Asian Journal of Epidemiology

ISSN 1992-1462

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Asian Journal of Epidemiology 2 (3): 49-58, 2009
ISSN 1992-1462

Prevalence of the Leptin and Leptin Receptor Gene Variants and Obesity Risk Factors among Malaysian University Students of Setapak, Kuala Lumpur

Suet-Fang Liew, Her-Swang Chuah, Chooi-Ling Lau,
Chean-Hui Lee and Yee-How Say
Department of Science, Faculty of Engineering and Science,
Universiti Tunku Abdul Rahman (UTAR), Jalan Genting Klang,
Setapak, 53300 Kuala Lumpur, Malaysia

Abstract: This study was to investigate the prevalence of the leptin gene (*LEP*) A19G and leptin receptor gene (*LEPR*) K109R, Q223R and K656N variants and their possible association with obesity and the prevalence of associated obesity risk factors in Malaysian university students of Setapak, Kuala Lumpur. Random convenience sampling was performed with informed consents, obesity risk factors were assessed by questionnaire and anthropometric measurements were taken. Mouthwash samples were obtained for DNA extraction and genotyping was performed using Polymerase Chain Reaction-Restriction Fragment Length Polymorphism. A Body Mass Index cut-off point of 27 kg m^{-2} for obesity was adapted; categorizing the 200 subjects (85 males, 115 females) into 143 non-obese and 57 obese. There was no significant difference in the genotype and allele frequencies of *LEP* A19G and *LEPR* K109R, Q223R variants between obese and non-obese subjects. Only the K656N genotype, but not the 656N allele, was associated with obesity. All the anthropometric measurements were significantly lower in the non-obese compared to the obese subjects, but the obesity risk factors were not significantly different between the two groups-except for physical activity. In conclusion, obesity is not prevalent among the sampled Malaysian university students and the genetic contribution of *LEP* and *LEPR* common polymorphism towards obesity seems to be insignificant.

Key words: Leptin, leptin receptor, single nucleotide polymorphism, obesity, Malaysia

INTRODUCTION

The prevalence of obesity, a multifactorial disease caused by an interaction of genetic factors with lifestyle and environmental factors, is rapidly increasing worldwide. The 2006 Third Malaysian National Health and Morbidity Survey (NHMS III) found that the prevalence of overweight had increased to 29.1% and that of obese-14.0%; compared to the 1996 NHMS II at 16.6 and 4.0%, respectively (Ministry of Health Malaysia, 2006). There is a pressing need to better understand the biochemical pathways that control energy intake and expenditure. In the last few years, a number of important signaling molecules that play

Corresponding Author: Yee-How Say, Department of Science, Faculty of Engineering and Science, Universiti Tunku Abdul Rahman (UTAR), Jalan Genting Klang, Setapak, 53300 Kuala Lumpur, Malaysia

important roles in obesity have been identified. One family of these molecules is the leptin-proopiomelanocortin system. This system plays a key role in the central nervous system control of food satiety and energy expenditure.

The obesity gene that encodes for leptin was originally identified in 1994 by Freidman's group at Rockefeller University (Zhang *et al*., 1994). Leptin is a 16 kDa peptide hormone with 167 amino acids, encoded by the leptin gene (*LEP*) found at chromosome 7q21.3 in humans. Leptin is secreted by white adipose tissue into the bloodstream, binds to leptin receptors (encoded by the leptin receptor gene, *LEPR*) in the hypothalamus and signals via the Janus kinases-start activators of transcription (JAK-STAT) signal transduction pathway to inhibit food intake and stimulate energy expenditure (Watowich *et al*., 1996). *LEPR* maps to chromosome 1p31 in humans and the protein has at least five short and long isoforms, which have identical extracellular and transmembrane domains but differ in the length of the cytoplasmic domain (Houseknecht and Portocarrero, 1998).

Several common polymorphism of the *LEP* and *LEPR* genes have been reported and the potential associations of these polymorphism with obesity have been evaluated in different populations. The *LEP* A19G variant is a polymorphism at the untranslated exon 1 in the promoter of *LEP* gene, which has been associated with a decrease in leptin levels and obesity (Hager *et al*., 1998). This variant has been studied for its association with obesity in separate studies of populations of Finland (Karvonen *et al*., 1998), France (Hager *et al*., 1998) and Italy (Lucantoni *et al*., 2000). Three Single Nucleotide Polymorphism (SNPs) in *LEPR* gene, all located in the extracellular binding domain of the receptor, have potential functional significance to the pathogenesis of obesity. These SNPs resulted in two non-conservative changes (changes in charge): glutamine to arginine at codon 223 (Gln223Arg or Q223R) in exon 6 as reported previously (Considine *et al*., 1996) and lysine to asparagine at codon 656 (Lys656Asn or K656N) in exon 14 and a conservative change: lysine to arginine at codon 109 (Lys109Arg or K109R) in exon 4, both as described earlier (Chung *et al*., 1997). Association studies of these variants with obesity have been carried out among the Caucasians (Gotoda *et al*., 1997), Pima Indians (Stefan *et al*., 2002), Japanese (Matsuoka *et al*., 1997) and Koreans (Koh *et al*., 2002) revealing different association findings.

To date, there is limited data on the association of these gene variants with the prevalence of obesity among the Malaysian population. The socio-demographic, diet, lifestyle factors and the genetic background of the Malaysian population are distinct from those previous populations studied. Hence, the data on previous association studies cannot be extrapolated for the Malaysian population. Meanwhile, dietary habits and lifestyle factors like salty food intake, vegetarian practice, coffee intake, alcohol drinking, smoking and physical activity, have been long established to play a role in the pathogenesis of obesity (Centers for Disease Control and Prevention, 2009). Therefore, in this pilot study among young Malaysian university students in Setapak, Kuala Lumpur, we performed genotyping for the *LEP* A19G and *LEPR* K109R, Q223R and K656N gene variants to find the prevalence of the mutated genotypes and alleles and to investigate if they had any association with obesity. We also took anthropometric measurements and assessed how likely selected dietary habits and lifestyle factors may contribute to the prevalence of obesity among the subjects.

MATERIALS AND METHODS

Subjects

Random convenience sampling was performed in this study. Booths were set up at the Kolej Tunku Abdul Rahman and Universiti Tunku Abdul Rahman campuses, two major

private institutions of higher learning in Setapak, Kuala Lumpur, from October to December, 2008. A short introduction of this study was given to subjects who passed by the booths. The subjects were healthy and unrelated college/university students and consisted of three major Malaysian ethnicities-Malays, Chinese and Indians. The institutional board approved this study, all individuals participating in this study signed informed consent forms and all samples were taken in accordance with the 1995 Declaration of Helsinki (as revised in Edinburgh, 2000).

Questionnaire and Anthropometric Measurements

A questionnaire was carried out to evaluate the demographic data, dietary habits, physical activity, smoking and drinking practices. The demographic data section delivered information of age, gender and race. The dietary habits section assessed eating habits related to obesity, namely the habit of consuming of salty foods, vegetarian practice and caffeine intake in the form of coffee consumption. The physical activity segment delivered information whether the subjects practiced physical activity (excluding occupational and household physical movements) and the frequency of physical activity was also assessed. The final section investigated the current status of tobacco smoking and alcohol drinking practices.

The systolic and diastolic blood pressures were taken using an automated blood pressure monitor (SEM-1, Omron) after the subjects have rested for 5 min. The height (in cm), waist and hip circumference (in inc.) of the subjects were measured using a measuring tape and their Waist-Hip Ratio (WHR) was calculated by dividing the waist circumference by the hip circumference. A bio-impedance body fat weighing scale (Salter Body Analyzer and Scale, UK) was used to determine the Body Mass Index (BMI) and Total Body Fat (TBF). Subjects with the BMI cut-off point of $\geq$27 kg m^{-2} were considered as obese (Yap *et al*., 2000).

DNA Extraction and Genotyping

Subjects were asked to rinse their mouths thoroughly using mineral water before DNA sampling of buccal cells. A 5 mL of 3% sucrose was then given to each subject and they were asked to use their tongues to rub the inner part of cheeks. After 1 min of gargling, the mouthwash was collected in a clean paper cup and was immediately poured into a 15 mL centrifuge tube containing 3 mL of TNE buffer [17 mM Tris/HCl (pH 8.0), 50 mM NaCl and 7 mM EDTA] diluted in 66% ethanol and stored at 4°C until further use. Genomic DNA was extracted using the isopropanol-ethanol precipitation method as described by Aidar and Line (2007).

The *LEP* A19G and *LEPR* K109R, Q223 and K656N gene variants were amplified by using a set of forward and reverse primers and PCR conditions according to Karvonen *et al*. (1998) and Gotoda *et al*. (1997) with the annealing temperatures of 71, 56, 54 and 47°C, respectively. Genotypes were determined by Restriction Fragment Length Polymorphism (RFLP), where restriction enzymes *Msp*A1I, *Hae*III, *MspI Bst*UI digested *LEP* A19G, *LEPR* K109R, Q223R and K656N into 175 and 29, 101 and 70, 80 and 58, 47 and 31 bp fragments, respectively. The homozygous wild-type genotypes were undigested. Fragments were resolved by 3% agarose gel electrophoresis at constant 100 V for 45 min before staining with ethidium bromide and viewed under an UV transilluminator.

Statistical Analysis

The data obtained was statistically analyzed by the SPSS for Windows® Version 16.0 (SPSS, Chicago, IL). The results for continuous variables are given as Means±SD. The

differences between the two groups (obese and non-obese) were assessed by the ANOVA for continuous variables and by the χ^2 test for non-continuous variables. The $p<0.05$ was considered as statistically significant.

RESULTS

Table 1 shows the socio-demographics and anthropometric measurements of the subjects in this study. There were 200 subjects recruited, consisting of 57 obese subjects (28 males and 29 females) and 143 non-obese subjects (57 males and 86 females), with the mean age of 21.22±2.85 years old. The BMI cut-off point of 27 kg m^{-2} for obesity used in this study was derived from a Singaporean study by Yap *et al.* (2000). There were more females (57.5%) in this study and Chinese comprised more than half of the subjects, followed by Indians, Malays and other ethnicities (Table 1). Student's t-test performed to compare the means of Systolic Blood Pressure (SBP), Diastolic Blood Pressure (DBP), Body Mass Index (BMI), Total Body Fat (TBF) and Waist-Hip Ratio (WHR) showed that all these means were significantly lower in the non-obese subjects ($p<0.001$).

The genotype frequencies and the allele frequencies are shown in Table 2. The total of subjects successfully genotyped was 112 for *LEP* A19G, 196 for *LEPR* K109R, 162 for Q223R

Table 1: Socio-demographics and anthropometric measurements of the subjects

Variable	Non-obese (N = 143)	Obese (N = 57)
Sex		
Male	57 (39.9)	28 (49.1)
Female	86 (60.1)	29 (50.9)
Ethnicity		
Chinese	96 (67.1)	32 (56.1)
Indian	36 (25.2)	15 (26.3)
Malay	8 (5.6)	9 (15.8)
Others	3 (2.1)	1 (1.8)
Anthropometric measurement		
Body Mass Index, BMI (Mean±SD, kg m^{-2})	21.660±2.84	31.900±4.50
Systolic Blood Pressure, SBP (Mean±SD, mmHg)	116.37±13.76	133.36±18.60
Diastolic Blood Pressure, DBP (Mean±SD, mmHg)	71.520±8.19	83.020±13.20
Total Body Fat, TBF (Mean±SD, %)	19.990±6.94	40.350±11.78
Waist-Hip Ratio, WHR (Mean±SD)	0.8400±0.07	0.9100±0.08

Brackets are percentages within BMI class, SD: Standard deviation

Table 2: Genotype and allele frequencies of *LEP* and *LEPR* gene variants in obese and non-obese subjects

	Genotypes			Alleles	
Gene variants	1	2	3	A	B
***LEP* A19G (N = 112)**					
Non-obese	6	71	10	83	91
Obese	4	17	4	25	25
Total	10	88	14	108	116
***LEPR* K109R (N = 196)**					
Non-obese	21	113	7	158	124
Obese	9	39	7	65	45
Total	30	152	14	223	169
***LEPR* Q223R (N = 162)**					
Non-obese	51	43	18	145	79
Obese	22	19	9	63	37
Total	73	62	27	208	116
***LEPR* K656N (N = 200)**					
Non-obese	84	41	18	209	77
Obese	18	32	7	68	46
Total	102	73	25	277	123

1: Homozygous wild-type genotype, 2: Heterozygous mutated genotype, 3: Homozygous mutated genotype, A: Wild-type allele, B: Mutated allele

Table 3: The frequencies of dietary habits and lifestyle factors that contribute to obesity among non-obese and obese subjects

	BMI class		
Variables	Non-obese	Obese	p‡
Salty food intake			
Yes	77 (53.8)	24 (42.1)	0.134
No	66 (46.2)	33 (57.9)	
Vegetarian practice			
Yes	56 (39.2)	27 (47.4)	0.288
No	87 (60.8)	30 (52.6)	
Coffee intake			
Yes	97 (67.8)	38 (66.7)	0.874
No	46 (32.2)	19 (33.3)	
Alcohol consumption (current status)			
Yes	53 (37.1)	21 (36.8)	0.284
No	90 (62.9)	36 (63.2)	
Tobacco smoking (current status)			
Yes	10 (7.0)	6 (10.5)	0.697
No	133 (93.0)	51 (89.5)	
Physical activity			
Yes	27 (18.9)	23 (40.4)	0.006*
No/Occ.	116 (81.1)	34 (59.6)	

‡p values by the χ^2 test, significant at $p<0.05$, Occ. Occasionally; brackets are percentages within BMI class

and 200 for K656N gene variants. The overall frequencies for the mutated alleles were 0.52 for *LEP* 19G and 0.43, 0.36 and 0.31 for *LEPR* 109R, 223R and 656N, respectively. For the *LEP* A19G variant, majority of the non-obese and obese subjects had the heterozygous mutated AG genotype with 52 and 50% of the mutant G allele, respectively (Table 2). The same was seen for the *LEPR* K109R variant; however, the mutant R allele was slightly less than half in both non-obese (44%) and obese (41%) subjects. This was also true for the *LEPR* K656N obese subjects, where the majority of them had the heterozygous KN genotype and 40% had the mutant N allele (Table 2). For the non-obese subjects, majority of them had the homozygous wild-type genotype QQ and KK for the *LEPR* Q223R and K656N gene variants, respectively. The wild-type allele of the above gene variants-Q and K also dominated the non-obese subjects, with 65 and 73%, respectively (Table 2). When analyzed by Chi-Square test, there was no significant difference ($p>0.05$) in the genotype and allele frequencies of *LEP* A19G and *LEPR* K109R, Q223R variants between obese and non-obese subjects ($\chi^2 = 2.576$, 95% *CI* 0.418-5.150, $p = 0.276$; $\chi^2 = 3.829$, 95% *CI* 0.098-0.198, $p = 0.147$ and $\chi^2 = 0.097$, 95% *CI* 1.24-1.38, $p = 0.953$ for genotypes, respectively). Only the K656N genotype was significantly associated with obesity ($\chi^2 = 14.324$, 95% *CI* 0.784-3.685, $p<0.01$), but not the 656N allele ($\chi^2 = 0.004$, 95% *CI* 0.453-2.321, $p = 0.953$).

Besides the *LEP* and *LEPR* gene variations, we also investigated how the dietary habits and lifestyle factors may contribute to obesity among the sampled Malaysian university students. We found that more than half of all subjects consumed salty food (50.5%) and drank coffee (67.5%), but vegetarian practice (41.5%), current habitual or social alcohol drinking (37%) or tobacco smoking (8%) and consistent physical activity excluding occupational and household physical movements (25%) were not prevalent among the subjects (Table 3). As the frequencies of these dietary habits and lifestyle factors were almost the same between the non-obese and obese groups, we found that there was no statistical significant difference in these factors between them ($p>0.05$ by Chi-Square test; Table 3), except only for physical activity ($p = 0.006$).

DISCUSSION

As random convenience sampling of the subjects was carried out at the Kolej and Universiti Tunku Abdul Rahman-two private institutions of higher learning in Setapak, Kuala Lumpur, the Malaysian subjects were young adults. The gender and ethnicity of the subjects were imbalanced with more females and majority of the subjects recruited were Chinese. This phenomenon is due to nature of these institutions designated to cater more for the Malaysian Chinese community, as they were established by the Malaysian Chinese Association (Wikipedia, 2009). In this study, we have adapted a lower BMI cut off point of 27 kg m^{-2} instead of 30 kg m^{-2} for obesity, as this former Singaporean cut-off point (Yap *et al*., 2000) is more relevant for the Malaysian context rather than the latter one recommended by the World Health Organization (2009a). However, obesity is not prevalent among the sampled subjects, as we could only recruit 57 obese subjects out of the total 200. Nevertheless, as expected, all the anthropometric measurements were significantly higher in obese subjects as indicated in Table 1. The systolic and diastolic blood pressure means of the obese subjects were still in the normal category according to the guideline by Chobanian *et al*. (2003) and this could be due to the subjects were still young. There seems to be a greater difference in the means of BMI and TBF compared to WHR between the non-obese and obese subjects (Table 1) and whether the WHR is a better predictor for cardiovascular risk compared to BMI (Yusuf *et al*., 2005) remains to be determined.

In this study, the allele frequencies of the gene variants were different from the previous studies in different populations. The 19G allele frequency of 0.52 for *LEP* was slightly higher than the overall frequency of 0.46 in 375 Caucasian subjects (France, Italy and Finland), as shown in the meta-analysis by Paracchini *et al*. (2005). Based on the same meta-analysis, the *LEPR* 109R allele frequency of 0.43 in this study was higher than the overall frequency of 0.25 in nine different Caucasian populations covering 2498 subjects, but still lower than the 0.82 frequency for Korean and Japanese subjects (Paracchini *et al*., 2005). Finally, in this study, the 0.36 frequency for the *LEPR* 223R allele was more similar to that of Pima Indians (0.32), compared to Caucasians (0.45) or Asians (0.85), whereas the frequency of the *LEPR* 656N allele (0.31) was higher in both the 2886 Caucasian (0.18) and 115 Japanese (0.12) subjects (reviewed in Paracchini *et al*., 2005). The discrepancy in allele frequencies in different populations, including the current study, is due to the genetic diversity that exists among different ethnic populations. Therefore, this further confirms the fact that the association in one population could not be extrapolated to another population.

The genetic component may not seem to play a role in the development of obesity in the current study, as only the *LEPR* K656N genotype was significantly associated with obesity, but not the 656N allele or other *LEP* and *LEPR* variants (Table 2). In support of this, two meta-analyses of association studies on Western and Eastern populations around the world, performed with stratification according to ethnicity, suggest no evidence of an association between these *LEP* and *LEPR* gene variants and obesity (Heo *et al*., 2002; Paracchini *et al*., 2005). This reported lack of association could be due to the complex pathogenesis of obesity, which involves many various genetic and environmental factors and the interaction between them. Besides that, the fact that these non-association findings were mostly based on studies using BMI as an indicator for the obesity phenotype, should also be taken into consideration. Therefore, studies including different measures of obesity, like waist circumference, WHR and TBF, could be beneficial in future association studies.

The mechanism by which the molecular variants of LEP A19G and *LEPR* K109R, Q223R and K656N are related to obesity is poorly understood. *In vitro* tests of *LEP* defects and

leptin production studies showed that the A19G variant affects the basal leptin level in human body (Hager *et al*., 1998). Thus this variant might lead to insufficient leptin levels and subsequently causes increase in body weight. Meanwhile, the Q223R substitution in exon 6 is located in the extracellular region of the leptin receptor within the highly conserved first cytokine domain (C domain), which represents a leptin-binding site. The single amino acid change from glutamine (Q) to arginine (R) will cause a change in charge from neutral to positive and therefore could affect the receptor's function and alter its signaling mechanism (Chagnon *et al*., 1999, 2000). This phenomenon can be similarly observed in the Zucker fatty rat model of obesity, where the fa mutation in Lepr is also located in the first C domain, resulting in a single amino acid substitution of Q to proline (P) in codon 269 (270 for humans) (Phillips *et al*., 1996). This substitution affects the receptor's function by significantly down-regulating its cell surface expression as well as changing signal transduction by constitutively activating STAT1 and STAT3 and strongly impairing ligand-induced STAT5B activation (White *et al*., 1997). Hence, this may lead to a condition called 'leptin resistance', which consequently leads to the observed obese phenotype in rats (Chua *et al*., 1996). Due to the close proximity and similarity of the *LEPR* Q223R to the Q269P variant, the leptin resistance phenomenon in rats could also be extrapolated for humans. Further *in vitro* and *in vivo* studies are needed to shed more light on the mechanisms of how SNPs in the human *LEP* and *LEPR* genes could contribute in the pathogenesis of obesity.

The study did not fully demonstrate the data from epidemiological studies that suggests that dietary habits and lifestyle factors play a major role in the genesis of obesity, as the frequencies were not significantly different between the non-obese and obese subjects-except for physical activity (Table 3). Dietary habits largely influence energy intake and may also affect the body metabolic rate, which may contribute to obesity. There has been evidence in the USA and UK that the increase of obesity was remarkably contributed by the high sodium and fat intake found in fast food (Stender *et al*., 2007). Previous studies also showed that vegetarians tend to have lower body mass compared to meat or fish eaters (Spencer *et al*., 2003) which could be due to the low fat and high fibre content in vegetarian diets (Barnard *et al*., 2005). Animal and epidemiological studies suggest that long-term caffeine and coffee consumption may decrease body weight in humans (Greenberg *et al*., 2006). However, the dietary habits were not significantly different between the non-obese and obese in this study, which was in contrast with the above findings. This may be due to the low amount of obese subjects and the small sample size, generally, which limits the power needed to make a statistical inference.

According to the 2006 NMHSIII survey (Ministry of Health Malaysia, 2006) out of the adults (≥18 years) surveyed, 21.5% were current smokers. The low prevalence of current smokers in this study (8%) could be due to the large amount of Chinese subjects, as the prevalence of smoking was highest in Malays (24.0%) and Other Bumiputras (24.8%) (Ministry of Health Malaysia, 2006). In another Malaysian study by Rampal *et al*. (2007) the prevalence of obesity was significantly higher among the non-smokers as compared to current smokers. The finding is consistent with respect to the effects of nicotine and nicotinic receptors in the regulation of appetite. Smokers are leaner and smoking cessation in the absence of nicotine replacement therapy typically results in significant and sustained hyperphagia and weight gain (Jo *et al*., 2002). Meanwhile, the prevalence of current drinkers was 37% in this study, compared to 7.4% among overall Malaysians in the 2006 NHMSIII survey (Ministry of Health Malaysia, 2006). The same survey found that Chinese were more prevalent drinkers, which could explain the high prevalence in the current study-as mainly Chinese subjects were recruited. The association of alcohol drinking habit with obesity

depends on the quantity and frequency of alcohol consumption per day. A study done by Tolstrup *et al.* (2005) proposed that for a given level of total alcohol intake, obesity was inversely associated with drinking frequency, whereas the amount of alcohol intake was positively associated with obesity. Finally, we also found that very few subjects had frequent or habitual physical activity (25%). Based on the World Health Organization (2009b) recommendation, a healthy adult must do at least exercise for 30 min of moderate-intensity physical activity 5 days per week, or 20 min of vigorous-intensity physical activity 3 days per week-in order to promote or maintain health and physical fitness of a person. Although genes play an important role in the development of obesity, physical activity seems to have an important independent preventive role in the development of obesity (Tammelin *et al.*, 2004).

This study could be further improved in a few aspects. We are aware of the limited samples successfully genotyped and the low prevalence of obesity in the current study. Therefore in the future, we plan to genotype more samples and to recruit more obese cases. A more balanced sampling of subjects covering different age groups and ethnic groups should also be performed. Besides BMI and other related anthropometric measurements, biochemical alterations like serum leptin, glucose and insulin levels could also be investigated as markers for the obesity phenotype-for the study of association with the *LEP* and *LEPR* genotypes.

In conclusion, present results show that obesity is not prevalent among the sampled Malaysian university students in Setapak, Kuala Lumpur. Besides *LEP* and *LEPR* gene variants, the surveyed environmental contribution towards obesity seems to be insignificant. These findings have to be interpreted with caution, due to the small amount of subjects successfully genotyped, the young age of the subjects and the low prevalence of obesity cases in this study. It may also suggest the existence of other possible factors contributing to the development of obesity among the sampled subjects, such as other genes besides *LEP* and *LEPR* and other dietary habits and lifestyle factors like high fat and sugar intake, stress and insufficient sleep.

ACKNOWLEDGMENTS

This project was funded by the Department of Science, Faculty of Science and Engineering, University Tunku Abdul Rahman. We also gratefully acknowledge all the volunteers who have participated in this study.

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