Association of Single Nucleotide Polymorphisms of Endothelin, Orexin and Vascular Endothelial Growth Factor Receptor Genes with Obstructive Sleep Apnea among Thai Ethnic

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Background: *Obstructive sleep apnea (OSA) is a complex disorder characterized by repetitive collapse of upper airway during sleep which strongly influenced by genetic factors, especially those affect regulation of the sleep-wake cycle and endothelial function.*
Objective: *This study investigated the association between single nucleotide polymorphisms (SNPs) in endothelin (EDNRA), orexin (OX1R, OX2R) and vascular endothelial growth factor (VEGFR1) receptor genes with risk of OSA in Thai population.*
Material and Method: *All subjects were diagnosed by overnight polysomnography (PSG) before divided into OSA (59) and NOSA (60) groups based on their apnea-hypopnea index (AHI). Serum lipid levels were examined by using enzymatic colorimetric and homogeneous methods. DNAs were extracted and genotyped the SNPs by polymerase chain reaction (PCR) and high-resolution melting (HRM) analysis. Genotype distribution were analyzed using Chi-square test of SPSS program version 15.0.*
Results: *The triglycerides level of OSA patients was significantly higher than NOSA (p-value = 0.002). The SNPs in EDNRA (rs5335), OX1R (rs2271933), OX2R (rs2292040, rs10456182) and VEGFR1 (rs11149523) genes showed no association with OSA. However, the SNP (rs17675063) in EDNRA gene showed significant differences in genotype distribution in the subjects with and without OSA (p-value = 0.002, odds ratio = 3.29 and 95% CI = 1.86-5.82).*
Conclusion: *The results suggested that SNP in EDNRA gene (rs17675063) associates with the risk of OSA in Thai population. It may be used as a marker for this disease.*

Keywords: *Obstructive sleep apnea, Single nucleotide polymorphisms, Endothelin receptor type A, Orexin receptor 1, Orexin receptor 2, Vascular endothelial growth factor receptor type 1*

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Obstructive sleep apnea (OSA) is a complex disorder caused by episodes of total or partial collapse of the upper airway leading chronic hypoxia, hypercapnia, sleep fragmentation and excessive daytime sleepiness (EDS)[1]. EDS leads to daytime fatigue, poor daytime performance, memory impairment and increased risk of traffic accidents. OSA is being increasingly as a likely cause of significant morbidity and mortality. Because there is evidence that it associated with risk for the development of many systemic diseases such as hypertension, cardiovascular disease, obesity and diabetes[2-4]. OSA can occur at any age, typically presents between the age of 40-60 years and increase with age[5]. In Asia, the prevalence of OSA in middle aged men and women are 4.1-7.5% and 2.1-3.2%, respectively. It is similar to the reported in Caucasian population[6]. In Thai population, the prevalence of OSA in men and women are 4.8% and 1.9%, respectively[7]. OSA is strongly influenced by genetic factors, especially those that affect regulation of the sleep-wake cycle, endothelial function, fat distribution, upper airway muscle tone, ventilatory

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control and craniofacial morphology. A percentage of 35 to 40% of its variance can be attributed to genetic factors[8].

Endothelins (EDN) are family of proteins known as the strongest vasoconstrictors. They are implicated in vascular diseases of several organ systems, including the heart, general circulation and brain[9]. Endothelins are composed of endothelin-1 (EDN1), endothelin-2 (EDN2) and endothelin-3 (EDN3). Each of these peptides is encoded by a separate gene, and the biosynthesis includes processing from prepro-form by a furin-like protease and processed by endothelin converting enzymes 1 and 2 (ECE-1 and ECE-2), respectively, to form EDN1, EDN2 and EDN3[10]. Endothelin exerts its actions through binding to specific receptors, included type A (EDNRA) and type B (EDNRB) receptors. Both of them are G protein coupled transmembrane proteins, with different molecular and pharmacological characteristics and functions based on their location. EDNRA are found in the smooth muscle of blood vessels. The binding of EDN1 and EDNRA leading to increased blood pressure[11].

Orexin was first described in 1998[12]. Mammalian prepro-orexin is composed of 130 amino acids. Cleavage and modification of one molecule of prepro-orexin produce orexin A and orexin B. Besides playing a role in the regulation of feeding, energy homeostasis[12,13], it also regulates the sleep-wakefulness[14]. Their effects are through binding to specific receptors, orexin receptor 1 (OX1R) and orexin receptor 2 (OX2R)[15]. There is an overall 64% sequence identity between OX1R and OX2R[16]. The rat and human receptors show highly conserved 94% and 95% homology for OX1R and OX2R, respectively. Orexin receptors were originally shown to be present only in the hypothalamus[12], but now their presence have been noted in many organs[17-19], implicating orexins and their receptors in an increasing number of physiological responses.

Vascular endothelial growth factor (VEGF) are important signalling proteins involved in both vasculogenesis and angiogenesis. The biological activity of VEGF is dependent on its reaction with specific receptors. Three different receptors have been identified including VEGFR1, VEGFR2, and VEGFR3. Both VEGFR1 and VEGFR2 have extracellular immunoglobulin like domains as well as a single tyrosine kinase transmembrane domain and are expressed in a variety of cells[20]. The VEGF ligand and receptor signalling system plays a key role in vascular development and permeability. It has been shown that serum and plasma VEGF levels are increased in patients with OSA compared to normal controls[21,22].

Because there is no report concerning to the association study of endothelin, orexin and VEGF receptor genes with OSA in the Thai ethnic. Therefore, this study aims to investigate the association between SNPs in endothelin, orexin and VEGF receptor genes with risk of OSA in Thai population.

Material and Method

Participants

All subjects were diagnosed by overnight polysomnography (PSG) before they were divided into OSA and control (NOSA) groups based on their apnea-hypopnea index (AHI). An AHI of less than 5 events per hour was considered to be control group, and an AHI of more than 10 events per hour considered to be OSA. Inclusion and exclusion criteria of the subjects were showed in the Table 1. The subjects were underwent overnight polysomnography at the ENT (ear, nose and throat) ward, HRH Princess Maha Chakri Sirindhorn Medical Centre, Faculty of Medicine, Srinakharinwirot University, Nakhon Nayok province. Blood samples were collected from 59 obstructive sleep apnea patients and 60 healthy controls between 07.00 and 08.00 AM. All subjects signed an informed consent which was approved by the Human Ethics Commitees of the Faculty of Medicine, Srinakharinwirot University before participating in this study.

DNA extraction

The genomic DNAs of patient and healthy control were extracted from peripheral blood by using Flexigene DNA kit (QIAGEN, German). The concentration of the DNA was determined by using a ND-2000C spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). The quality of genomic DNA was determined by gel electrophoresis using 1% agarose gel. All the purified samples were stored at -80°C until used. Prior to genotype, the DNAs were diluted and adjusted to a concentration of 25 ng/µl.

Determination of serum lipids level

Level of serum triglycerides and total cholesterol were determined by the enzymatic colorimetric method[23,24] whereas HDL-C and LDL-C were analysed by using homogeneous method[25]. The analysis was performed at laboratory of HRH Princess Mahachakri Sirindhorn Medical Center, Faculty of Medicine, Srinakharinwirot University, Nakhon Nayok

province. All lipid profile tests were run on Dimension RXL chemistry analyzer (Dade Behring, USA).

SNP primers and genotyping

All SNPs of endothelin, orexin and VEGF receptor genes were selected from JSNP/NCBI and of minor allele frequency (MAF) exceeding 0.2 in population of Japan descent[26]. PCR primers were designed from website http://snp.ims.u-tokyo.ac.jp/search.html. The sequence of PCR primers of each SNPs were shown in Table 2. Real-time PCR reaction was performed in 20 µl reaction mixture composed of 2 µl of DNA (25 ng/µl), 0.6 µl of 10 µM forward primer, 0.6 µl of 10 µM reverse primer, 10 µl of Supermix (2X) and 6.8 µl of sterile-distilled water. PCR reaction was carried out for 40 cycles of 2 steps amplification: pre-denaturing at 98°C for 3 min, denaturation at 98°C for 10 s, annealing at x°C (depend on primer) for 15 s. HRM analysis was performed at temperature ramping from 65-95°C using a CFX96 Touch real-time PCR detection system (California, USA). Nucleotide sequence of selected DNAs were confirmed by sequencing to validate the genotypes.

Statistical analysis

The demographic characteristics of group variables were expressed as mean $\pm$ standard deviation (SD). The significance of variables between the two groups were tested by using the t-test. The goodness-of-fit test of Hardy-Weinberg equilibrium (HWE) was performed using the equation p2+2pq+q2, where p and q represented the wild-type and variant allele of a gene. Association analyses were performed using Pearson's Chi-square test implemented in SPSS program version 15.0 for Windows. Significant level was set at $p<0.05$. Odds ratio and 95% confidence interval were calculated for significantly SNP.

Results

Demographic characteristic of the subjects

The subjects were divided into OSA and NOSA based on their apnea-hypopnea index (AHI); 60 were controls (NOSA) and 59 fulfilled the criteria for OSA. The demographic characteristic and metabolic

Table 1. Inclusion and exclusion criteria for selection the subjects

Inclusion	Exclusion
Age 30-60 years	Age less than 30 or more than 60 years
Sex; male and female	Refuse to sign the informed consent
Subjects must diagnostic by overnight PSG	
OSA group AHI $\geq$10 events/h	
Control group AHI <5 events/h	
Sign informed consent	

Table 2. SNP primers of endothelin, orexin and VEGF receptor genes used in PCR amplification

Genes	db SNPs ID	Location	Forward sequence 5' .....3'	Reverse sequence 5' .....3'	Product size (bp)
EDNRA	rs5335 C/G	3'UTR	CCATAATCCTCTCGGAG AAAAA	TCTTGGGTGTGGGAGTG AA	95
	rs17675063 A/G	intron 2	GCTCCTGCTCTTGCTCC A	CCCGCTTGTTAATCTCT TCC	94
OX1R	rs2271933 C/T	CDS	TAGTCCCCGCTCCTCTG C	TGTGGTGACGCTGGTGA G	100
OX2R	rs2292040 A/G	intron 5	TGACCTGATTTGGCTTT TGA	TTTCTGACCCCATAATT GGTTT	90
	rs10456182 A/G	intron 1	TCTCTGCTGAAAATGAC AGGA	CCCAGCCTATCTCACCT CAC	98
VEGFR1	rs11149523 A/G	intron 10	TTCATTTGTTTGGGCTG GAT	CAGCAGCTCCTCCACCA TT	92

data of these subjects are shown in Table 3. The AHI was significantly higher in the OSA group (p<0.001). Triglycerides of OSA patient was significantly higher than NOSA (p-value = 0.002). However, there were no differences in cholesterol, HDL and LDL levels between the two groups.

Single nucleotide polymorphisms of EDNRA, OX1R, OX2R, and VEGFR1 genes

The result of goodness-of-fit test was equal to 1 indicated that all of SNPs tested were in Hardy-Weinberg equilibrium. The distributions of allele and genotype frequencies of SNPs in endothelin, orexin and VEGF receptor genes were summarized in Table 4. The rs17675063 in EDNRA gene was significantly associated with OSA (p-value = 0.002, odds ratio = 3.29 and 95% CI = 1.86-5.82). However, the other SNPs in EDNRA (rs5335), OX1R (rs2271933), OX2R (rs2292040, rs10456182) and VEGFR1 (rs11149523) showed no association with OSA.

Discussion

Obstructive sleep apnea (OSA) is the most common type of sleep apnea, characterized by repetitive collapse of the upper airway during sleep. The severity of sleep apnea based on AHI (the total number of apneas and hypopneas occurring per hour of sleep)[27]. It is considered as a multifactorial disease in which multiple genes, environmental influences, and development factors are related. Obesity is the major risk factor for the development of OSA (10-14 folds). More than 60% of the OSA patients reported with obesity[28]. In previous study, in severe OSA patients showed a significant increase in waist circumference, triglycerides, and a decrease in HDL cholesterol levels[29]. Similarly to our study that the level of triglycerides of OSA was significantly higher than NOSA. OSA is an important risk factor for cardiovascular disease[30]. The persons with an OSA relative have been shown increase the relative risk for OSA 1.5-fold[31]. The disease is strongly influenced by genetic factors, a percentage of 35 to 40% of its variance can be attributed to genetic factors[8]. Genetic variability of several genes have been reported to associate with OSA. EDN1 SNP (rs5370), Lys198Asn (G/T), is associated with the severity of OSA in obese European subjects[32]. A genome-wide association study (GWAS) showed that the rs2071943 and rs9296344 of EDN1 were associated with OSA in European Americans and African Americans[33]. In knock-out mice models with a lack of EDN1 and/or lack of EDNRA gene showed characteristic traits occurring in OSA such as hypertension, respiratory failure, ventilator control abnormalities and craniofacial dysmorphisms[34,35]. An increased expression of EDNRA which mediate a potent vasoconstrictor response, plays an important role in the pathogenesis of chronic intermittent hypoxia[36]. In addition, genetic polymorphisms in the EDNRA gene have been found to increase susceptibility for OSA in adults[37]. Furthermore, VEGF levels significantly correlated with the severity of OSA, represent a response to hypoxia which occurs during sleep[21,22]. The plasma orexin-A levels were decreased in parallel with the arousal index (AI) and AHI in patients with OSA[38]. Previous study in the Cleveland Family Study (CFS) found evidence for linkage to AHI at 6p12, the location of the orexin receptor 2 (OX2R) in European Americans[39].

This study, statistical significant of EDNRA (rs17675063) with G allele was found in OSA patients more than control group (p-value = 0.002). This SNP is in intron region which may be affected with RNA processing, leading to abnormalities in protein synthesis. Individuals with G allele had 3.29 times higher risk in develop OSA when compare to those with A

Table 3. The demographic characteristic of controls (NOSA) and OSA patient

Variables	NOSA (n = 60)	OSA (n = 59)	p-value
AHI (events/h)	1.86±1.32	28.19±21.48	<0.001
Cholesterol (mg/dl)	197.85±38.84	208.25±42.89	0.180
Triglycerides (mg/dl)	124.00±72.96	195.28±97.99	0.002
HDL (mg/dl)	50.55±14.18	50.42±12.87	0.490
LDL (mg/dl)	128.80±30.98	129.28±35.34	0.480

Data are means ± SD

Table 4. Distributions of allele and genotype frequencies of endothelin, orexin and VEGF receptor SNPs in subjects with and without OSA

Genes	SNP	Allele	NOSA		OSA		*p*-value	OR	95%CI
EDNRA	rs5335 C/G		n = 60		n = 46				
			n	%	n	%			
		CC	23	38	18	39	0.712	0.89	0.51-1.60
		CG	32	54	26	57			
		GG	5	8	2	4			
		Allele C	78	65	62	67			
		Allele G	42	35	30	33			
EDNRA	rs17675063 A/G		n = 60		n = 52				
			n	%	n	%			
		AA	39	65	21	40	0.002	3.29	1.86-5.82
		AG	14	23	10	20			
		GG	7	12	21	40			
		Allele A	92	77	52	50			
		Allele G	28	23	52	50			
OX1R	rs2271933 C/T		n = 54		n = 48				
			n	%	n	%			
		CC	2	4	5	10	0.276	0.77	0.44-1.33
		CT	44	81	39	81			
		TT	8	15	4	9			
		Allele C	48	44	49	51			
		Allele T	60	56	47	49			
OX2R	rs2292040 A/G		n = 60		n = 46				
			n	%	n	%			
		AA	25	42	25	54	0.427	0.62	0.36-1.06
		AG	2	3	1	2			
		GG	33	55	20	44			
		Allele A	52	43	51	55			
		Allele G	68	57	41	45			
OX2R	rs10456182 A/G		n = 60		n = 59				
			n	%	n	%			
		AA	3	5	1	2	0.412	0.93	0.51-1.72
		AG	20	33	25	42			
		GG	37	62	33	56			
		Allele A	26	22	27	23			
		Allele G	94	78	91	77			

Table 4. Cont.

Genes	SNP	Allele	NOSA		OSA		*p*-value	OR	95%CI
VEGFR1	rs11149523 A/G		n = 60		n = 59				
			n	%	n	%			
		AA	1	2	2	3	0.454	0.80	0.41-1.59
		AG	20	33	14	24			
		GG	39	65	43	73			
		Allele A	22	18	18	15			
		Allele G	98	82	100	85			

allele (odds ratio = 3.29 and 95% CI = 1.86-5.82). This finding is the first study that report a strong association of EDNRA (rs17675063) G allele with risk of OSA.

Conclusion

The SNPs in EDNRA (rs5335), OX1R (rs2271933), OX2R (rs2292040, rs10456182) and VEGFR1 (rs11149523) showed no association with OSA. However, the rs17675063 in EDNRA gene showed significant association with OSA. Therefore it may be used as a biomarker for the risk of this disease in Thai population.

What is already known on this topic?

OSA is strongly influenced by genetic factors especially those affect regulation of the sleep-wake cycle, endothelial functional, fat distribution, upper airway muscle tone, ventilator control and craniofacial dysmorphisms. The genetic polymorphisms in the EDNRA gene (rs1801708) have been found that it increased susceptibility for OSA in adults.

What this study adds?

This study analysed serum lipid profile between OSA and control; NOSA. Furthermore, we investigated the association between SNPs in endothelin (EDNRA), orexin (OX1R, OX2R), and vascular endothelial growth factor receptor (VEGFR1) genes with risk of OSA in Thai population. We found that the triglycerides level of OSA patients was significantly higher than those of NOSA. The rs17675063 in EDNRA was associated with the risk of OSA in Thai population.

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Potential conflicts of interest

None.

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การหาความสัมพันธ์ระหว่างซิงเกิลนิวคลีโอไทด์โพลีมอร์ฟีซึมของจีนตัวรับเอนโดทีลิน โอรีซินและ เว็จเจฟ กับโรคนอนกรนที่มีภาวะหยุดหายใจขณะหลับในประชากรไทย

กันต์กนิษฐ รัตนธนาวรรณ, ภนารี บุษราคัมตระกูล, พลเพ็ชร ทองเกตุ, ชัยรัตน์ นิรันตรัตน์, วาสนา สุขุมศิริชาติ

ภูมิหลัง: *ภาวะหยุดหายใจขณะหลับจากการอุดกั้นเป็นความผิดปกติที่มีความซับซ้อน โดยมีลักษณะสำคัญ คือ การยุบตัวของทางเดินหายใจส่วนต้นบางส่วนหรือทั้งหมดขณะนอนหลับ ซึ่งได้รับอิทธิพลจากปัจจัยทางพันธุกรรม โดยเฉพาะอย่างยิ่งจีนที่เกี่ยวกับการควบคุมการนอนหลับและการทำหน้าที่ ของหลอดเลือด*

จุดประสงค์: *เพื่อหาความสัมพันธ์ระหว่างซิงเกิลนิวคลีโอไทด์โพลีมอร์ฟีซึม (สนิปส์) ของจีนตัวรับเอนโดทีลิน (EDNRA), โอรีซิน (OX1R, OX2R), และเว็จเจฟ (VEGFR1) กับความเสี่ยงต่อการเกิดโรคนอนกรนที่มีภาวะหยุดหายใจขณะหลับในคนไทย*

วัสดุและวิธีการ: *อาสาสมัครทั้งหมดได้รับการตรวจความผิดปกติในการนอนหลับก่อนแล้วแบ่งออกเป็น 2 กลุ่ม คือกลุ่มคนไข้ที่มีภาวะหยุดหายใจ ขณะหลับ (59 คน) และกลุ่มปกติ (60 คน) โดยอาศัยค่าความถี่ของการหยุดหายใจและหายใจน้อยที่เกิดขึ้นระหว่างการนอนหลับ (เอเอชไอ) หาระดับ ไขมันในซีรัมโดยใช้วิธี enzymatic colorimetric และ homogeneous methods ทำการสกัดดีเอ็นเอและหาจีโนไทป์ของสนิปส์ในจีนเหล่านี้ โดยเทคนิคพีซีอาร์และตามด้วยไฮเรสโซลูชั่นเมลติ้ง (เอชอาร์เอ็ม) จากนั้นวิเคราะห์ความถี่ของจีโนไทป์ในกลุ่มคนไข้และกลุ่มปกติโดยใช้ Chi-square test โปรแกรม SPSS รุ่น 15.0*

ผลการศึกษา: *จากการเปรียบเทียบระดับไขมันในซีรัม พบระดับไตรกลีเซอไรด์ในกลุ่มคนไข้ที่มีภาวะหยุดหายใจขณะหลับสูงกว่ากลุ่มปกติอย่างมีนัยสำคัญ ทางสถิติ (p = 0.002) การวิเคราะห์สนิปส์ในจีนตัวรับเอนโดทีลินชนิด เอ (rs5335) โอรีซินชนิดที่หนึ่ง (rs2271933) โอรีซินชนิดที่สอง (rs2292040, rs10456182) และเว็จเจฟ (rs11149523) ไม่พบความสัมพันธ์กับภาวะหยุดหายใจขณะหลับ อย่างไรก็ตามการศึกษาครั้งนี้พบความสัมพันธ์ อย่างมีนัยสำคัญทางสถิติของสนิปส์ตำแหน่ง rs17675063 ในจีนตัวรับเอนโดทีลินชนิด เอ (p = 0.002, odds ratio = 3.29 และ 95% CI ของอัลลีล G = 1.86-5.82)*

สรุป: *สนิปส์ตำแหน่ง rs17675063 ในจีนตัวรับเอนโดทีลินชนิด เอ มีความสัมพันธ์กับโรคนอนกรนที่มีภาวะหยุดหายใจขณะหลับในประชากรไทย ซึ่งอาจใช้เป็นตัวบ่งชี้ถึงความเสี่ยงของการเกิดโรคนี้ได้*