Data were analyzed by SPSS (Statistical Product and Service Solutions, Chicago, IL, USA) 22.0 for Windows. Categorical data were expressed as number and continuous data as

mean ± SD. Chi-square test (Fisher's exact test) was used to examine the relation between qualitative variables. T-independent, T-dependent, Pearson correlation were used to evaluate quantitative variables. The significance was taken at 95% with a *p*-value <0.05.

Results

During the study period, from June to October 2015, there were 90 babies who were born with a low birth weight. Two mothers were not willing to participate in KMC. Some newborns were excluded from the study. There were 45 newborns who needed NICU care; of these, 1 had a congenital anomaly, 1 with a cardiovascular disorder, and 1 was a newborn who was dead. 40 newborns were eligible for the study and were successfully enrolled. There was no loss to follow up or from withdrawal from the study.

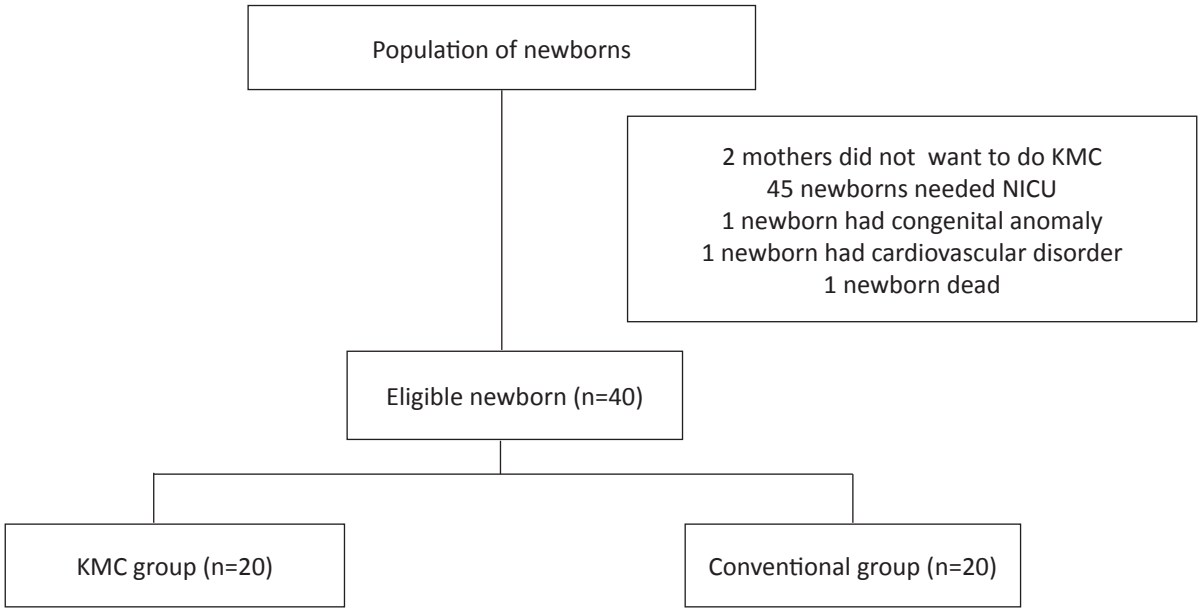

Figure 1: Flow chart of the study

Maternal and fetal demographic characteristics were shown in Table 1. No differences were found between maternal age, parity, gestational age, maternal education, mode of delivery, fetal sex, and the Apgar score between KMC and conventional group. The initially measured weight, length, and head circumferences were similar in both groups (p=0.100; p=0.353; p=0.088, respectively) making the study results more accurate. The last measured weight was significantly higher in the KMC group than in the conventional group (2187.5 ± 371.04 vs 1899 ± 242.55; p=0.015). The difference of the mean, the initial and the last weight was also higher in the KMC group than the conventional group (205.5 ± 147.451 vs 96 ± 68.702; p=0.001). To determine the accuracy of the weight parameter, we calculated the weight gain velocity. The accurate weight gain velocity was 5.098 ± 2.155 g/day, and the estimated weight gain velocity was 5.112 ± 2.168 g/day. Accurate weight gain velocity, estimated weight gain velocity, and absolute difference percentage were found to be significantly higher in the KMC group than in the conventional group (p=0.01; p=0.009; p<0.001). But, there were no differences in the last measured weight Z score between both groups (p=0.364).

We also found no differences of the last measured length and head circumference Z-score between both groups (Figure 2). Although the mean difference of initial and last head circumference (cm) showed a significant difference (p=0.004), other parameters regarding head circumferences did not show any difference. Duration of hospital stay was higher in the conventional group than in the KMC group, but this was not significantly different (28.4 ± 5.020 vs 23.15 ± 5.184; p=0.42).

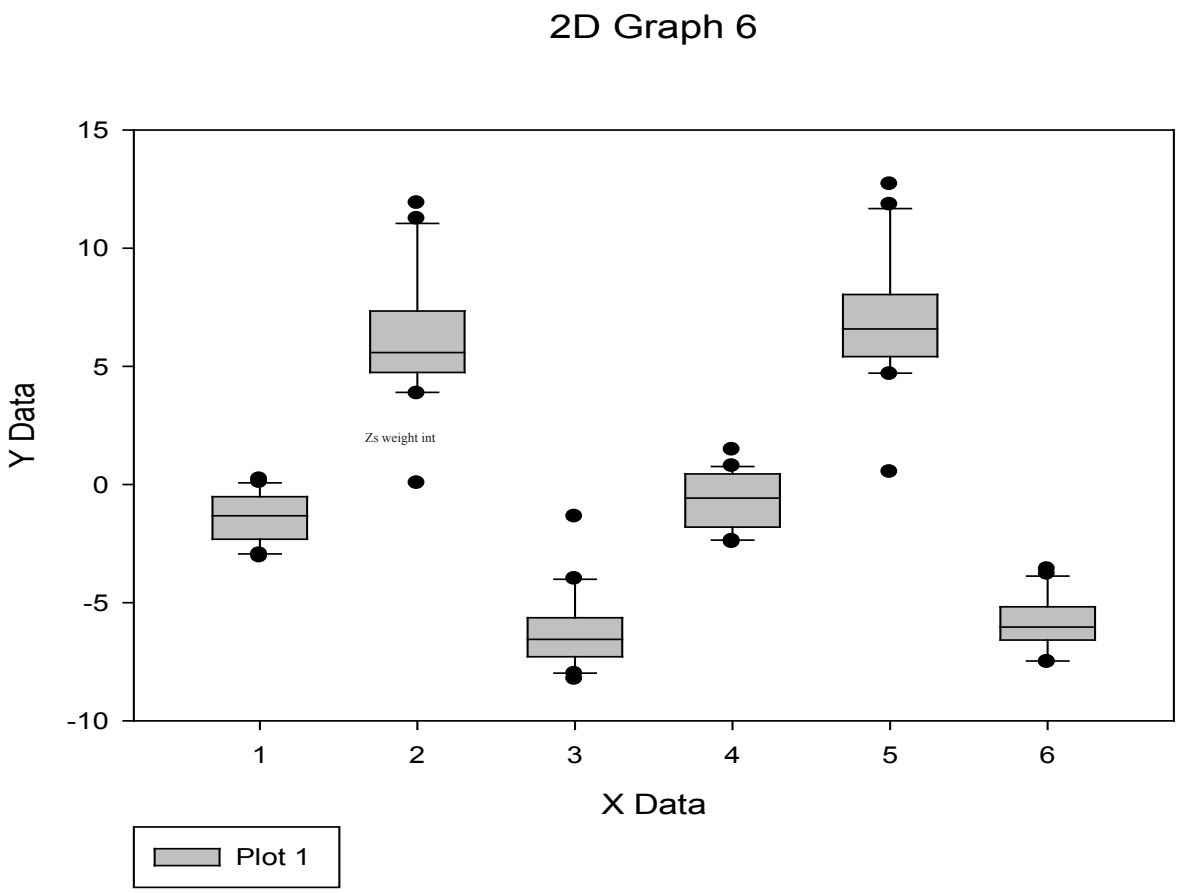

Figure 2: Distribution of Z-score for babies' weight, length, and head circumference (HC) at initial (int) and day (last) 30 measurement

Table 1: Demographic characteristics of maternal, fetal, and measured parameters.

Characteristics	KMC group	Conventional group	p
Maternal characteristics			
Maternal age (years old)	28.6 ± 2.703	28.7 ± 4.485	0.263
Parity (n)	2.25 ± 0.786	2.1 ± 0.852	0.765
Gestational age			
<37 weeks	7 (35%)	5 (25%	0.490
>37 weeks	13 (65%)	15 75	
Maternal education			
Non-educated	5 (25%)	4 (20%)	0.705
Educated	15 (75%)	16 (80%)	
Maternal morbidity			
Preeclampsia	3 (15%)	5 (25%)	0.429
Placenta previa	1 (5%)	0 (0%)	
Gestational diabetes	0 (0%)	0 (0%)	
Other causes	16 (80%)	15 (75%)	
Mode of delivery			
Spontaneous delivery	9 (45%)	5 (25%)	0.185
Vaginal delivery	11 (55%)	15 (75%)	

Characteristics	KMC group	Conventional group	p
Fetal characteristics			
Fetal sex			
Male	11 (55%)	15 (75%)	0.320
Female	9 (45%)	5 (25%)	
Initial Apgar score	8.2 ± 0.696	8 ± 0.795	0.672
Measured parameters			
Initial measured weight			
Weight (kg)	1882 ± 293.125	1803 ± 217.234	0.100
Weight Z score	-1.385 ± 1.019	-1.483 ± 1.070	0.901
Last measured weight			
Weight (kg)	2187.5 ± 371.014	1899 ± 242.55	0.015*
Weight Z score	6.316 ± 2.232	5.414 ± 1.814	0.364
Initial measured length			
Length (cm)	41.025 ± 2.993	39.75 ± 2.653	0.353
Length Z score	-6.121 ± 1.613	-4.076 ± 11.878	0.117
Last measured length			
Length (cm)	42.28 ± 2.938	40.625 ± 2.665	0.340
Length Z score	-0.623 ± 1.165	-1.305 ± 1.030	0.290
Initial measured head circumference (HC)			
HC (cm)	30.325 ± 3.001	29.225 ± 1.824	0.088
HC Z score	7.134 ± 2.202	6.008 ± 1.83	0.444
Last measured head circumference (HC)			
HC (cm)	30.96 ± 1.762	30.035 ± 1.719	0.800
HC Z score	-5.86 ± 1.088	-6.351 ± 1.418	0.394
Accurate weight gain velocity (g/day)	5.098 ± 2.155	1.763 ± 1.220	0.010*
Estimated weight gain velocity (g/day)	5.112 ± 2.168	1.764 ± 1.221	0.009*
Absolute difference percentage (%)	0.214 ± 0.162	0.032 ± 0.029	<0.001*
Mean difference of initial and last weight (kg)	205.5 ± 147.451	96 ± 68.702	0.001*
Mean difference of initial and last length (cm)	1.255 ± 0.305	0.875 ± 0.215	0.513
Mean difference of initial and last head circumference (cm)	0.635 ± 2.494	0.81 ± 0.354	0.048*
Duration of hospital stay	23.15 ± 5.184	28.4 ± 5.020	0.420

*Significant difference

From the maternal characteristics, only gestational age was found to influence the mean differences of initial and last head circumference (-0.314 ± 3.746 vs 1.415 ± 1.064; p=0.035). However, gestational age was not associated with other parameters. There were no differences in maternal age, parity, maternal education, mode of delivery, fetal sex, and initial Apgar score with accurate weight gain velocity, estimated weight gain velocity, absolute difference percentage, and mean differences of initial and last weight, length, and head circumferences (Table 2).

We plotted a mean weight gain graph. Peak weight gains were shown on day 16, 18, and 29. Although the graph showed irregular weight gain, on the last day, the increase in weight was greater than the initial weight (Figure 2).

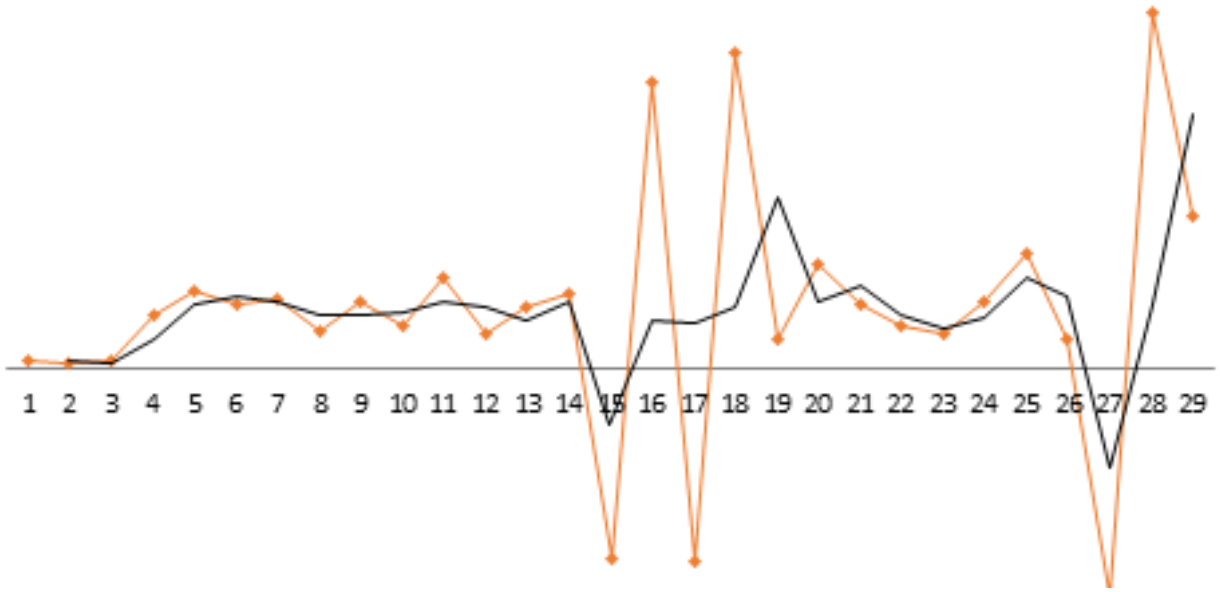

Figure 3: Mean weight gain in KMC group (red line showed the real weight gain and black line showed smoothed out curve)

Discussion

This is the first study that specifically determined the effect of maternal factors on the successful use the of Kangaroo Mother Care in low birth weight babies compared with conventional therapy. Pratiwi et al. (2015) showed that high levels of maternal education had no effect (p=0.408) (22) and Rodriguez et al. (2007) showed no difference in weight gain with the application of KMC in LBW newborns, whether with spontaneous delivery or cesarean section (23).

The maternal-fetal skin-to- skin contact therapy of KMC is applied as soon as possible after birth (24), helping mothers in their role as providers of biological and psycho-emotional needs for their child (25, 26). According to WHO guidelines, continuous KMC is indicated in infants with BBL below 2000 g. Intermittent KMC can also be useful in infants hospitalized in NICU, but this still needs further research (27).

The importance of KMC is in the enhanced bonding of mother and child. Feldman et al. (2002) successfully demonstrated the positive impact in the relationship between mother and child (28). Athanasopolou et al. (2014) showed that KMC resolved negative moods of maternal anxiety and depression (29). KMC also increased the confidence and competence of the mother to care for her baby and significantly reduced stress. These all contribute to the improvement in the growth and development of the newborns (30).

In this study, an assessment of growth was based on the infant's weight, length, and head circumference, which are the important markers of a child's health (31,32). Bera et al. (2014) showed that in infants receiving KMC, the growth parameters and mental development were better than in infants who were treated conventionally (33). However Ali et al. (2009) showed that infants treated in an incubator had a higher weight gain per day (19.3 vs.10.4 g, p<0.001), shorter duration of stay (6.9% vs. 23.2% p = 0.014), lower infection rate (6.9% vs. 23.2% p=0.014), than babies who received KMC (19). Palencia et al. (2009) in 115 LBW infants who were followed up until 13 months, showed that the growth in height for age was higher within the percentiles of weight for age (p=0.0001). Male gender had a higher weight than females (p=0.031) (34). This study was similar to the study in Indonesia reported previously. In that study, we showed that only the last measured weight, the difference in the initial and the last weight, and the weight gain velocity were higher in the KMC group than the conventional group. No differences were found in the length and head circumferences. Haksari (2004) and Rahmayanti (21) in Indonesia also found that there was no difference between weight/age, length/age, head circumference/age in LBW infant treated with KMC and conventional therapy. On the other hand, Rao et al. (20) showed that infants treated in incubators had a higher weight gain, head circumference (0:49 vs 0.75 cm, p = 0:02), and body length (0.99 vs. 0.7 cm, p=0.008) compared with infants who received KMC.

It had been observed that weight gain should approximate an intrauterine growth rate of 16.8 to 30.7 g/day (32). In this study, the accurate weight gain velocity was 5.098 ± 2.155 g/day and the estimated weight gain velocity was 5.112 ± 2.168 g/day. These were still far below the recommended growth rate. The Z-score of weight was still below the expected average. Newborns were still categorized as small for gestational age at the end of the study.

The parameters of weight and length were not affected by maternal age, parity, maternal education, mode of delivery, fetal sex, and the initial Apgar score. Newborns

Table 2: Difference accurate weight gain velocity, estimated weight gain velocity, mean differences of initial and last weight, length, and had circumference between subgroups of maternal and fetal characteristics

Characteristics	Accurate weight gain velocity	Estimated weight gain velocity	Absolute difference percentage	Mean differences of initial and last weight	Mean differences of initial and last length	Mean differences of initial and last head circumference
Maternal characteristics						
Maternal age (years old)	r 0.309 p 0.186	r 0.309 p 0.185	r -0.325 p 0.163	r 0.155 p 0.515	r -0.325 p 0.163	r 0.155 p 0.515
Parity (n)	r 0.134 p 0.574	r 0.134 p 0.572	r -0.417 p 0.068	r 0.350 p 0.130	r -0.417 p 0.068	r 0.350 p 0.130
Gestational age						
<37 weeks	5.793 ± 1.898	5.810 ± 1.913	0.258 ± 0.156	338.57 ± 137.165	1.286 ± 0.474	-0.314 ± 3.746
>37 weeks	4.680 ± 2.289	4.691 ± 2.304	0.189 ± 0.168	283.85 ± 158.248	1.192 ± 0.236	1.415 ± 1.064
p value	0.810	0.812	0.980	0.815	0.271	0.035*
Maternal education						
Non educated	3.819 ± 1.223	3.824 ± 1.229	0.111 ± 0.078	208.33 ± 79.352	1.050 ± 0.197	1.600 ± 1.580
Educated	5.604 ± 2.309	5.622 ± 2.324	0.257 ± 0.173	343.57 ± 156.579	1.300 ± 0.351	0.471 ± 2.683
p value	0.061	0.08	0.015	0.074	0.487	0.774
Mode of delivery						
Vaginal delivery	4.773 ± 1.541	4.782 ± 1.552	0.175 ± 0.1257	281.25 ± 114.697	1.300 ± 0.417	-0.025 ± 3.471
Caesarean section	5.267 ± 2.565	5.283 ± 2.581	0.238 ± 0.186	317.50 ± 172.844	1.175 ± 0.263	1.517 1.048
p value	0.059	0.059	0.85	0.346	0.723	0.073
Fetal characteristics						
Fetal sex						
Male	5.408 ± 2.535	5.426 ± 2.549	0.248 ± 0.170	315.27 ± 169.534	1.063 ± 0.1912	1.482 ± 1.155
Female	4.654 ± 1.699	4.664 ± 1.713	0.171 ± 0.153	285.56 ± 129.722	1.422 ± 0.3632	-0.011 ± 3.298
P value	0.069	0.07	0.271	0.142	0.266	0.142
Intial Apgar score	r -0.041 p 0.864	r -0.041 p 0.865	r 0.064 p 0.788	r -0.141 p 0.554	r 0.292 p 0.211	r 0.018 p 0.938

*Significant difference

at birth who were <37 weeks of gestational age, had delayed the growth of head circumference. KMC had been proved to increase cerebral blood flow, acting as nutritional support, mainly to the brain. Korraa et al. (2014) found that newborns who received KMC had a lower cerebral blood flow resistive index ($p<0.05$), indicating an improved cerebral blood flow (35). The influence of immature organ in preterm babies need to be considered, and this would require further research (36).

The strength of this study is the detailed follow-up parameters which are better than those reported in other studies. The better follow-up rate in the KMC group could be due to the active involvement of the mother in the care of her LBW baby. The limitations of this study are the small sample size conducted in two institutions only. Further research is needed to confirm these findings.

Conclusion

There were no maternal and fetal differences in the growth parameters of the two groups of the study, except for the delayed growth of head circumferences in preterm infants.

Acknowledgements

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Conflict of interest

The author declared no conflict of interest in this study.

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VEGFR OVEREXPRESSION AS A PROMISING PREDICTIVE AND PROGNOSTIC BIOMARKER FOR BREAST CANCER

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ABSTRACT

Background:

The majority of breast cancer cases are presented in an advanced stage; hence, there is a need to have a biomarker that is able to function both as a predictive and prognostic tool for breast cancer. Since angiogenesis has been found to be closely related to the invasiveness of breast cancer, angiogenic marker such as vascular endothelial growth factor (VEGF) may be a promising marker for this cancer.

Objective:

The aim of this study is to determine the association between the VEGF receptor (VEGFR) expression with human epidermal growth factor receptor-2 (HER-2)/ neu and estrogen receptor (ER) /progesterone receptor (PR) expression in an attempt to clarify the role of VEGFR as a potentially novel predictive and prognostic biomarker for breast cancer.

Materials and Methods:

This study examined 40 tissue biopsies taken from patients diagnosed with breast cancer in H. Adam Malik Hospital Medan, Indonesia. Samples were analyzed by immunohistochemistry to determine the histopathology, grading, lymphovascular invasion and expression of VEGFR, HER-2/neu and ER/PR. Association between dependent and independent variables was conducted using chi-square test and logistic regression.

Results:

The majority of the cases in this study were infiltrating ductal carcinoma (90%), in stage III (70%), and showed positive TIL (75%). VEGFR expression was found to be upregulated in 21 samples (52.5%). HER-2/neu was positive in 14 patients (35.0%) and ER/PR was positive in 22 patients (55%). The expression of VEGFR positively correlated with HER-2/neu expression (p= 0.002) and negatively correlated with ER/PR expression (p= 0.012).

Conclusion:

Overexpression of VEGFR is a potential valuable predictive and prognostic biomarker for breast cancer. Antagonising VEGFR may serve as the future target therapy for the disease.

Background

Breast cancer remains a major problem in many countries. The reported worldwide incidence of breast cancer in women is 126 for every 100,000 cases, whilst its incidence in the male population is 0.6 for every 100,000 cases (1). In the United States, breast cancer is the most common malignancy with 180,000 new cases reported annually (2). In developing countries such as Indonesia, there are approximately 20,000 new cases of breast cancer each year, of which more than 50% of these cases are found to be in an advanced stage (3).

The invasiveness and rate of metastasis of breast cancer cells have been found to be closely related to angiogenesis (4). This is a mechanism that helps to maintain the metabolism, growth, and nutritional supply of tumor cells (5). Vascular endothelial growth factor (VEGF) is an angiogenic factor that plays an important role in maintaining the growth of tumors (6, 7). Various studies have shown that mRNA and serum VEGF levels increase along with the rate of metastasis because VEGF aid in the formation of new vessels, which bring nutrients and oxygen for cancer cells to grow and spread. Hence, VEGF may potentially be a suitable biomarker to predict the prognosis and response to chemotherapy in patients with breast cancer (8, 9). The level of VEGF is better represented by the quantity of its receptor because aggregated platelet, activated neutrophil, and lymphocytes can ameliorate its serum level (10). VEGFR-l/Flt-1 is a tyrosine kinase receptor, which binds to VEGF-A, VEGF-B, and PIGF, and plays a role in collagen breakdown and hematopoiesis. On the other hand, VEGFR-2/KDR is a domain kinase receptor that binds

to VEGF with high affinity and plays a role in angiogenesis and hematopoiesis (6, 11).

Breast cancer can be classified into luminal type A, luminal type B, HER-2 type, and basal type, based on different gene expressions. In developing countries, many of the more technologically advanced tests cannot be performed because of financial constraints and relatively poor health care facilities. Therefore, there is a need to use a biomarker that can function as a general screening tool for all types of breast cancer. Various studies have confirmed the role of HER-2/neu and ER/PR as a very valuable rapid proliferation marker of cancer cells, which function both as a predictive and prognostic factor in breast cancer. However, the relationship between HER-2/neu and ER/PR expression with vascular endothelial growth factor (VEGF) has not been fully defined. Thus, the aim of the study is to determine the level of VEGFR and its association with HER-2/neu and ER/PR expression in a localised population of patients with breast cancer. Any association found between those markers will support the use of VEGFR as a novel predictive and prognostic biomarker for breast cancer.

Materials and Methods

Samples

Biopsies were collected from 40 patients diagnosed with breast cancer who were scheduled for elective surgery in H. Adam Malik Hospital, Medan, from October 2013 to December 2013. Patients who had received both radiotherapy and chemotherapy, and who suffered from other cancers, and metabolic syndromes, were excluded from the study. This study was approved by the Ethics Committee of the Faculty of Medicine, University of Sumatera Utara. Written informed consent was obtained from each participant prior to the commencement of the study.

Medical record, biopsy collection, and processing of paraffin blocks were collected by different observers blinded to the study. Specimens were processed in the Department of Pathology, University of Sumatera Utara, by hematoxylin eosin staining to examine the histopathology, grading, vascular invasion and lymphatic invasion. Samples were further sent to the Department of Pathology, Gadjah Mada University, Yogyakarta, to determine the expressions of VEGFR, HER-2/neu and ER/PR.

Study criteria

The expressions of vascular endothelial growth factor receptor (VEGFR), HER-2/neu, estrogen (ER) and progesterone (PR) receptors in the primary tumor were also determined by immunohistochemistry. VEGFR was regarded as being overexpressed if the positively stained cells were > 42.5% of the total cell population, and unexpressed if the stained cells were <42.5%. HER-2/neu was regarded as positive if the positively stained cells were > 10%, and negative if the stained cells were <10%. ER was considered positive if the stained cells were > 10%, and negative if the stained cells < 10%. PR was regarded as positive if the positively stained cells were > 10%, and negative if the stained cells < 10%.

VEGFR analysis

Paraffin blocks were sliced by ±1-2 microns thick, placed in special glass object, deparafinnated, then washed with Phosphate Buffer Saline (PBS), 3-5 times for 5 minutes. Antigen retrieval was carried out using citrate buffer pH 6 0 and heated to 95°C, two times for 5 minutes, then allowed to cool, and washed again with PBS, 3-5 times, each lasting for 5 minutes. Specimens were then treated with normal rabbit serum for 10 minutes, followed by monoclonal VEGFR with 1: 100 dilution (Kione TLC-9 Novocastra) overnight at room temperature. Samples were then washed again with PBS, 3-5 times, 5 minutes each time. Secondary antibody was added for 30 minutes. Samples were rewashed again with PBS, 3 times, followed by addition of streptavidin bioin complex (SABC from Daco) for 30 minutes, and washed again with PBS, 3 times for 5 minutes. Diamine benzidine was added for 3 minutes, and then washed with sterile water. The specimens were stained with Mayer's hematoxylin solution for 20 seconds to 1 minute and washed with running water. Specimens were then examined under the microscope, at a magnification of 100 x 400. Stained cells were calculated by 10 times sliding and the averaged results were collected.

Statistical analysis

Statistical analysis was done by SPSS 17.0, Inc (Chicago, IL). Association between dependent and independent variables was conducted using chi-square test and logistic regression. Significance level is set at $p \leq 0.05$

Results

Patients and tumor biopsies

The characteristics of patients in this study are shown in Table 1. The primary locations of the breast cancer were in the right breast (77.5%) and in both lateral upper quadrant and central quadrant (30.0%), respectively. Infiltrating ductal carcinoma accounts for 90% of the breast cancer cases. The majority of the patients were in stage III (70%); 30 patients (75.0%) obtained a positive TIL, and 30 patients (75.0%) show no lymphovascular invasion.

VEGFR, HER-2/neu, and ER/PR expression

On immunohistochemistry examination, VEGFR expression was found to be overexpressed in 21 samples (52.5%) and unexpressed in 19 samples (47.5%). HER-2/neu was found to be positive in 14 patients (35.0%) and ER/PR was positive in 22 patients (55%). VEGFR was overexpressed in 12/24 HER-2/neu positive compared to only 9/26 in HER-2/neu negative samples. Chi square analysis revealed a significant association between overexpression of VEGFR and positivity of HER-2/neu expression (X^2 9.52,

Table 1: Characteristics of breast cancer patients in this study

Characteristics	Total number of patients	Percentage of patients (%)
Age		
< 25	1	2,5%
26 – 40	12	30%
41 – 65	26	65%
> 65	1	2,5%
Stage		
I	2	5%
II	2	5%
III	28	70%
IV	8	20%
Histopathology		
LDC	36	90%
Lobular Ca	2	5%
Tuular. Ca	1	2,5%
Mucinous Ca	1	2,5%
Grading		
Grade I	6	15%
Grade II	18	45%
Grade III	16	40%
Tumor location		
Upper lateral	12	30%
Lower lateral	7	17,5%
Upper median	5	12,5%
Lower median	4	10%
Cental	12	30%
TIL		
Positive	30	75%
Negative	10	25%
Lymphovascular invasion		
Positive	10	25%
Negative	30	75%
HER-2/neu		
Positive	14	35%
Negative	26	65%
P53		
Positive	13	32,5%
Negative	27	67,5%
ER/PR		
Positive	22	55%
Negative	18	45%
VEGFR		
Overexpressed	21	52,5%
Unexpressed	19	47,5%

p= 0.002; OR: 11.33; 95% CI: 2.06 – 62.1). On the other hand, VEGFR was overexpressed in 16/18 ER/PR negative, compared to only 5/22 in HER-2/neu positive samples. A significant association between overexpression of VEGFR and negativity of ER/PR expression was also demonstrated (X^2 6.32, p= 0.002; OR: 0.182; 95% CI: 0.04 – 0.71).

Discussion

Breast cancer is a solid cancer that is difficult to treat due to its rapid behavior of metastasis. In line with the tremendous development of biomolecular research, diagnosis and therapy of breast cancer are now improved. Since metastasis is largely influenced by angiogenesis, VEGFR may have an important role as a predictive biomarker for breast cancer. Malignant transformation of cells in culture condition is associated with an increased expression of VEGFR to support their growth (12).

VEGF binds to tyrosine kinase receptors i.e. VEGFR-1 (Flt-1), VEGFR-2 (Flk-l/KDR) and VEGFR-3 (Flt-4). Receptors are then deformed, dimerized, and autophosphorylated. This will induce signal transduction, resulting in endothelial cell proliferation, activation of mitogen-activated protein kinase (MAPK), protein kinase C (PKC) and Akt pathway. This will lead to migration of endothelial cells, involving the induction of MMP activation of focal adhesion kinase and phosphatidylinositol 3'-kinase (PI3-K). Nitric oxide serves as a mediator for vasodilation, proliferation, and migration of endothelial cells in response to VEGF.

Table 2: Association of VEGFR and HER-2/neu expression

	Her2/Neu		Total	p
	Positive	Negative		
Overexpressed	12	9	21	0.002
Unexpressed	2	17	19	
Total	14	26	40	

Table 3: Association of VEGFR and ER/PR expression

	EKSP.ER/PR		Total	p
	Positive	Negative		
Overexpressed	5	16	21	0.002
Unexpressed	17	2	19	
Total	22	18	40	

Angiogenesis-induced VEGF does not only promote the growth of endothelial cells but also inhibit apoptosis through activation of Akt-PI3-K pathway. VEGF also regulates expression of several anti-apoptotic proteins, such as Bcl-2. This protein ultimately inhibits caspase activation and regulates the expression of X-chromosome-linked inhibitor of apoptosis family of proteins. This mechanism is caused by the destruction of immature blood vessels as an effect of low VEGFR expression (13).

VEGFR has been previously tested as a biomarker that can function generally for all types of breast cancer (12). HER2/neu and ER/PR have been confirmed as the promising marker in breast cancer. If there were associations between HER-2/neu and ER/PR with VEGFR, then VEGFR can be a valuable biomarker in general types of breast cancer. Changes in estrogen and progesterone levels induce remodelling of breast cancer epithelial, stroma, and vascular tissues (13). A previous study involving the San Antonio patient database showed that negativity of ER/PR in terminal ductal breast cancer cells induced cancer cells differentiation and lymphovascular invasion (14). McPherson *et al.* found that ER negative-breast cancer was linked to rapid tumor cell growth and angiogenesis, which was shown by increased thymidin labeling index, mitotic index, and microvessel density (15). In this study, there was a significant association between the overexpression of VEGFR with negativity of ER/PR. Linderhaim *et al.* also found an association between these parameters in progressive proliferated and invasive, mainly on invasive ductal breast carcinoma.

In a previous study, Bilious *et al.* showed that gene amplification and overexpression of HER-2/neu was a poor prognostic factor for breast cancer (16), as well as for disease recurrence and patients' survival rates (17). High cell proliferation and grading with HER-2/neu overexpression requires angiogenesis to maintain sufficient nutrition and metabolism for tumor cell development (18). In this study, a significant association between the overexpression of VEGFR with positivity of HER-2/neu was found. A study by the American Association for Cancer Research also showed that high VEGF levels correlated with high HER-2/neu (19).

VEGFR overexpression was also found to be closely associated with tumor invasion into the lymphatic vessels. Lymphovascular invasion is correlated with tumor size, histopathology grading, and lymph node metastasis (20). Clinical trials in cancer patients with VEGFR inhibitors, including anti-VEGFR monoclonal humanized antibody (mAb VEGFrhu) showed promising results. Phase III of randomized controlled trial study in metastatic colorectal cancer demonstrated significant increase in overall rate of survival with VEGF mAb rhu therapy (21).

This study was conducted in one of the tertiary referral centers in Indonesia; hence, it has an advantage of representing a larger population of breast cancer cases in Indonesia. However, the limitation of this study was that the majority of breast cancer types was invasive ductal carcinoma, in which the usual tumor sizes were larger and histological grading and mitotic indices were higher, so that logically, the levels of VEGFR tend to be higher.

Conclusion

Overexpression of VEGFR showed significant association with positivity of HER-2/neu expression and negativity of ER/PR expression. Thus, VEGFR might serve as a suitable new biomarker for breast cancer. Further research with larger sample size is needed to improve the reproducibility of the study.

Abbreviations

ER/PR: Estrogen receptor/Progesteron receptor, HER-2/neu: Human Epidermal growth factor Receptor 2/neu, MAPK: Mitogen-activated protein kinase, PI3K: Phosphatidylinositol 3'-kinase, PBS: Phosphate Buffer Saline, PKC: Protein kinase C, TIL: Tumor infiltrating lymphocyte, VEGF: Vascular endothelial growth factor, VEGFR: Vascular endothelial growth factor receptor

Competing interests

The author declares that he has no competing interests.

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