THE STUDY OF SIX SNPS IN LCT REGULATORY REGION AND ITS ASSOCIATION TO LACTOSE INTOLERANCE IN SABAH

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FACULTY OF SCIENCE AND NATURAL RESOURCES

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2014

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THESIS SUBMITTED IN FULFILLMENT FOR THE MASTER DEGREE

FACULTY OF SCIENCE AND NATURAL RESOURCES
UNIVERSITI MALAYSIA SABAH

2014

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UNIVERSITI MALAYSIA SABAH

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DEGREE	:	**MASTER DEGREE (BIOTECHNOLOGY)**
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ACKNOWLEDGEMENT

First of all, I would say Alhamdulillah to Allah S.W.T. for giving me the strength and health to complete this research. I also want to express my sincerest gratitude to my supervisor, Assoc. Prof. Dr Lee Ping Chin and my co-supervisor, Prof. Peter Barling and Dr Yasmin Beng Houi Ooi, who has supported me throughout my research and thesis with patience and knowledge whilst allowing working in my own way. I attribute the level of my Masters degree to their encouragement and effort and without them this thesis, too, would not have been completed or written.

Apart from that, I would like to thank a few medical lab technologist who were willing to squeeze their time from their work and busy schedule to give their helping hands while I am conducting subjects recruitment and collecting blood sample in rural area. A special thanks to my colleagues, Eric Chong and Dg Nur Azierah who always encourage me to finish my laboratory works and thesis writing. Last but not least, I'm also indebted to my dearest friend, Siti Noraniza, who gives me hearty support when I am depressed.The simple phrase "thank you" cannot represent how much their friendship means to me.

Thanks to my parents for providing a carefree environment for me so that I can concentrate to my study. Although they hardly understand what writing thesis is and what I research on, my parents are willing to support any decision I make. I am so lucky to have them be my parents. Thanks also to any party that assist me either directly or indirectly throughout this research. The research and this dissertation will not be successful without the help and support from all of you.

Nur Ashikhin Gandah

22 August 2014

ABSTRACT

Lactose intolerance is a clinical condition such as bloating, flatulence and diarrhea that affects lactase non-persistence (LNP) individuals following ingestion of lactose. LNP individuals are unable to digest lactose due to transcriptional down-regulation of lactase (*LCT*) gene, which results in reductionof lactase-phlorizinhyrolase (LPH) level required for lactose digestion. However, there are also individuals known as lactase persistence (LP)who retain continuous production of LPH throughout their adult life. This phenotype has been associated with the presence of six single nucleotide polymorphisms (SNPs) within particular region of *MCM6* gene that regulates the promoter of *LCT* gene. These six SNPs (G/A-22018, G/C-14010, T/G-13915, C/G-13907, C/T-13910 and T/C-3712) have been extensivelyused in genotype test for determination of LNP and LP in non-Asian population. To date, there is no report of these polymorphisms and its association with lactose intolerance in Malaysia. Therefore, this study was conducted to determine the presence of these SNPs and its associations with LP and LNP traits mainly in Sabah. A total of 188 subjects were diagnosed using 50 g-Hydrogen Breath Test (HBT) and Lactose Tolerance Test (LTT). Changes in breath H_2 and glucose level were measured every 30 minutes interval for 3 hours test period. Subject who has increased breath H_2 of > 20 ppm and blood glucose of < 1.5 mmol/L was grouped as LNP. The data showed that the distribution of LNP in Sabahwas extremely high at 94% (N=176) whereas the LP was only 6% (N=12). Whole blood samples were collected for DNA extraction using modified alkaline lysis method. Target sequences were amplified using specific primers in Polymerase Chain Reaction (PCR) and subjected to direct sequencing. The sequencing results showed that99.4% (N=176) of LNP and 83.3% (N=10) of LP carryhomozygous wild-type genotype associated with LNP as reported elsewhere (GG-22018, GG-14010, TT-13915, CC-13907, CC-13910 and TT-3712). Chi-square analysis showed no significant association (p>0.05) between these six LNP genotypes towards LNP/LP phenotype.Heterozygote CT-13910 and GA-22018 associated with LP in non-Asian population were also found in one LNP and two LP subjects. Since there was no genotype difference between LNP and LP, this study suggests that thesix SNPs associated with LNP/LP reported in non-Asian population are not suitable to be used as a genetic marker for determination of LNP/LP in Sabah.

ABSTRAK

KAJIAN TERHADAP ENAM SNP DALAM KAWASAN PENGAWALSELIAAN LCT DAN HUBUNGKAITNYA TERHADAP INTOLERANSI LAKTOSADI SABAH

Intolerasi laktosa adalah satu keadaan klinikal dimana kembung perut, buang angin dan cirit-birit dialami oleh individu yang tidak mempunyai laktes (Lactase non-persistence, LNP) selepas pengambilan laktosa. Individu LNP tidak berupaya mencerna laktosa kerana kurangnya regulasi transkripsi pada gen laktes (LCT), seterusnya menyebabkan penurunan tahap enzim laktes (lactase-phlorizin hyrolase, LPH) yang diperlukan untuk pencernaan laktosa. Walaubagaimanapun, terdapat individu yang mempunyai laktes (Lactase persistence, LP) dimana individu ini mampu menghasilkan LPH secara berterusan hingga dewasa. Fenotip ini telah dikaitkan dengan kehadiran enam polimorfi neuklotida tunggal (SNPs) pada sesuatu tempat dalam gen MCM6 yang mengawal promoter gen LCT. Semua SNPs ini (G/A-22018, G/C-14010, T/G-13915, C/G-13907 dan T/C-3712) telah digunakan secara meluas dalam ujian genotip untuk mengenalpasti LNP dan LP dalam populasi bukan Asia. Setakat ini tiada lagi laporan mengenai polimorfi ini di Malaysia dan hubungkaitnya terhadap intoleransi. Oleh yang demikian, kajian ini dijalankan untuk mengenalpasti kehadiran SNPs ini dan hubungkaitnya terhadap tret kumpulan LP dan LNP di Sabah. Seramai 188 subjek telah diuji dengan 50g-Ujian Pernafasan Hidrogen (HBT) dan Ujian Toleransi Laktosa (LTT). Perubahan pada pernafasan H_2 dan kepekatan glukosa dalam darah diukur setiap 30 minit selama 3 jam. Subjek yang menunjukkan peningkatan H_2> 20 ppm dan glukosa darah < 1.5 mmol/L dikelaskan sebagai LNP. Data menunjukkan bahawa taburan LNP di Sabah adalah sangat tinggi iaitu 94% (N=176) manakala LP hanya 6% (N=12) sahaja. Sampel darah telah diambil untuk pengekstrakan DNA menggunakan lisis kaedah alkali yang telah diubahsuai. Jujukan target telah diamplifikasi menggunakan primer yang spesifik dalam tindakbalas rantai polimeras (PCR) diikuti dengan penjujukan. Keputusan penjujukan menunjukkan bahawa 99.4% (N=176) LNP dan 83.3% LP membawa genotip homozigot wild-type yang berkait rapat dengan LNP di lain-lain tempat(GG-22018, GG-14010, TT-13915, CC-13907, CC-13910 and TT-3712).Analisis Chi-Square menunjukkan tiada hubungkait yang ketara (p>0.05) antara kesemua genotip ini terhadap fenotip LP/LNP.Heterozigot CT-13910 dan GA-22018 yang berkait rapat dengan LP pada populasi bukan Asia telah dijumpai pada seorang LNP dan dua orang LP. Oleh kerana tiada perbezaan genotip antara LP dan LNP, kajian ini mencadangkan bahawa keenam-enam SNP yang dikaitkan dengan LNP/LP di populasi bukan Asia tidak sesuai digunakan sebagai penanda genetik untuk mengenalpasti LNP/LP di Sabah.

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α	-	Alpha
ß	-	Beta
X^2	-	Chi-square
CFU	-	Colony forming unit
DNA	-	Deoxyribonucleic acid
dNTP	-	Deoxyribonucleotide triphosphates
EDTA	-	Ethylenediaminetetraacetic acid
g	-	Gram
g	-	Gravity forces
-OH	-	Hydroxyl
kb	-	Kilo base
kDa	-	Kilo Dalton
<	-	Less than
L	-	Liter
MgCl	-	Magnesium chloride
mRNA	-	Messenger ribonucleic acid
µl	-	Micro liter
ml	-	Milli liter
mmol	-	Milli mol
mM	-	Milli molar
>	-	More than
ng	-	Nano gram
ppm	-	Parts per millions
%	-	Percent
s	-	second
NaCl	-	Sodium chloride
SDS	-	Sodium dodecyl sulphate
X	-	Times
TAE	-	Tris-Acetate-EDTA
TE	-	Tris-EDTA
U	-	Unit

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CHAPTER 1

INTRODUCTION

Lactose is a principal sugar of milk and can be found in wide range of dairy product. Human milk contains the highest amount of lactose (7.1g/dL) followed by cow milk (4.7g/dL) and only traces in marine mammalian milk (Solomon, 2002). In small intestine, lactose which is in the form of disaccharides is hydrolyzes into small galactose and glucose monosaccharide by the enzyme called lactase-phlorizin hydrolase (LPH) so that it can be easily absorbed by the body. LPH which encoded by the lactase gene (*LCT*) is highly expressed during the third trimester of gestation (Grand *et al.*, 2003)and begin to decrease at weaning to a fraction of that found in adult human. Hence, most of the adult human have reduced ability to digest lactose because of decreasing LPH levels (Buller*et al.*, 1990). When lactose was consumed, fraction of those lactose remain undigested, accumulated in the small intestine and subjected to bacterial fermentation which then leads to the occurrence of symptoms such as flatus, diarrhea, cramping etc.All these symptoms are referred as lactose intolerance and the type of intolerance is known as primary lactose intolerance.

Incidence of primarylactose intolerance can be detected in children as early as two or three years old in some Asian countries (Keusch*et al.*, 1969; Yang *et al.*, 2000). This implies that in Asian population, the level of LPH is already become deficient during childhood and not necessarily begins during adulthood. While most of the human populations undergo developmental decrease of LPH (lactase non-persistence, LNP), there are some individuals who produce sufficient LPH throughout their adult life and the phenotype is known as lactase persistence (LP). LP phenotype is very frequent in the Northern Europe population and is strongly related to a single mutation C→T-13910 within intron 13 of minichromosome maintenance complex component 6 gene (*MCM6*) neighboring the *LCT* gene (Enattah*et al.*, 2002).

*MCM6*plays an essential role for the initiation of eukaryotic genome replication and its function may seem to be unrelated with the production of LPH. Extensive research however has showed that any single mutation in particular region of this gene can induce different transcriptional activation of the *LCT* gene (Wang *et al*., 1998; Lewinsky *et al*., 2005; Jensen *et al*., 2011).

Other than C/T-13910, Enattah and colleagues (2002) also found another single mutation, G→A-22018 within intron 9 of *MCM6.* Although not completely associated with LP in Finnish population, this single nucleotide polymorphism (SNP) was found to be a better biomarker in determining LP/LNP in Japanese-Brazillian population compared to C/T-13910 (Mattar*et al*., 2010). Prevalence of LP is also reported to be high in Africa and Middle East populations however neither C/T-13910 nor G/A-22018 was found to be associated with the existence of LP within these populations (Tishkoff*et al*., 2007; Ingram *et al*., 2009b). Meanwhile multiple variants (G/C-14010, T/G-13915 and C/G-13907) were found in LP of Africa. These variants were located in close proximity to the European C/T-13910. In the study of LP in Saudis population, Enattah and colleagues (2008) found another SNP, T/C-3712 as compound allele with T/G-13915 located at exon 17 of *MCM6* gene.The existence of different SNPs associated with LP in different populations confers high probability that there might be other SNP(s) that can be associated with LP in other population.

1.1 Statement of Problems

Research conducted by commercial milk industries, Dutch Lady in 2012 summarized that fresh milk and dairy product consumption in Malaysia has increased in the past few years although the average consumption of milk was still relatively low compared to other Asian countries. Furthermore, Malaysia has an outstanding milk distribution program for primary school since 1983 to ensure that every school children received nutritional benefits of milk for their health. Thus, there is a concern rises whether the milk-drinking culture really benefits children and adulthood of Malaysia population. In a study of three different ethnic of Malaysia, Asmawi*et al.,* (2006) found that most of the Malaysian adults are lactose intolerant, indicating the importance of low lactose

dairy product consumption. Consumption of lactose that exceeds the amount of available LPH can induce lactose intolerance symptoms. Specific dietary guidelines and milk formulation for primary school can be established to overcome this problem but unfortunately there is only trace of information regarding to the incident of lactose intolerance in Malaysia.

Detection of LP/LNP in non-Asian population has move to genotyping level, focusing on the six SNP associated that have been reported to be associated with the LNP/LP phenotype which is G/A-22018 G/C-14010, T/G-13915 C/T-13910 C/G-13907 and T/C-3712. Among all these six SNPs, the European C/T-13910 is the most extensively used as the biomarker in determining the LP/LNP globally. While the reliability of this test is still under investigation, extensive research has been conducted in different populations to determine the specific SNP that can be used as a marker to distinguish the LP/LNP phenotype for the particular population studied (Table 1.1).In Asian population, especially in Southeast Asia, genotype testing for determination of LNP/LP has never been done before. Most of the data on incidence of lactose intolerance in based on phenotypic test, which is hydrogen breath test (HBT), lactose tolerance test (LTT), intestinal biopsy, or by observing the occurrence of lactose intolerance symptom upon consumption of milk or lactose bolus.

Table 1.1: Past finding in identification of LNP/LP in different populations

	Studied Population	No of Subjects	Phenotype Test (PT)/Genotype Test (GT)	Reference
Non-Asian Population	Israelis	439	GT	Raz *et al.*, 2013
	Northern Russian	518	GT	Khabarova *et al.*, 2011
	Brazilians	567	GT	Mattar *et al.*, 2009
	Poland	200	PT & GT	Madry *et al.*, 2010
	Tanzanian, Kenyan, Sudanese (Africa)	470	PT & GT	Tishkoff *et al.*, 2007
	Sardinia (Italy)	240	PT & GT	Obinu *et al.*, 2010
	Colombia	367	GT	Mendoza *et al.*, 2010
	Finnish (Northern Europe)	252	PT & GT	Rasinpera *et al.*, 2004
	Germany	166	PT & GT	Buning *et al.*, 2005
	Sweden	51	PT & GT	Ridefelt & Hakansson, 2005
Asian Population	Russians	484	GT	Borinskya *et al.*, 2006
	Saudi Arabia	432	PT & GT	Imtiaz *et al.*, 2007
	Indian	2264	GT	Romero *et al.*, 2011
	Tibetan (Chinese)	495	GT	Peng *et al.* , 2012
	Myanmar	324	PT	Lwin *et al.*, 2010
	Singaporeans	77	PT	Yap *et al.*, 1989
	Indonesians	85	PT	Yohmi *et al.*, 2004
	Thailand	45	PT	Densupsoontorn *et al.*, 2004
	Malaysians	300	PT	Asmawi *et al.*, 2006

*PT: Hydrogen breath test, glucose plasma test, endoscopic duodenal biopsy, self-reported intolerance survey, etc; GT: PCR-RFLP, direct sequencing, etc.

Previous study on lactose intolerance in Malaysia was conducted by Asmawi*et al.,*(2006).The data suggested that the frequency of LNP in Malaysia was ranged from 83% to 91%. This finding implies that the distribution of LNP in Malaysia was relatively high. However, result from that study does not represent the distribution of LNP in Malaysia as a whole since it does not include the population living in East Malaysia particularly in Sabah. Hence, there is a need to determine the distribution of LNP in Sabah as this will be useful in understanding the lactose intolerance in Malaysia as a whole.

Nevertheless, Asmawi*et al.*, (2006) did not provide any genetic basis underlying the existence of the lactose intolerant or specifically LNP. In fact, there is no knowledge on LNP/LP genetic polymorphism in Malaysia or even in Sabah.

Identification of population-specific SNPs associated with LNP phenotype is important due to the fact that certain individuals can be misdiagnosed as LNP as they develop lactose intolerance symptoms following ingestion of lactose. Moreover, occurrence of symptoms do not necessarily reflect LNP since individuals who have secondary loss of LPH due to intestinal injury also develops lactose intolerance symptoms especially when they were diagnosed using conventional method such as hydrogen breath test (HBT) or lactose tolerance test (LTT). In addition, different population was found to carry different SNP for LNP/LP phenotype. Thus there is a need to determine whether the previously reported SNPs is present within Sabah population and most importantly to determine whether these SNPs can be used for identification of LNP/LP phenotype in Sabah.

1.2 Objectives of Study

a. To determine the distribution of LNP and LP in Sabah.

b. To determine the common six SNPs in *LCT* regulatory region of subject with LNP and LP phenotype.

c. To determine the association of the SNPs towardsLNP and LP phenotype.

CHAPTER 2

LITERATURE REVIEW

2.1 Lactose

Lactose (β-D-galactopyranosyl-1,4-D-glucose) is a carbohydrate that can be found in significant quantities in human diets primarily in milk and dairy products. Human milk for instance contains the highest concentration of lactose (7%) in mammals (Solomons, 2002). Lactose exists in the form of disaccharide of two carbohydrate monomers which are β-galactose and glucose. Lactose has two isomeric (α-lactose or β-lactose) which determined by the form of glucose it contains. Lactose can be produced from simple condensation reaction, but synthesis of lactose in mammary glands is somewhat complicated. Briefly during the condensation, the β-anomer of galactose will form a bond known as β-1,4-glycosidic linkage with a –OH group on carbon number 4 of glucose as shown in Figure 2.1.

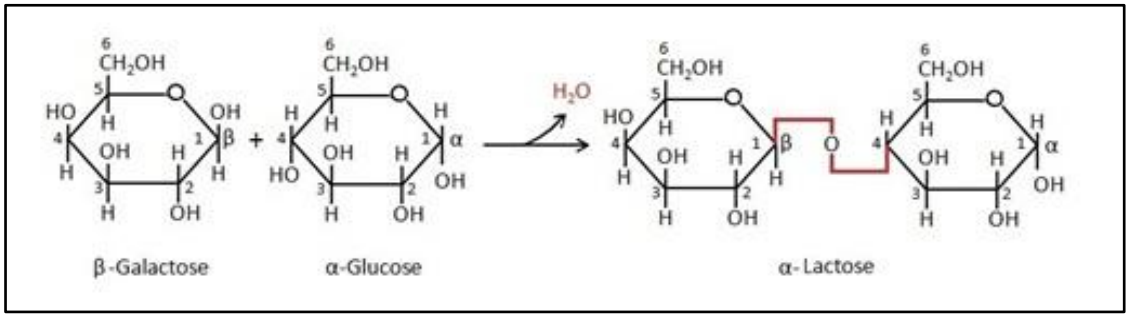

Figure 2.1: **Condensation reaction involves in the production of α-lactose.** Along the process, water molecule was removed, resulting in formation of glycosidic bond (red line) between β-galactose and α-glucose.

Source : Campbell and Farrel (2006).

Information on lactose synthesis from mammary glands has already been established for a long time and discussed vastly in academic books. Based on Cupps (1991) and Norman and Henry (2014), the synthesis of lactose occurs inside the Golgi complex of mammary gland cells. Each lactose molecule synthesized requires two glucoses; one glucose molecule is converted to glucose-1-phosphate first before being converted to UDP-galactose and transported into the Golgi complex via an antiporter called UDP galactose translocator. Meanwhile, the other glucose is transported directly to Golgi complex via glucose transporter (GLUT-1) without modification.

Once in the Golgi complex, an enzyme complex called lactose synthase removed the galactose from the UDP and joining it with glucose to form lactose. Lactose synthase is made up of two components which are a protein, α-lactalbumin and an enzyme, galactosyltransferase. The α-lactalbumin defines specificity of the galactosyltransferase so that it will synthesize lactose (Brew *et al.*, 1967). The gene for α-lactalbumin is only expressed in the mammary gland, thus lactose synthesis occurs only in the mammary gland (Brew *et al.*, 1967).

Metabolism of lactose occurs in small intestine where an enzyme called lactase-phlorizin hydrolase, (LPH) hydrolyzed lactose into glucose and galactose. LPH is present predominantly along the brush border membrane of differentiated enterocytes of small intestine (Olds and Sibley, 2003). These monosaccharides then pass through the intestinal epithelium and transported into the bloodstream. Glucose then is used for generating energy through cellular metabolism. Meanwhile galactose is used to make cellular components such as glycoprotein and glycolipid (Lomer *et al.*, 2008) or converted back to glucose through Leloir Pathway (Holden *et al.,* 2003).

2.2 Nutritional Value of Lactose

Lactose tastes less sweet compared to sucrose (table sugar) and glucose (Vesa *et al.*, 2000). Despite of it taste, lactose have the same number of calories as any other carbohydrate. Since lactose shares so much in common with other carbohydrates, the

nutritional value of lactose is often underestimated. Lactose was thought to have no special dietary importance since any galactose (monomers of lactose) required for cellular metabolism can be made in the human liver by using glucose as precursor.

However, lactose can be beneficial for diabetic patients since it has relatively low glycemic index (GI). A lower GI of lactose describe a slower rate of lactose digestion and absorption which requires lower insulin demand, thus may improved long-term glycemic control in type-2 diabetic patient (Jenkins *et al*., 2008). Slow hydrolysis of the lactose also helps to generates longer energy supply for the body (Walzem, 2004). Furthermore, diets that emphasize low GI foods may help reduce the risk of heart disease (McBean, 2004).

Lactose was found to increase the absorption of minerals such as calcium, magnesium and zinc in laboratory animals and human infant (Greger*et al*., 1989; Heijnen*et al*., 1993; Obermeyer-Pietsch*et al*., 2007). However little is known about the ability of lactose in enhancing calcium absorption in human adults. In addition, lactose has been found to have a low cariogenicity (McBean, 2004) and promotes a healthy intestinal flora by stimulating the growth of beneficial intestinal bacteria such as Bifidobacterium and Lactobacilli (Szilagyi, 2002). Henceforth, by consuming lactose, a person can maintain a healthy intestinal flora as well as enhancing their immunity against intestinal infection (McBean, 2004).

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2.3 Lactose Intolerance and Its Clinical Features

Lactose intolerance is a clinical condition or symptom that occurs following ingestion of lactose or lactose-containing food substances such as milk and dairy products (Heyman, 2006). The typical symptoms of lactose intolerance include diarrhea, abdominal pain, flatulence, bloating, borborygmi (rumbling stomach), cramps and sometimes nausea and vomiting (Heyman, 2006). Other extra-intestinal symptoms also have been observed such as muscular and articular pain, cardiac arrhythmia, short term memory lost, headache, tiredness, irritability, drowsiness, mouth ulcers, sore throat, allergic manifestations, increase in micturition frequency, acne and depression (Lomer*et al.*, 2008). There are several categories of lactose intolerance describe below;

a. Congenital Lactose Intolerance

This type of lactose intolerance is a rare recessive disorder, characterized by watery diarrhea in infants from the first exposure to maternal milk. These infants can be severely ill because of dehydration and electrolyte loss unless they were given milk which is lactose-free (Roberson, 2005).In a study of patient with congenital LPH deficiency, Kuokkanen*et al.* (2006) found that 84% (27/32) of these subjects carry homozygous nonsense mutation, T/A-4170. The data suggested that the intolerance is caused by mutations which affecting the structure of the LPH, thus inactivate it.

b. Primary Lactose Intolerance

This type of lactose intolerance is very common in most population and considered as a wild type condition. It is an ancestral condition for almost all humans and indeed for all mammals (Swallow, 2003) thus it should not be thought of as a disease (Swargerty*et al.*, 2002). Primary lactose intolerance (also referred as adult-type hypolactasia or lactase non-persistence) is characterized by the absence or insufficient amount of LPH due to down-regulation of lactase *LCT* gene (Ingram *et al.*, 2009a; Heyman, 2006). The present research was focused to this type of intolerance.